



Early View

Original article

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Bioengineered airway epithelial grafts with mucociliary function based on collagen IV- and laminin-containing extracellular matrix scaffolds

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Take home message: Collagen IV- and laminin-rich decellularised dermis scaffolds support a mucociliary airway epithelial graft but in vivo transplantation in pre-clinical models is challenging.

Running title: ECM-guided bioengineered airway mucosa

Abstract

Current methods to replace damaged upper airway epithelium with exogenous cells are limited. Existing strategies use grafts that lack mucociliary function, leading to infection and the retention of secretions and keratin debris. Strategies that regenerate airway epithelium with mucociliary function are clearly desirable and would enable new treatments for complex airway disease. Here, we investigated the influence of the extracellular matrix on airway epithelial cell adherence, proliferation and mucociliary function in the context of bioengineered mucosal grafts. *In vitro*, primary human airway epithelial cells adhere most readily to collagen IV. Biological, biomimetic and synthetic scaffolds were compared in terms of their extracellular matrix protein content and airway epithelial cell adherence. Collagen IV and laminin were preserved on the surface of decellularised dermis and epithelial cell attachment to decellularised dermis was greater than to the biomimetic or synthetic alternatives tested. Blocking epithelial integrin $\alpha 2$ led to decreased adherence to collagen IV and to decellularised dermis scaffolds. At air-liquid interface, bronchial epithelial cells cultured on decellularised dermis scaffolds formed a differentiated respiratory mucosal layer with mucociliary function. Using *in vivo* chick chorioallantoic membrane and rabbit airway models, we showed short-term preservation of the differentiated cell layer following transplantation. Our results demonstrate the feasibility of generating human airway epithelial cell grafts on clinically applicable decellularised dermis scaffolds and identify matrix proteins and integrins important for this process. The long-term survivability of pre-differentiated epithelia and the relative merits of this approach against transplanting basal cells should be assessed further in pre-clinical airway transplantation models.

1. Introduction

The respiratory mucosa lines the internal surface of the trachea and bronchi and consists of a pseudostratified, multiciliated epithelium containing mucus-secreting goblet cells [1]. The respiratory mucosa performs a vital array of functions, including acting as a barrier against infection and clearing secretions from the lower airways via the mucociliary escalator [2, 3]. Existing methods to restore respiratory mucosa following airway reconstruction and cancer resection, rely on the transfer of muscle on a vascularised pedicle and skin grafting. Whilst these can re-epithelialise small sections of airway, they are not suitable for reconstruction of larger areas as the epithelium retains stratified squamous histology and thus lacks the ciliated and mucosecretory cells required for normal functionality [4]. The epidermis also has a higher rate of epithelial turnover than respiratory epithelium, which may contribute to airway sloughing and obstruction in these patients [5]. Buccal epithelium has been used in mucosal grafts and successfully applied to restore small sections of tracheal mucosa [6] but, due to limitations in the extent of donor tissue that can be harvested, this approach is also not suitable for extensive proximal airway repair.

The ability to regenerate a transplantable respiratory mucosal layer with mucociliary function would be a significant step forward in the field of airway regenerative medicine; it would enable new therapies to treat long-segment mucosal diseases of the upper airways, including complex scarring and granulomatous conditions. Such a technique would also be highly relevant to gene editing approaches to treat genetic disorders such as cystic fibrosis, where cell engraftment poses a major challenge [7]. Examples of bioengineered tracheal replacements have been limited by slow mucosalisation following implantation [8-10] and bioengineered respiratory mucosal grafts might improve the safety and efficacy of such procedures.

Current reports of *in vitro* bioengineered upper airway mucosa have mainly focused on regenerating the mucosal layer on tracheal scaffolds [11, 12]. However, the *in vivo* application of these techniques is limited by the time taken for revascularization to occur following transplantation. To overcome this, we envisage the use of a two-stage procedure

[13] whereby a mucosal layer composed of respiratory cells (rather than cells from other epithelia, e.g. buccal [14, 15]), is generated *in vitro* that can be used to re-epithelialise a pre-vascularised implanted airway scaffold or be grafted directly onto the airway to replace damaged mucosa. This methodology more closely follows the principles of free tissue transfer, where well-vascularised graft beds are essential for successful outcomes [16].

In formulating a method to regenerate respiratory mucosa, careful consideration needs to be given to the extracellular matrix (ECM) environment. The ECM is a complex network of macromolecular proteins that are bound by specific cation-dependent cell surface receptors, the integrins, on the basolateral surface of epithelial cells [17]. Integrin-ECM binding leads to cascades of intracellular signalling that influence multiple cellular processes including attachment, proliferation, polarity and programmed cell death [18]. Evidence from investigations of the ECM in stratified epithelia, along with proteomic data examining the composition of the upper airway basement membrane indicate that collagen I, collagen IV, laminin, vitronectin and fibronectin play important roles in modifying epithelial cell behavior [19-21]. Here, the effect of these ECM proteins on respiratory epithelial cell attachment, expansion and differentiation *in vitro* was investigated with a view to optimizing the ECM environment for bioengineered airway mucosa.

2. Materials and Methods

2.1 Primary cell culture

Primary human bronchial epithelial cells (HBECs) were isolated from endobronchial biopsies from the human adult upper airways or from the bronchi of patients undergoing lobectomy (Supplementary Table 1). Ethical approval was obtained from a Research Ethics Committee (REC references: 06/Q0505 and 11/LO/1522). HBECs were maintained in bronchial epithelial growth medium (BEGM; Lonza, Slough, U.K.) for attachment and proliferation experiments. For differentiation experiments, HBECs that had been isolated and maintained on mitomycin C-treated 3T3-J2 feeder layers with 5 μ M ROCK inhibitor Y-27632 (3T3+Y; Enzo Life Sciences, Exeter, U.K.) were used as previously described [22-24]. Cells isolated and expanded in BEGM were used between passage one to three, while cells isolated and expanded in 3T3+Y were used between passage one to six. Primary human lung fibroblasts were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Hemel Hempstead, U.K.) containing 10% foetal bovine serum (FBS) and were used no later than passage ten.

2.2 Coating of tissue culture plastic with extracellular matrix protein

Extracellular matrix (ECM) proteins were used to coat tissue culture plastic using the recommended manufacturer's concentrations and, in Figure 1A, a value above (high) and below (low) that concentration (medium). The ECM proteins used were collagen I from human neo-natal fibroblasts (Advanced Biomatrix, San Diego, U.S.A.) at 38.7 μ g/ml, 387 μ g/ml and 968 μ g/ml; collagen IV from human placenta (Sigma-Aldrich, Dorset, U.K.; #C8374) at 10 μ g/ml, 250 μ g/ml and 500 μ g/ml; vitronectin from human plasma (Sigma-Aldrich, Dorset, U.K.; #V8379) at 0.5 μ g/ml, 1.25 μ g/ml and 2.5 μ g/ml; laminin from human placenta (Sigma-Aldrich, Dorset, U.K.; #L6274) at 1 μ g/ml, 2 μ g/ml and 4 μ g/ml and 0.1% fibronectin from human plasma (Sigma-Aldrich, Dorset, U.K.; #F0895) at 1 μ g/ml, 25 μ g/ml and 250 μ g/ml. Non-adherent 96-well plates (Fisher Scientific, Hemel Hempstead, U.K.; #10554961) were coated with ECM proteins following the relevant manufacturer's protocol.

2.3 Integrin blocking experiments

Antibodies previously reported to have blocking function against integrin subunits [25-32] were added at a concentration of 1:200 in BEGM and the antibody-cell suspension was kept on ice for 20 minutes before adding to ECM-coated or decellularised dermis scaffold-containing wells for experiments examining cell attachment. Untreated cells were used as controls. In cell expansion experiments, cells were allowed to adhere for two hours before they were washed with PBS and the medium replaced with BEGM containing the blocking antibodies. For experiments examining differentiation, blocking antibodies were refreshed with medium changes following air lift as indicated in figure legends. The antibodies used to block integrin subunits were: α 1 (5E8D9; Novus Biological, U.S.A.; #NBP2-29757), α 2 (P1E6-C5; Biolegend, San Diego, U.S.A.; #359304), α 3 (ASC-1; Merck Millipore, Watford, U.K.; #MAB2056Z), α v (NKI-M9; Biolegend, San Diego, U.S.A.; #327904), α 5 (NKI-SAM-1; Biolegend, San Diego, U.S.A.; #328004), α 6 (GoH3; Biolegend, San Diego, U.S.A.; #313614), α 9 β 1 (Y9-A2; Bio-Rad, California, U.S.A.; #OBT1715Z) and β 1 (P5D2; Merck Millipore, Watford, U.K.; #MAB1959Z).

2.4 Attachment, proliferation and metabolic assays

For assays involving ECM-coated plates, non-adherent 96-well plates were coated with ECM as described above and cells were seeded in BEGM before 30 minutes incubation at 37°C, 5% CO₂. Wells were washed three times with phosphate buffered saline (PBS) to remove unbound cells and plates were frozen at -80°C. To quantify the number of cells attached, plates were thawed to room temperature and a cell lysis buffer containing green fluorescent DNA dye (CyQUANT® GR, Life Technologies, Hemel Hempstead, U.K.) was added according to the manufacturer's protocol. Fluorescence was measured using a microplate reader (excitation 480 nm, emission 520 nm). Background fluorescence was subtracted from these readings. To determine cell expansion on ECM substrates, the above steps were performed at two hours and 48 hours post-cell seeding. Cell number was calculated using a reference standard curve of known cell number.

To assess HBEC attachment to candidate scaffold materials, cells were seeded in 96-well ultra-low attachment plates (Sigma-Aldrich, Dorset, U.K.; #CLS3471) containing the scaffolds. After 1 hour, the wells were washed four times with PBS to remove unbound cells and trypsin was applied to detach the adhered HBECs. Scaffolds were assessed by light microscopy to ensure detachment of adhered cells, collected and plated into a separate 96-well plate. A DNA dye (CyQUANT® GR, Life Technologies, Hemel Hempstead, U.K.) was used to compare cell attachment as described above. To assess the viability of epithelial cells seeded on decellularised dermis or tracheal scaffolds, a cell viability assay (alamarBlue, Life Technologies, Hemel Hempstead, U.K.) was used. Cells were seeded onto decellularised scaffolds at the bottom of a 96-well plate and incubated for 1 hour. Scaffolds were then washed with PBS, transferred to a new well and alamarBlue cell viability reagent was added in medium (1:10 ratio). After two hours, fluorescence (excitation 560 nm, emission 590 nm) was recorded and a final value calculated by subtracting values generated from negative control wells containing scaffolds but no epithelial cells.

2.5 Air-liquid interface culture and trans-epithelial electrical resistance

For standard air-liquid interface cultures on PET membranes, 1×10^6 HBECs were seeded in 0.4 μ M, 12 mm PET membrane transwells (Corning, Flintshire, U.K) and incubated at 37°C with 5% CO² supply [33]. Air-liquid interface (ALI) conditions, in which medium was removed from the apical well, were applied after 2 days. Cells were fed with ALI medium containing BEGM and DMEM at a ratio of 1:1 supplemented with 100 μ g/ml streptomycin and 100 Units/ml penicillin as well as freshly prepared retinoic acid equivalent to 100 nM final concentration. Trans-epithelial electrical resistance (TEER) was measured using an EVOM2 resistance meter and Endohm chamber (World Precision Instruments, U.S.A.). Both the apical and basolateral sides of cultures were filled with BEBM (Lonza, Slough, U.K.) and three replicate readings were taken for each well, allowing values to stabilize for 5-10 seconds each time.

2.6 Production of candidate scaffolds

To produce decellularised dermis, glycerol-preserved cadaveric human skin was sourced from the Euro Tissue Bank (Beverwijk, The Netherlands). Decellularization was carried out by washing three times in PBS over a 48-hour period and incubating the skin for 24 hours at 37°C in 1M NaCl as described by Deshpande et al [34]. The epidermis was then peeled off and the decellularised dermis either used directly or stored for up to one month at 4°C in DMEM. POSS-PCU was provided by the Department of Nanotechnology (University College London, U.K.), where it was produced using previously published methods [35]. Biomimetic collagen I scaffolds were prepared using custom-made absorbers following the manufacturer's protocol (RAFTTM, Lonza, Slough, U.K.). In brief, rat tail collagen I (First Link, Wolverhampton, U.K.), DMEM and MEM 10x (Thermo Fisher, Hemel Hempstead, U.K.), were combined in an 8:1:1 ratio, neutralised with 5M sodium hydroxide and incubated for 15 minutes to allow the solution to gel. Absorbers were placed onto the surface of each well for 15 minutes to dehydrate the gel and produce translucent collagen scaffolds [36].

Tracheae for decellularization were obtained from terminated New Zealand white rabbits (Envigo; Huntingdon, U.K.). Tracheae were stripped of all fascia and washed three times in PBS containing penicillin and streptomycin (1X; Gibco, Hemel Hempstead, U.K.) over 24 hours. Tracheae were decellularised using a two-cycle enzymatic detergent decellularization protocol previously published by Conconi et al. [37]. Briefly, tracheae were then placed in a 4% sodium deoxycholate (Sigma #S1827) solution for 4 hours with luminal agitation at room temperature. Tracheae were then washed in PBS for 30 minutes with luminal agitation at room temperature. Following this, tracheae were placed in a solution of 1 L of 1M NaCl containing 22.5 mg deoxyribonuclease I (Sigma #9003989) for three hours. Tracheae were then washed in Milli-Q water overnight at 40°C before repeating the above steps for the second cycle. After completing the second cycle, the tracheae were washed in Milli-Q water for three days at 40°C with the water being changed daily. Finally, tracheae were sterilised using ionizing irradiation.

2.7 Assembly of tissue-engineered respiratory mucosa

Decellularised dermis prepared using the method described above was clipped into CellCrowns™ (Sigma-Aldrich, Dorset, U.K.) fitting either 24-well plates for *in vitro* experiments and 12-well plates for *in vivo* experiments. HBECs and lung fibroblasts were co-seeded onto the dermis at densities of $1 \times 10^6/\text{cm}^2$ and $1 \times 10^4/\text{cm}^2$, respectively in 1 ml epithelial cell culture medium. Scaffolds were maintained submerged in epithelial cell culture medium containing 5 μM Y-27632 [23] and medium was changed daily. After 7 days, the CellCrowns™ were removed from the plates and CellCrowns™, containing the scaffolds, were placed into PET membrane transwell inserts (0.4 μm ; Corning, Flintshire, U.K.) for air-liquid interface (ALI) culture [38]. Medium was changed to ALI medium and was changed three times per week. Beyond two weeks at ALI, scaffolds were cleared of mucus by gentle aspiration every other day. After three to four weeks at ALI, high-speed videos were taken using an inverted microscope system (Nikon Ti-U 1000, Japan) with a 40X objective and a 1.5X magnifier to assessing ciliary beat frequency and pattern. The digital camera (Motion Pro 4x; IDT, Pasadena, U.S.A.) was set for 512 x 512 regions of interest and recorded at 250 frames/second for ciliary beat frequency and 500 frames/second for ciliary beat pattern. Ciliary beat frequency was analysed using CiliaFA [39] and also calculated by determining the number of frames required for five full sweeps of the ciliary tip. Ciliary dyskinesia was assessed by defining the uncoordinated ciliary beat pattern across a strip of ciliated epithelium. The dyskinesia score is calculated by the percentage of dyskinetic ciliated cells against the total number of ciliated cells.

2.8 Histology

Samples for histology were washed twice in PBS and fixed in 10% neutral buffered formalin (NBF) for 1 hour. Epithelialised scaffolds were fixed for 30 minutes and embedded in HistoGel (Thermo Fisher, Hemel Hempstead, UK) to protect the top layer of epithelium from detachment during processing. After fixation, samples were stored in 70% ethanol at 4°C before dehydration using an automated system (Leica TP 1050). Formalin-fixed, paraffin-embedded samples were sectioned at 5 μm thickness. Haematoxylin and eosin (H&E) staining was performed using an automated staining machine (DRS-601, Sakura). Samples

were mounted using a Sakura Coveraid automatic cover-slipping machine with Tissue-Tek cover slip film (Bayer Diagnostics, Reading, U.K.).

2.9 Immunofluorescence

Slides were dewaxed for immunofluorescence using an automated process (DRS-601, Sakura). Slides were washed in distilled water and blocked with PBS containing 10% FBS (block solution) for 2 hours. Primary antibodies were applied in block solution and incubated at 4°C overnight. Following three PBS washes, species-specific secondary antibodies (AlexaFluor, Thermo Fisher, Hemel Hempstead, U.K.) were applied in block solution at a concentration of 1:200 for one hour at room temperature. Slides were washed a further two times in PBS and stained with DAPI (5 mg/ml stock, 1:10,000 in PBS; Life Technologies; Hemel Hempstead, U.K.) for 10 minutes. Following a final PBS wash step, slides were coverslipped using ImmuMount (GeneTex, Irvine, U.S.A.) and sealed with nail varnish. Slides were imaged on a Zeiss 700 confocal microscope.

2.10 Immunocytochemistry

For top-down images, scaffolds were washed twice in PBS and fixed in 10% NBF for 30 minutes. Scaffolds were washed twice in PBS for five minutes each and incubated in block solution for 2 hours at room temperature. Primary antibodies were applied in block solution and incubated overnight at 4°C. After three 5-minute washes in PBS, species-specific secondary antibodies were applied at 1:200 in block solution at room temperature for 1 hour. Scaffolds were washed twice in PBS and DAPI (5 mg/ml stock, 1:10,000 in PBS; Life Technologies, Hemel Hempstead, U.K.) was applied. Samples were washed in PBS before imaging, either *in situ* or following removal from wells and coverslipping using ImmuMount (GeneTex, Irvine, U.S.A), using a Zeiss 700 confocal microscope.

To assess cell viability on the surface of scaffolds, a live/dead cell viability assay was used (Thermo Fisher, Hemel Hempstead, U.K.). 3.4 µl/ml calcein AM and 4 µl/ml ethidium homodimer-1 were mixed with medium and added to scaffolds following cell adhesion. The scaffolds were incubated for 45 minutes and mounted on glass slides for top-down whole-mount imaging on a Zeiss 700 confocal microscope.

2.11 Scanning electron microscopy

Scaffolds were fixed in 2.5% glutaraldehyde then washed with 0.1M phosphate buffer (pH 7.4) and distilled water. Scaffolds were dehydrated in a graded ethanol-water series to 100% ethanol and dried using carbon dioxide. Using sticky carbon tabs, the scaffolds were mounted on aluminium stubs and coated with a 2 nm layer of Au/Pd using a Gatan ion beam coater. The scaffold surface was imaged using a Jeol 7401 field emission scanning electron microscope. Ciliary length measurements were made at 20,000 times screen magnification by electron microscopy.

2.12 Chick chorioallantoic membrane (CAM) assay

Fertilised Bovan brown chicken eggs were supplied by Henry Stewart & Co (Norfolk, U.K.). In brief, at embryonic day (E) 3, eggs were cleaned with 70% ethanol and 3-4 ml of albumin was removed via needle aspiration at the apex of the shell. A 2 x 2 cm window was excised from the upper aspect of the shell to reveal the embryo and CAM blood vessels. Windows were sealed with adhesive tape and the eggs incubated for a further five days. At E8 the CAM was re-exposed and minutia pins used to disrupt several of the smaller blood vessels. 2 mm² sections of test scaffold were placed gently onto the CAM with the epithelial side upwards and excess blood drained. The window was re-sealed and the eggs incubated until E10. Following imaging with a Leica Zoom 2000 stereomicroscope with camera adaptor (MagnifiTM, Arcturus Labs, Kansas, U.S.A.), grafted CAMs were harvested and fixed overnight in 4% PFA. Tissue-engineered respiratory mucosa bathed in vascular endothelial growth factor (VEGF; Sigma-Aldrich, Dorset, U.K) was used as a positive control, a section of sterile plastic casing as a negative control and acellular decellularised dermis to examine the effect of including human cells. Comparison of epithelialised scaffolds was performed in two separate experiments involving at least six eggs in each experimental group. Comparison of acellular dermis was performed in one experiment with at least six eggs in each experimental group.

2.13 Engraftment of tissue-engineered respiratory mucosa on decellularised trachea in rabbits

Male New Zealand white rabbits age 9-10 weeks old weighed 2.5-3 kg on arrival. General anaesthesia was induced using 0.5 ml/kg ketamine intramuscularly and 0.2 ml/kg of xylazine and maintained with sevoflurane. All drugs were supplied by the National Veterinary Services (Stoke-on-Trent, U.K.). 5 cm sections of decellularised trachea were pre-implanted in a lateral thoracic muscle flap raised from the chest and tunnelled under the cervical skin into the neck in order to create a vascularised scaffold on which to graft engineered mucosa. A Foley catheter (size 16F) was inserted through the lumen, cut to size and sutured in place at either end with 4.0 Prolene sutures (Ethicon, Berkshire, U.K.) to act as a stent and prevent scaffold collapse. The trachea was then wrapped in the muscle flap and secured within the neck using a 3.0 silk suture (Ethicon, Berkshire, U.K.) and the wound closed. Concurrently, HBECs and fibroblasts were seeded onto decellularised dermis to fabricate the respiratory mucosal grafts as described above. After four weeks of pre-vascularisation, the cervical incision was re-opened and the scaffold identified in the muscle wrap. The trachealis was identified and opened by incision directly through muscle. The tracheal rings were visually assessed for evidence of vascularization, structural integrity and signs of infection before the newly fabricated tissue-engineered mucosa was grafted onto the luminal surface. A Foley catheter stent or alginate-based dressing (Kaltostat, Convatec, Flintshire, U.K.) was placed over the graft to protect the epithelial layer and a stent placed to prevent displacement of the graft within the muscle flap. The tracheal and cervical incisions were closed and animals were terminated either 24 hours or 1-week post-transplantation.

Immunosuppression of rabbits was required to reduce the probability of acute rejection of the transplanted human cells [40]. Tacrolimus (Cambridge Bioscience, Cambridge, U.K.) was administered subcutaneously at 0.3 mg/kg for three consecutive days, beginning 7 days before the day of surgery and then on alternate days until the end of the experiment. Dexamethasone was co-administered in later experiments at a dose of 2 mg/kg three times daily beginning 24 hours before grafting (Supplementary Table 2). Blood samples were

taken on the day of graft implantation and sent to the Royal Veterinary College (Hatfield, U.K.) for quantification of white cells, lymphocytes and neutrophils.

2.14 Engraftment of tissue-engineered respiratory mucosa in immunosuppressed mice

NOD SCID mice (Envigo, Huntingdon, U. K.) were anaesthetised and maintained with sevoflurane (National Veterinary Services, Stoke-on-Trent, U.K.). A 5 cm transverse incision was made on the back of the mouse and a subcutaneous pocket made. Decellularised dermis-based respiratory mucosal sheets were then laid onto the surface of the muscle and a layer of silicone dressing (Mepitel, Mölnlycke Healthcare, Oldham, U. K.) was laid over the top to prevent shear. After 7 days, the mice were sacrificed and the wound was reopened and the graft and underlying mouse muscle explanted for histological processing.

2.14 Statistics

Statistical analyses were performed using GraphPad Prism (GraphPad Software, CA, U.S.A.) as indicated in figure legends. Statistical significance was assigned when $p < 0.05$ and p values are reported rounded to three decimal places. All *in vitro* experiments were carried out in at least technical triplicates unless otherwise indicated and repeated as described in legends. Data are presented as mean values \pm SEM.

3. Results

3.1 ECM-dependent human airway epithelial cell attachment, proliferation and differentiation.

We first assessed the attachment of cultured primary human bronchial basal epithelial stem/progenitor cells (HBECs) [41-43] to extracellular matrix (ECM) proteins found in the native human airway (Supplementary Figure 1), finding that collagen IV-coated wells performed best with significantly more cells attaching than to laminin- or vitronectin-coated wells (Figure 1A). To determine the integrin mediators of this ECM attachment, we performed attachment assays to ECM proteins in the presence of blocking antibodies against various integrin α subunits. Blocking integrin $\alpha 2$ led to significantly lower levels of epithelial cell attachment to collagen IV but blocking integrin subunits $\alpha 3$, αV , $\alpha 5$ and $\alpha 6$ had no effect (Figure 1B). Other than a trend towards decreased attachment to Collagen I in the presence of an integrin $\alpha 2$ blocking antibody, attachment to collagen I or fibronectin was variable between experimental replicates but unaffected by the presence of these blocking antibodies (Figure 1B). To determine the effect of ECM proteins on early epithelial proliferation after attachment, HBECs were seeded onto wells coated with matrix proteins that supported epithelial cell attachment and cell number was determined after 2 and 48 hours. No significant change in HBEC expansion was seen when these proteins were combined with laminin to more closely mimic the native ECM (Figure 1C) but blocking antibodies against integrin $\beta 1$ significantly curtailed early HBEC expansion on collagen IV- and laminin-coated wells (Figure 1D). No inhibition of proliferation was seen in the presence of blocking antibodies against integrins $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$ or $\alpha 9\beta 1$ (Figure 1D).

To determine the effect of integrin blockade on HBEC differentiation, blocking antibodies against integrins $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$ or $\beta 1$ were added to air-liquid interface cultures at the point of air exposure (and at medium changes thereafter). Epithelial integrity increases during the establishment of a polarised, differentiated epithelial layer through the formation of tight junctions. To examine this process, trans-epithelial electrical resistance (TEER) was measured on days 0, 7, 14, 21, and 28 after air exposure. Epithelial integrity was disrupted by inhibition of integrin $\alpha 1$ and integrin $\alpha 3$, both of which are expressed in native

human airway epithelium (Supplementary Figure 2), indicating a failure of cells to differentiate appropriately. TEER values were unaffected in the presence of blocking antibodies against integrins $\alpha 2$, $\alpha 5$, $\alpha 6$ or $\beta 1$ (Figure 1E) and epithelial differentiation towards multiciliated and mucosecretory cells was not obviously altered by these blocking antibodies as evidenced by high-speed video microscopy at day 21 and immunofluorescence staining of independent cultures incubated with blocking antibodies (Supplementary Figure 3). In wells treated with $\alpha 1$ and $\alpha 3$ integrin blocking antibodies, no evidence of ciliation was detected and cell adherence to the PET membrane had largely failed, consistent with TEER value readings (Figure 1E).

3.2 ECM proteins and attachment in tissue-engineered scaffolds

To move our experiments towards airway mucosal regeneration, we investigated attachment of epithelial cells to three relevant scaffolds of varying ECM complexity (Figure 2A): a complex biological scaffold which retained multiple ECM proteins including collagen IV and laminin after decellularization (decellularised dermis; Figure 2B); a simpler collagen I matrix; and a synthetic POSS-PCU scaffold which does not contain ECM proteins. Epithelial cell attachment to the complex decellularised scaffold was greater than to collagen I and POSS-PCU scaffolds (Figure 2C) suggesting that the functionality of the ECM is retained despite possible alterations in its structure caused by decellularization. We further investigated how decellularised dermis performed compared to decellularised trachea. After live/dead staining, abundant live cells were seen on the surface of decellularised dermis scaffolds, whilst only sparse coverage was seen on the decellularised tracheal scaffold (Figure 2D). Correspondingly, in alamarBlue cell viability assays, there was a trend towards higher fluorescence intensity two hours following cell seeding onto decellularised dermis scaffolds compared to decellularised tracheal scaffolds (Figure 2E); this indicated better adherence and/or cell survival on decellularised dermis, consistent with the two decellularization protocols preserving matrix to different extents (Supplementary Figure 4). Similar to in experiments on collagen IV (Figure 1B), epithelial cell attachment to decellularised dermis was lower following blockade of integrin $\alpha 2$ (Figure 2F).

3.3 Culture of tissue-engineered respiratory mucosa

Based on these findings, we investigated the use of decellularised dermis as a substrate for respiratory mucosal regeneration. HBECs and primary human lung fibroblasts [44, 45] were cultured on decellularised dermis at an air-liquid interface for three weeks to induce differentiation of basal cells to mature epithelium containing mucosecretory and ciliated cells [38], as is seen in the native human airway (Figure 3A). Consistent with previous observations using this scaffold material [46], mucus was observed on the surface of the scaffold after two weeks and by three weeks ciliary beat could be observed by top-down, high-speed digital video (Supplementary Video). Haematoxylin and eosin (H&E) staining of sections taken three weeks post-ALI demonstrated an intact epithelial layer across the extent of the scaffold with ciliated cells and mucus-containing secretory cells. The fate of fibroblasts remained uncertain as, although fibroblasts had not convincingly repopulated the lamina propria (Figure 3B), very rare cells with a fibroblastic morphology were observed by immunofluorescence (Supplementary Figure 5). The height of the pseudostratified layer was variable along the length of the scaffold but immunofluorescence staining demonstrated the expression of keratin, the mucin MUC5AC, indicating the presence of mucosecretory cells, acetylated tubulin (ACT) in cilia and the basement membrane ECM proteins collagen IV and laminin (Figure 3C). Expression of integrin $\alpha 2$, identified earlier as an important mediator of epithelial attachment (Figure 1B), was also seen throughout the differentiated epithelium (Figure 3C). Top-down immunofluorescence confirmed the presence of ACT-positive ciliated cells and MUC5AC-positive mucosecretory cells across the surface of the scaffold (Figure 3D). Electron microscopy of the mucosal sheets further confirmed the presence of ciliated cells on the apical surface of the dermis-based scaffold (Figure 3E). Ciliary length measurements were made on between 7 to 10 cilia per cell from six different ciliated cells from five separate strips of epithelium. Cilia from three of the cells were of normal length (5.5 μm , SD 0.3; 5.6 μm , SD 0.3; 5.7 μm , SD 0.4) and cilia from three cells were slightly shorter (5.4 μm , SD 0.3; 5.0 μm , SD 0.2; 5.0 μm , SD 0.3) suggesting ciliogenesis might be on-going or incomplete in some cells. Importantly, analysis of ciliary beat frequency showed that it was comparable to that seen using a standard PET ALI culture substrate (Figure 3F). Finally, the ciliary dyskinesia score of cells on decellularised dermis scaffolds was within the anticipated range for ciliated ALI cultures (Figure 3F).

3.4 Grafting of tissue-engineered respiratory mucosa onto chick chorioallantoic membrane

Having determined that the decellularised dermis matrix was capable of supporting a differentiated respiratory epithelium *in vitro*, we next assessed short-term engraftment of decellularised dermis scaffolds seeded with HBECs and lung fibroblasts onto a vascularised surface in a chick chorioallantoic membrane (CAM) assay (Figure 4A). The CAM assay has the advantages of immune naivety, rapid neo-vascularisation and exposure of engrafted tissue to an air interface. 48 hours after grafting, top-down digital photography showed small blood vessels penetrating the scaffold indicating neo-vascularization (Figure 4B), in contrast to plastic negative controls, which showed no such vascularization (Figure 4B). Sections of acellular decellularised dermis also demonstrated neo-vascularisation (Figure 4B) indicating that this process was not dependent on the inclusion of transplanted cells. H&E sections of the engineered respiratory mucosa at this time-point demonstrated preservation of the epithelium (Figure 4C). Immunofluorescence staining confirmed their human (STEM121; Figure 4D) and epithelial (pan-keratin; Figure 4D) origin and showed rare cells expressing the mucosecretory cell-associated MUC5AC (Figure 4D). However, an absence of ACT+ cilia suggested epithelial remodelling occurred in the *in vivo* environment.

3.5 Grafting of tissue-engineered respiratory mucosa *in vivo*.

Finally, we investigated whether we could apply these decellularised dermis-based respiratory mucosal sheets to a pre-vascularised, decellularised trachea in an *in vivo* rabbit model [47] (Figure 5; Supplementary Table 2). If successful mucosal regeneration were achieved in this manner, the construct could be rotated on its vascular pedicle to replace a section of damaged trachea, providing a re-mucosalised and vascularised section of airway at the point of transplantation. Segments of decellularised rabbit trachea, 5 cm in length, were implanted into muscle flaps in the necks of New Zealand White rabbits. We allowed four weeks for scaffold vascularization and initiated immunosuppression before surgical grafting. To monitor immunosuppression, venous blood was sampled on the day of mucosal grafting. In our initial experiment, immunosuppression consisted of tacrolimus only and

lymphocyte counts were within normal range (Figure 5B). Grafts on tracheae after 7 days had integrated macroscopically (Figure 6A) but did not show a preserved epithelial layer (2/2; Figure 6B). An intense inflammatory reaction was observed and subsequently the immunosuppressive regime was modified to include dexamethasone. The combination of dexamethasone and tacrolimus caused lymphopenia (Figure 5C) and led to a less intense inflammatory reaction within the implanted dermis layer at 7 days (Figure 6C). Very sparse areas of keratin-positive epithelial cells could be found in these rabbits (2/3; Figure 6C). To counteract the possibility of mechanical shear damaging the engrafted epithelial layer, we finally implanted decellularised dermis-based airway mucosal sheets with a protective layer of soft alginate dressing in place of a silicone stent. 24 hours after engraftment, the epithelial layer was preserved (1/2; Figure 6D) and demonstrated appropriate expression of markers associated with mucosecretory and ciliated differentiation (Figure 6D). However, vascular channels did not appear to penetrate to the surface and the mucosal layer was detached from the underlying stromal layer following processing and sectioning, possibly indicating weak integration (Figure 6D). After five days, the bioengineered graft was well-integrated (Figure 6E) with some evidence of re-vascularization (Figure 6E) but the epithelial layer had been lost (2/2; Figure 6E). Blood cells and diffuse inflammatory cell infiltration were observed within the decellularised dermis at this time point (Figure 6E).

Due to the possibility of incomplete immunosuppression leading to these results in rabbits, we further investigated *in vivo* integration in NOD SCID mice. Decellularised dermis-based airway mucosal sheets were grafted onto the back muscle with a silicone dressing overlaid to protect the epithelial layer (Figure 7A). After seven days, the grafts were retrieved and histology demonstrated retention of epithelial cells (3/3; Figure 7B), although coverage of scaffolds varied among replicates. Retained epithelial cells expressed basal cell-associated keratin 5 (Figure 7C) suggesting that remodelling had occurred, perhaps because of the lack of polarity signals available to the epithelial cells in this experimental model.

4. Discussion

Collagen IV and laminin anchor multiple epithelia – including airway, skin [21], small intestine [48], and cornea [49] – to the underlying stroma *in vivo* [50]. Supporting their active role in epithelial remodelling, collagen IV expression is reduced in partial thickness dermal wounds as epithelial cells migrate [51] and laminin guides respiratory [52], epidermal [53], and corneal [49] epithelial cell migration. Our study suggests that collagen IV is a key mediator of airway epithelial cell attachment and proliferation on bioengineered scaffolds and that integrins $\alpha 2$ and $\beta 1$ are mediators of these processes, consistent with the known role of integrin $\alpha 2/\beta 1$ as a receptor for both ECM proteins [54].

We believe that the presence (and native presentation of) collagen IV and laminin in bioengineered airway constructs and/or manipulation of integrin $\alpha 2$ -mediated adhesion, may improve epithelial cell attachment efficiency and permit lower cell seeding densities to achieve scaffold coverage [22]. The benefits of this are manifold as overall cell culture time, the number of population doublings experienced by cells in culture and manufacturing costs could be reduced. Although this could take many forms, we identified decellularised dermis as a promising scaffold material for airway regeneration based on its retention of collagen IV and laminin. Compared to collagen I-based scaffolds, synthetic and decellularised tracheal scaffolds, primary human airway epithelial cells adhered well to decellularised dermis *in vitro*. It is likely that the more extensive decellularization process required for whole trachea disrupts the collagen IV-rich basement membrane, thus limiting epithelial adherence. Further research might aim to deploy decellularization methods that preserve key matrix proteins on the luminal surface or to develop methods to replace these when they are lost through decellularization.

We generated bioengineered respiratory mucosa with beating cilia and mucus secretion by culturing primary human airway epithelial cells on decellularised dermis at an air-liquid interface *in vitro*, consistent with a previous report [46]. We found that the length of cilia was comparable to those in native airways and ciliary beat frequency and dyskinesia scores were comparable to healthy human nasal cilia assessed by high-speed video microscopy

either directly [55] or after culture at ALI [22]. Such scaffolds are surgically manipulable and could be a means to restore mucociliary coverage in respiratory mucosa that has been lost or damaged. To this end, we examined the performance of the scaffolds in CAM assays, finding that the epithelium was retained two days post-engraftment but that cilia were no longer present. The fate of these cells is unknown but our data suggest that the CAM assay might act as an accessible short-term epithelial remodelling assay in the window before 15 days when immune reactions to implanted material become limiting [56].

In a second *in vivo* study, we used a two-stage transplantation procedure in rabbits to test decellularised dermis-based bioengineered respiratory mucosal graft. The combination of tacrolimus and dexamethasone resulted in a lymphopenia with preserved neutrophil count, which limits immune-mediated remodelling of the scaffold whilst preventing immunosuppression-associated opportunistic infections. We saw engraftment with short-term epithelial retention but were unable to demonstrate longer-term retention of the differentiated epithelial layer. Delayed re-vascularization may explain this observation as penetration of new vascular channels approximately 1 mm into the dermis scaffold is likely to take several days, during which the epithelium relies on diffusion of nutrients from the underlying graft bed [57]. Our teams experience in producing 3D tissue-engineered skin and oral mucosa, including clinical engraftment, shows that grafts placed on poorly vascularised beds often fail to survive [58]. The inclusion of endothelial cells and angiogenic growth factors have been shown to shorten the time for neo-vascularisation in synthetic and biological scaffolds and may be a potential strategy to more rapidly restore a blood supply to the epithelial layer [59, 60]. An alternative strategy would be to differentiate respiratory epithelial cells on a thinner scaffold that enables the delivery of nutrients and oxygen to the epithelial layer from the underlying capillaries by diffusion. Diffusion is limited to a distance of approximately 150 μm from the capillary and therefore the scaffold would need to be of similar thickness [61-63]. Whether taking a 150 μm shave of dermis to include the collagen- and laminin-rich basement membrane and the underlying lamina propria would shorten the time taken for revascularization and enhance epithelial survival is a clear target for future investigations.

The frequent loss of the epithelial layer in our *in vivo* experiments highlights the technical challenges of grafting a new mucosal layer into the airway. In order to prevent displacement of a mucosal graft, a stent is deployed that retains the graft on the graft bed whilst maintaining airway patency. However, the dynamic properties of the airway inevitably result in mechanical shear of the stent against the engrafted epithelial layer that is likely to result in further epithelial loss [64]. Future developments might adopt custom-made stents with a supportive soft outer layer containing a nutrient supply that could enable engraftment and maintain airway patency while simultaneously supporting and protecting the epithelium. Alternatively, developing pro-angiogenic adhesives might allow grafting of the tissue-engineered respiratory mucosa onto the trachea without the need for a stent.

Conclusions

Tissue-engineered whole organ transplants in the airways, but also in the bladder and bowel, are currently limited by the inability to regenerate a functioning mucosal lining. Our findings indicate that the extracellular matrix proteins collagen IV and laminin are important for respiratory epithelial adherence and expansion *in vitro*, and informed the development of bioengineered scaffolds that contain a differentiated respiratory mucosal layer on a decellularised dermis scaffold. The delivery of these scaffolds is feasible *in vivo* but, while epithelium was retained in the short-term, further research is required to improve long-term *in vivo* survival. Our work emphasises the importance of cell delivery methods in airway cell therapy research and suggests the merit of pre-clinical studies that directly compare differentiated cell transplantation to basal cell-only methods: although the success of the former would restore tissue function rapidly, undifferentiated basal stem cells might be more robust for transplantation.

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Figure Legends

Figure 1: The role of extracellular matrix proteins in human bronchial epithelial cell behaviour.

(a) 5×10^4 HBECs were seeded on non-adherent tissue-culture plastic coated with ECM proteins and attachment was determined at 30 minutes using CYQUANT GR with background fluorescence subtracted. Attachment to collagen IV was greater than to laminin (two-way ANOVA with Holm-Sidak's test for multiple comparisons; * $p = 0.018$) and vitronectin-coated wells (* $p = 0.018$). The experiment was repeated three times in technical triplicate, including using two independent donor cell cultures. Data are presented on a log scale. **(b)** HBECs were incubated in blocking antibodies against integrins $\alpha 2$, $\alpha 3$, αV , $\alpha 5$ and $\alpha 6$ for 20 minutes before 2×10^4 cells were seeded onto non-adherent tissue culture plastic wells coated with either collagen IV, collagen I or fibronectin. Fluorescence with background subtracted was measured using CYQUANT GR. Blocking integrin $\alpha 2$ led to significantly less HBEC attachment in the collagen IV- (one-way ANOVA with Holm-Sidak's test for multiple comparisons; ** $p = 0.002$) but not collagen I- ($p = 0.141$) or fibronectin-coated wells ($p = 0.881$) compared to control wells. The experiment was repeated three times in technical triplicate (indicated by black, grey and white colour), including using two independent donor cell cultures. **(c)** HBEC cell number was determined using CYQUANT GR and a standard curve of known cell number at 2 hours and 48 hours after seeding 1×10^4 HBECs onto non-adherent tissue culture plastic coated with ECM proteins. No statistical difference was found between the groups (one-way ANOVA with Holm-Sidak's test for multiple comparisons; $p > 0.25$ for all comparisons). The experiment was repeated three times in technical triplicate, including using two independent donor cell cultures. **(d)** Blocking antibodies were added to wells 2 hours after seeding 5×10^3 HBECs onto collagen IV- and laminin-coated wells. Fluorescence with background subtracted was recorded at 48 hours using CYQUANT GR and compared to a control well without blocking antibody. Significantly less epithelial cell expansion was observed compared to the control in the presence of integrin $\beta 1$ blocking antibody (one-way ANOVA with Holm-Sidak's test for multiple comparisons; ** $p = 0.006$). The experiment was repeated six times in technical triplicate, including using two independent donor cell cultures. **(e)** Integrin blocking antibodies were added to air-liquid interface cultures at the point of air-liquid interface creation and at every feed thereafter. Trans-epithelial resistance, as a measure of epithelial integrity, was

recorded at five timepoints after air-lift (1 = 0 days, 2 = 7 days, 3 = 14 days, 4 = 21 days and 5 = 28 days). Blockade of $\alpha 1$ and $\alpha 3$ integrins inhibited trans-epithelial resistance (two-way ANOVA with Holm-Sidak's test for multiple comparisons; **** $p < 0.001$). The experiment was performed by making three repeated measurements from duplicate wells from two independent donor cell cultures (i.e. 4 independent wells at each timepoint). Data are presented on a log scale.

Figure 2: Attachment of HBECs to tissue-engineered scaffolds.

(a) Digital photographs of the three scaffolds tested: decellularised dermis (biological), POSS-PCU (synthetic), dehydrated collagen I (biomimetic). **(b)** Whole-mount top-down immunofluorescence staining of the basement membrane proteins collagen IV and pan-laminin in decellularised dermis indicates that these matrix proteins are preserved following decellularization. **(c)** 5×10^4 human bronchial epithelial cells (HBECs) were seeded into each scaffold-containing well. After one hour, the wells were washed and trypsin applied to detach the HBECs that had adhered to the scaffolds. In a separate 96-well plate, CyQUANT GR was used to measure fluorescence of attached cells with values from negative control scaffolds subtracted. Fluorescence was significantly greater in the decellularised dermis group compared with collagen I (one-way ANOVA with Holm-Sidak's test for multiple comparisons; * $p = 0.029$) and POSS-PCU scaffolds (* $p = 0.015$). The experiment was repeated three times in technical triplicate, including using two independent donor cell cultures. **(d)** Top-down whole-mount confocal images showing live/dead staining of decellularised dermis and decellularised tracheal scaffolds two hours post-epithelial cell seeding. **(e)** Cell viability of 1.5×10^5 epithelial cells seeded on decellularised dermis or decellularised trachea was compared using an alamarBlue cell viability assay. Fluorescence intensity was measured at two hours following adherence and values from negative control scaffolds were subtracted. Fluorescence intensity was greater in the dermis group compared to the tracheal group but the finding did not reach statistical significance (paired t-test, two-tailed; $p = 0.062$). The experiment was repeated three times in technical triplicate, including using two independent donor cell cultures. **(f)** 1.5×10^5 HBECs were seeded onto decellularised dermis scaffolds with and without pre-treatment with an integrin $\alpha 2$ blocking antibody. Cells were washed at one hour and an alamarBlue cell viability assay was performed with background fluorescence subtracted. Blocking integrin

$\alpha 2$ resulted in significantly less attachment of cells to decellularised dermis (paired t-test; ** $p=0.006$). The experiment was performed four times in technical triplicate, including using two independent donor cell cultures. All scale bars = 50 μm .

Figure 3: HBEC differentiation on decellularised dermis scaffolds at air-liquid interface.

(a) Haematoxylin and eosin (H&E) stained section of adult human tracheal mucosa. A pseudostratified layer of ciliated epithelium with mucus-secreting goblet cells can be seen overlying the basement membrane. The lamina propria lies underneath the basement membrane. **(b)** H&E stained section of the respiratory mucosa cultured on decellularised dermis; a differentiated epithelial layer is seen along the length of the scaffold and cells with the morphological appearance of mucus-secreting goblet cells and ciliated cells are seen. **(c)** Immunofluorescence staining of sections of bioengineered respiratory mucosa. The DAPI positive cell layer stained positively for the epithelial marker pan-keratin (green), the mucosecretory cell-associated mucin 5AC (MUC5AC; red), the ciliary protein acetylated tubulin (ACT; red), the basement membrane proteins collagen IV (yellow) and laminin (blue) and integrin $\alpha 2$ (red). **(d)** Top-down confocal microscopy for markers of differentiation on decellularised dermis-based respiratory mucosa demonstrated ACT (red) and MUC5AC (yellow) positivity. **(e)** Electron microscopy of decellularised dermis-based respiratory mucosa. Cilia can be seen on the surface of the scaffold. **(f)** High-speed digital video analysis of one donor HBEC culture differentiated on either standard PET ALI substrates (controls; $n = 5$ videos) or decellularised dermis scaffolds ($n = 10$ videos). Analysis of ciliary beat frequency (Hz) and dyskinesia (%) demonstrated that on decellularised dermis, cilia exhibited a comparable beat frequency to the control substrate. The dyskinesia score was slightly higher than in controls but remained within normal range. Scale bars in (a)-(d) = 50 μm , in (e) = 10 μm .

Figure 4: Engraftment of decellularised dermis-based airway mucosal grafts in the chick chorioallantoic membrane assay.

(a) Timeline of the chick chorioallantoic membrane (CAM) assay. The egg is incubated, windowed at E3, and then the scaffold is grafted at E8 before harvest at E10. **(b)** Digital photographs of CAM assays after two days. **(c)** Haematoxylin & eosin (H&E) stained sections of the respiratory mucosal layer grafted onto CAM assay at E10. An epithelial layer (Epi) is

demonstrated on the surface of the dermis (De) overlying the CAM. Higher magnification of the epithelial layer demonstrates preservation of an epithelium but apparent loss of cilia.

(d) Immunofluorescence staining of sections of decellularised dermis-based respiratory mucosa. The DAPI positive cell layer stained positively for the epithelial marker pan-keratin (green), the human cell marker STEM121 (yellow) and rarely the mucosecretory cell marker MUC5AC (red). The CAM experiment involving epithelialised scaffolds was performed twice including six eggs in each experimental group on each occasion. The decellularised dermis CAM experiment was performed once with six eggs in each experimental group. All scale bars = 50 μm .

Figure 5: Timeline for engrafting decellularised dermis-based airway mucosal grafts onto a pre-vascularised segment of decellularised trachea in rabbits.

(a) Cadaveric rabbit trachea was decellularised using a series of enzymatic and detergent washes. Decellularised tracheae were pre-implanted in the lateral thoracic muscle of New Zealand White rabbits to promote vascularization. At this stage, HBECs and lung fibroblasts were seeded onto decellularised dermis and cultured at an air-liquid interface. After 4 weeks, the muscle and tracheal composite was opened and the trachea visually assessed for signs of vascularization, infection and for structural integrity ahead of grafting the cultured respiratory mucosal graft onto the luminal surface. In some rabbits, alginate dressing was placed over the mucosal scaffold to protect the epithelial cells and then stents were placed within the lumen to secure the graft in place. Immunosuppression began prior to transplantation of bioengineered mucosal grafts. **(b)** Immunosuppression efficacy with tacrolimus in rabbits (n=3; 0.3 mg/kg subcutaneously for three days and then on alternative days, blood sampled on day 7). **(c)** Immunosuppression efficacy with combined tacrolimus and dexamethasone in the rabbit model (n=3; 0.3 mg/kg tacrolimus subcutaneously for three days and then on alternative days, three doses of 2 mg/kg dexamethasone intramuscularly 24 hours prior to blood sampling on day 7).

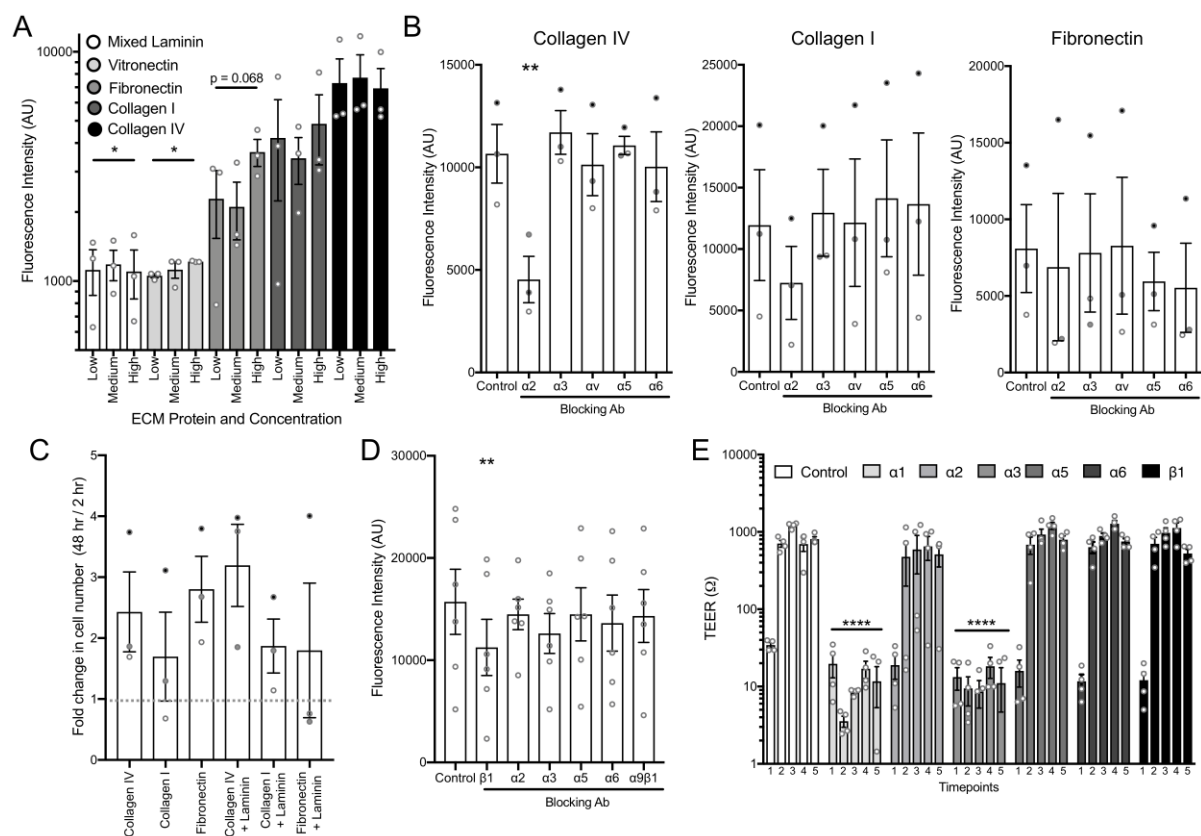
Figure 6: Engraftment of decellularised dermis-based airway mucosal grafts onto pre-vascularised decellularised tracheae in rabbits.

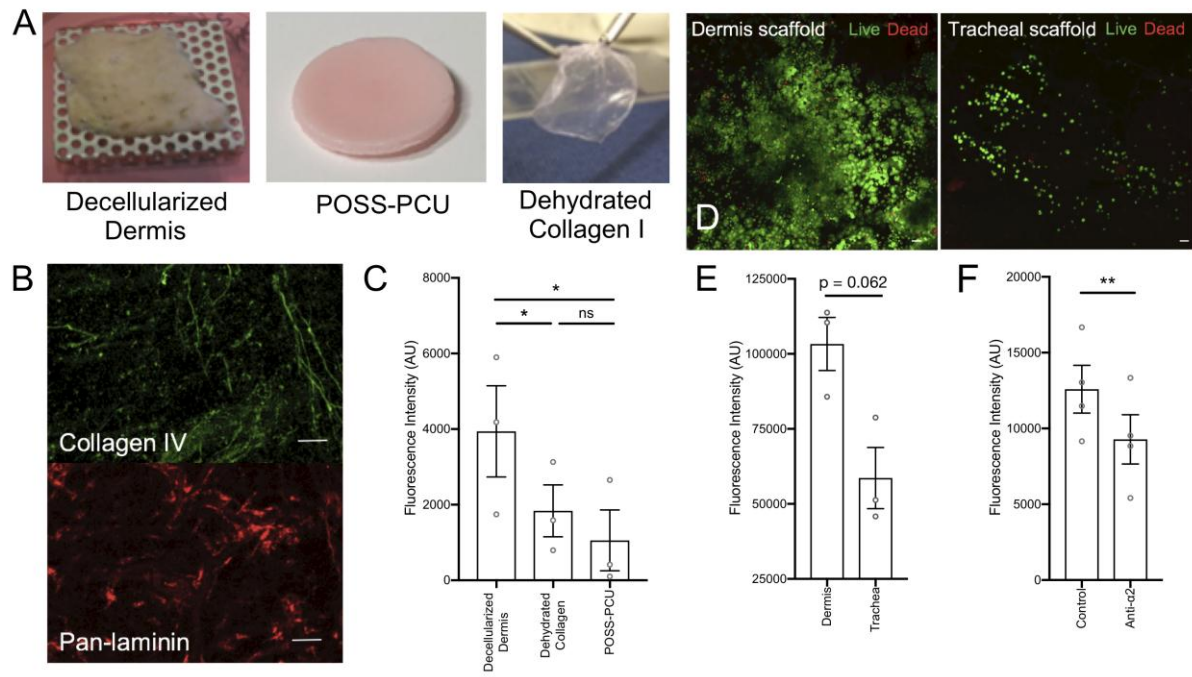
(a) Digital photograph of an explanted trachea seven days after grafting with bioengineered airway mucosa. The grafted scaffold can be seen on the luminal surface as a distinct layer

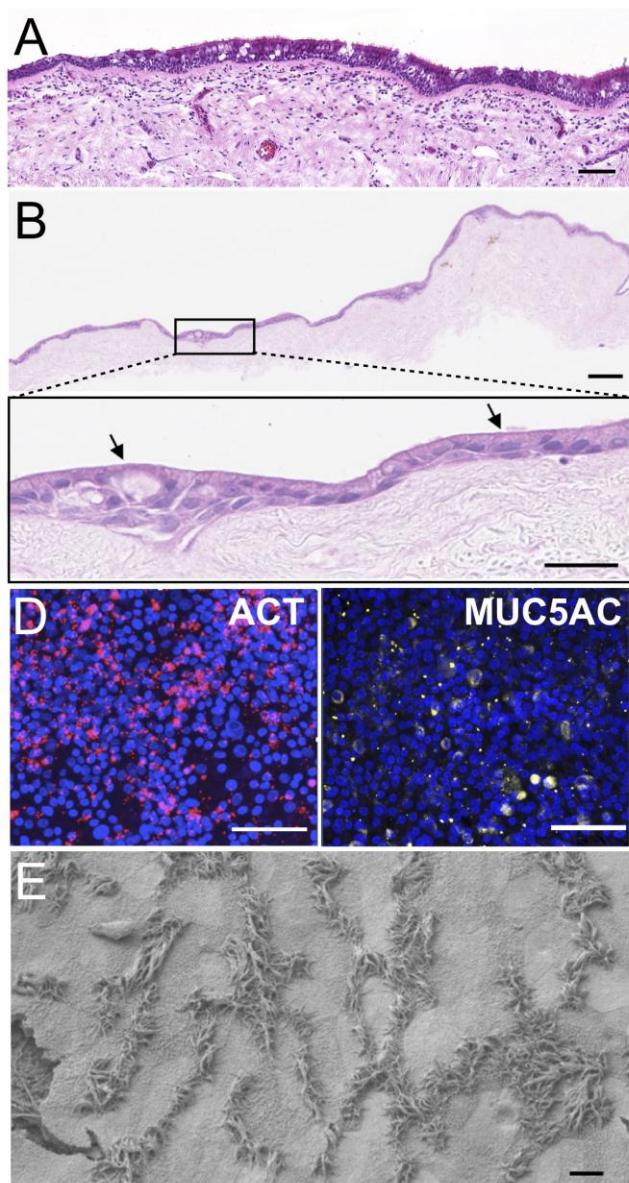
overlying the cartilage rings. **(b)** Haematoxylin & eosin (H&E) staining of explanted rabbit trachea 7 days after grafting TERM around a silicone stent with the use of tacrolimus immunosuppression. The grafted TERM can be seen on the luminal surface with an intense inflammatory infiltrate and loss of all epithelium (magnified section). Scale bars = 1 mm (left) and 250 μ m (right). **(c)** Rabbit trachea seven days after grafting TERM around a silicone stent with the use of tacrolimus and dexamethasone immunosuppression. The grafted TERM can be seen on the luminal surface with less inflammatory infiltrate and some overlying epithelium which was incomplete over large portions (magnified area). Scale bars = 1 mm (left) and 100 μ m (upper right). Sections were stained for keratin 5 (green) and DAPI (blue). Keratin 5+ cells were found over small areas of the trachea. Scale bar = 50 μ m (lower right). **(d)** H&E staining of an explanted rabbit trachea 24 hours after grafting with an alginate dressing and dual immunosuppression. An epithelial layer was observed on the surface of one of the grafts but the grafts had detached from the underlying trachea, perhaps during processing. Immunofluorescence staining for the human cell marker STEM121, the mucin MUC5AC and the ciliary protein acetylated tubulin (ACT) is shown. Scale bars = 50 μ m. **(e)** H&E staining of explanted trachea five days after grafting with an alginate dressing and dual immunosuppression. The epithelial layer had been lost from the decellularised dermis. The magnified image shows evidence of blood cells within the dermis layer (red arrows) and the presence of inflammatory cells (black arrows). Scale bars = 50 μ m.

Figure 7: Engraftment of decellularised dermis-based airway mucosal grafts in NOD SCID mice.

(a) A 5 cm transverse incision was made on the back of three NOD SCID mice. A subcutaneous pocket was developed and the decellularised dermis scaffolds – pre-seeded with differentiated human airway epithelial cells and lung fibroblasts – were implanted onto vascularised muscle and overlaid with a silicone-based dressing (*). **(b)** Hematoxylin & eosin (H&E) staining shows integrated scaffold after seven days. A continuous monolayer epithelium was seen suggesting that differentiated cells may have been lost. **(c)** Immunofluorescence staining confirmed that the monolayer was keratin 5-expressing (green). Slides were counterstained with DAPI (blue). Scale bars = 50 μ m.

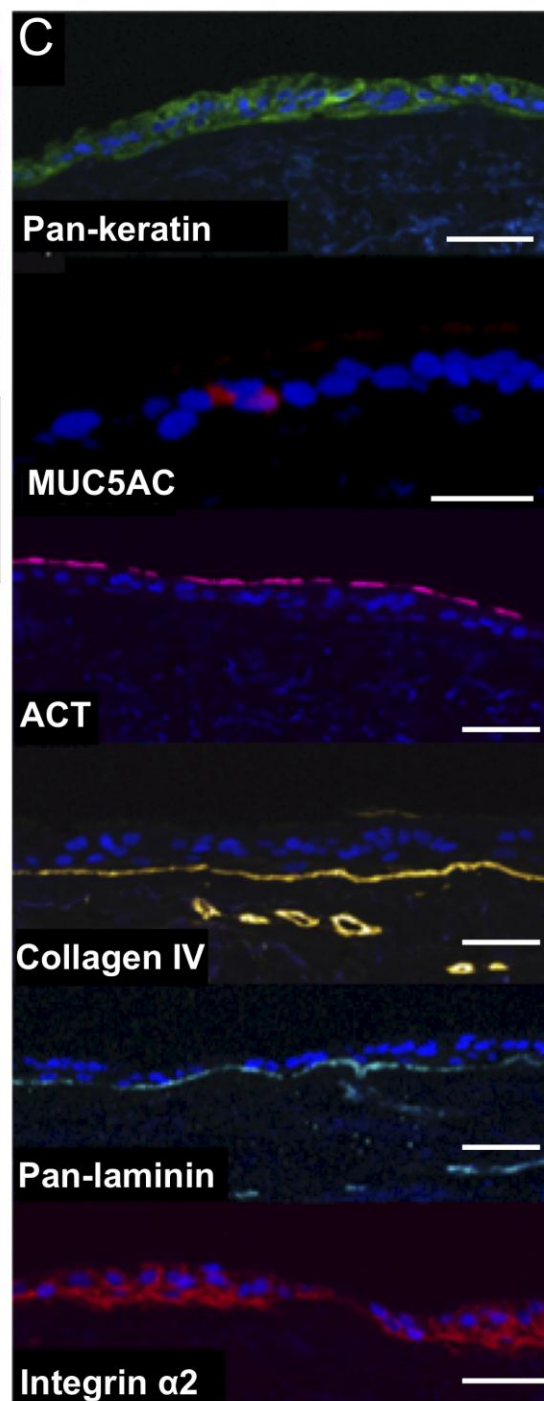


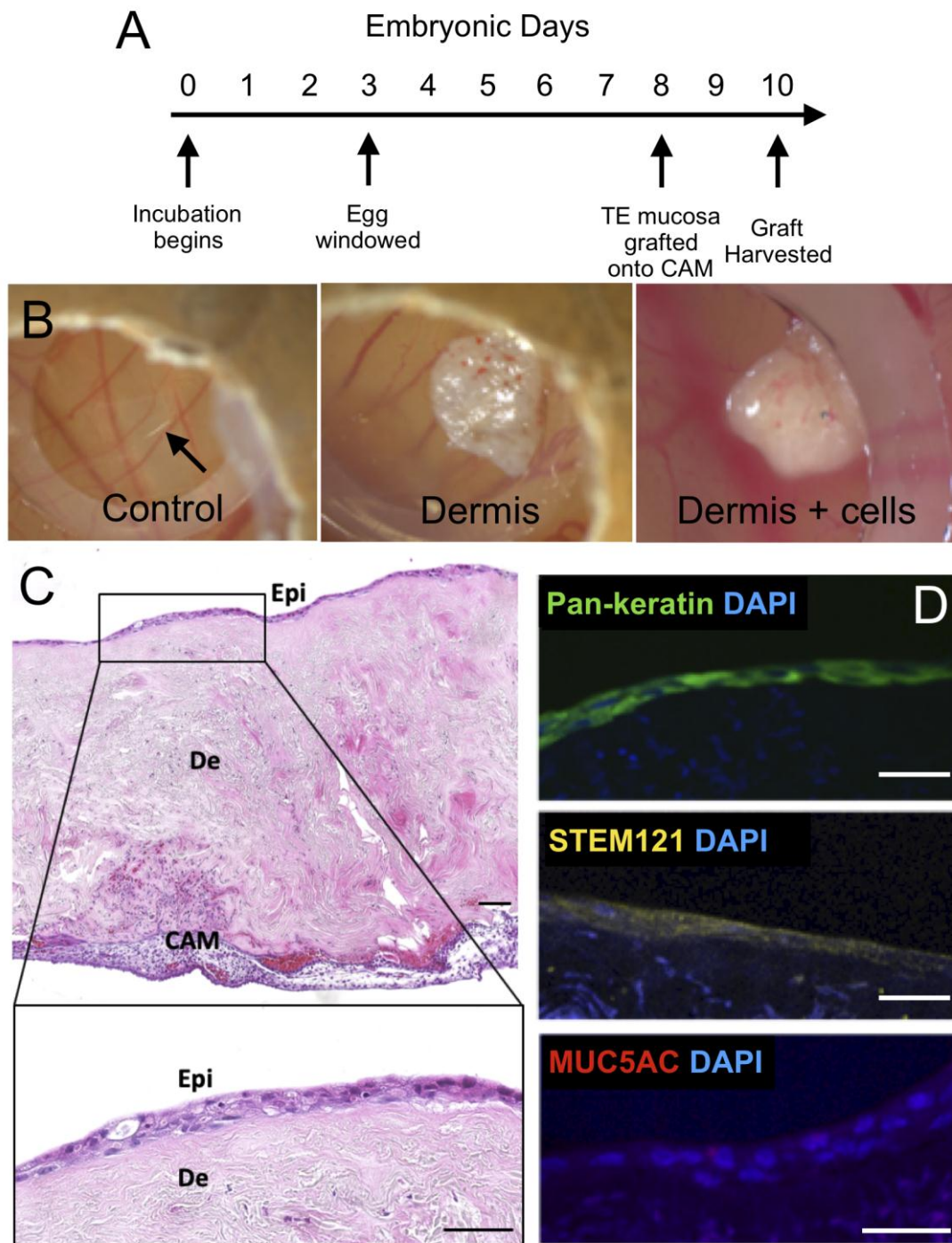


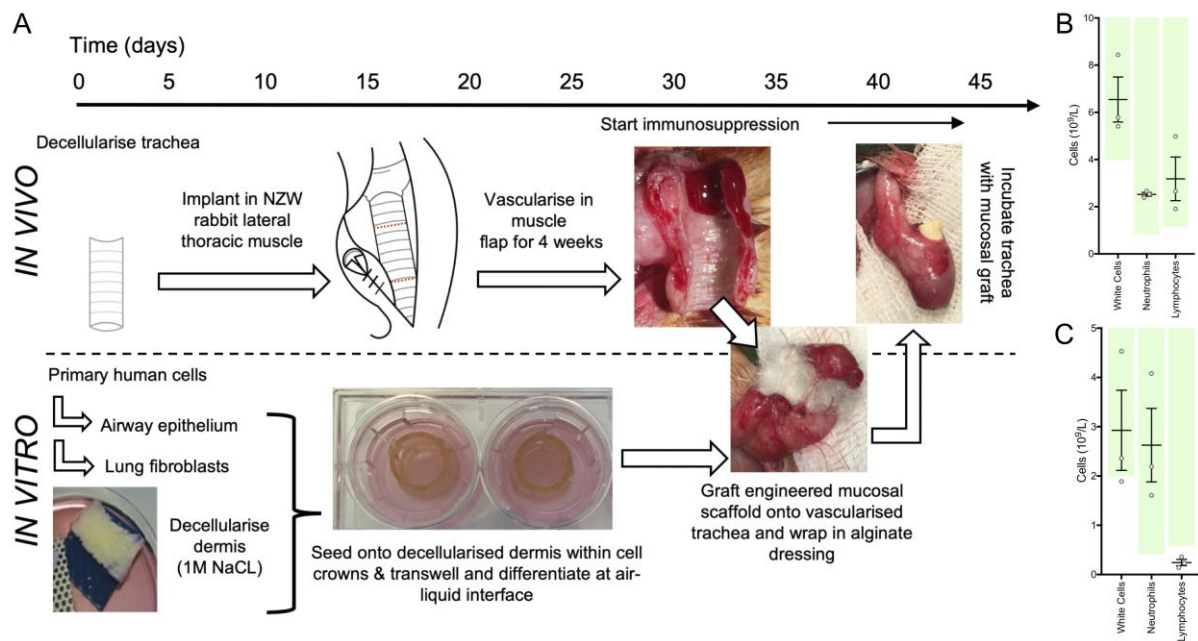


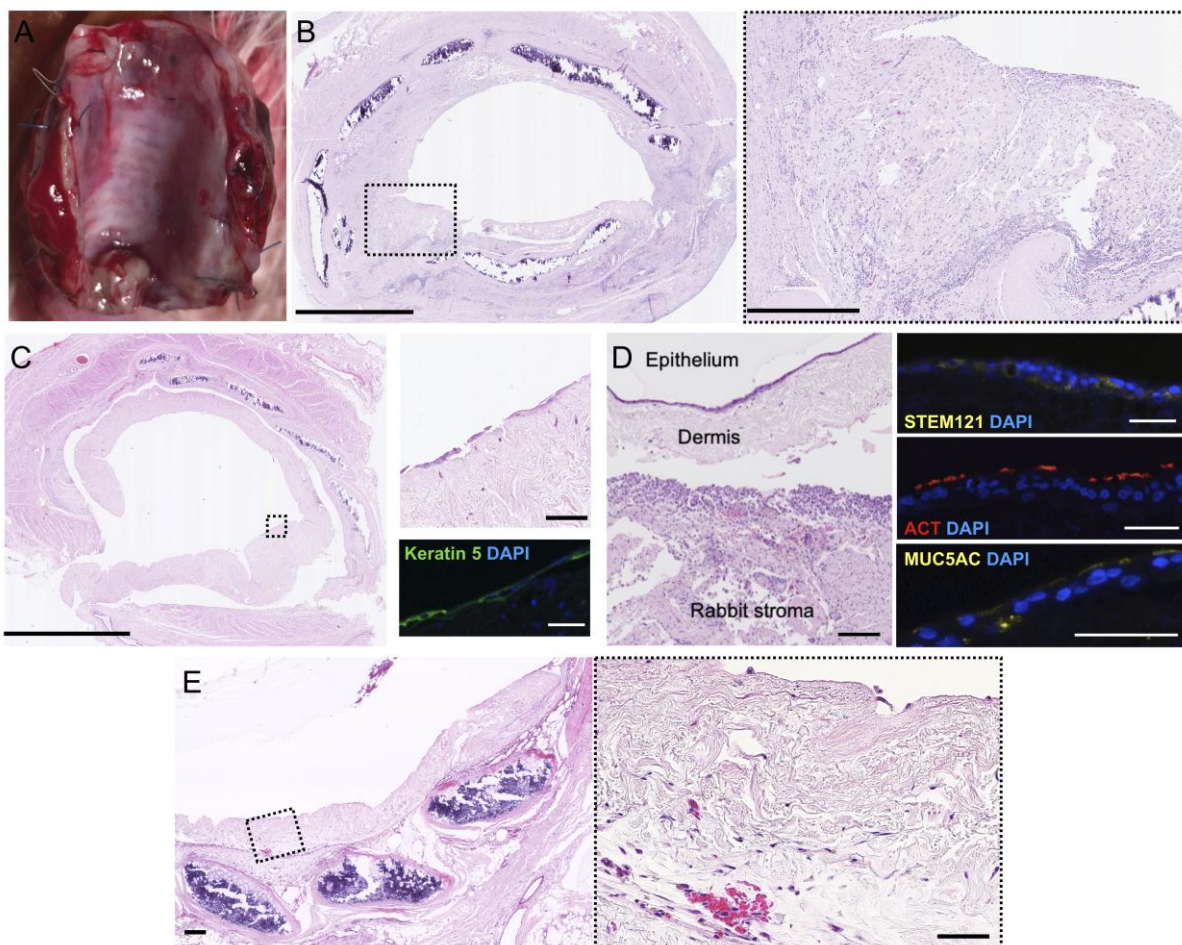
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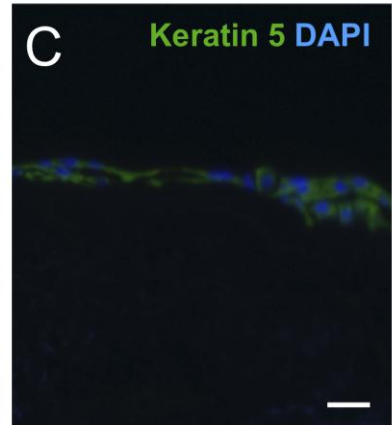
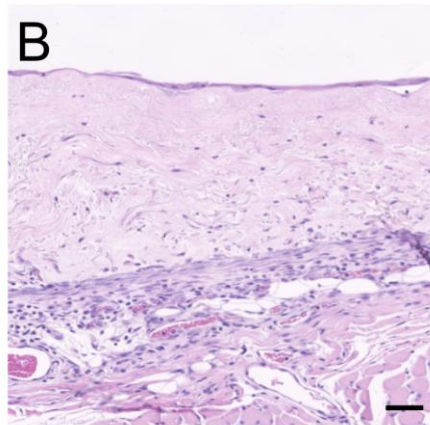
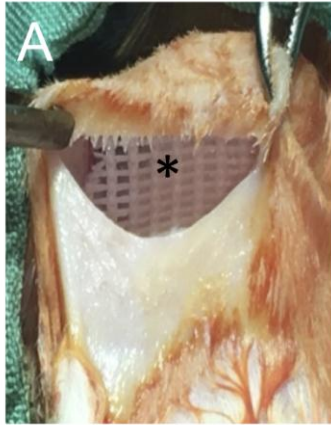
	Control	Scaffold	Normal Range
Ciliary Beat Frequency	14.056 +/- 1.859	14.837 +/- 0.719	7-16
Dyskinesia Score	2 (33% ciliation)	5 (69% ciliation)	0-10











Data Supplement

Bioengineered airway epithelial grafts with mucociliary function based on collagen IV- and laminin-containing extracellular matrix scaffolds

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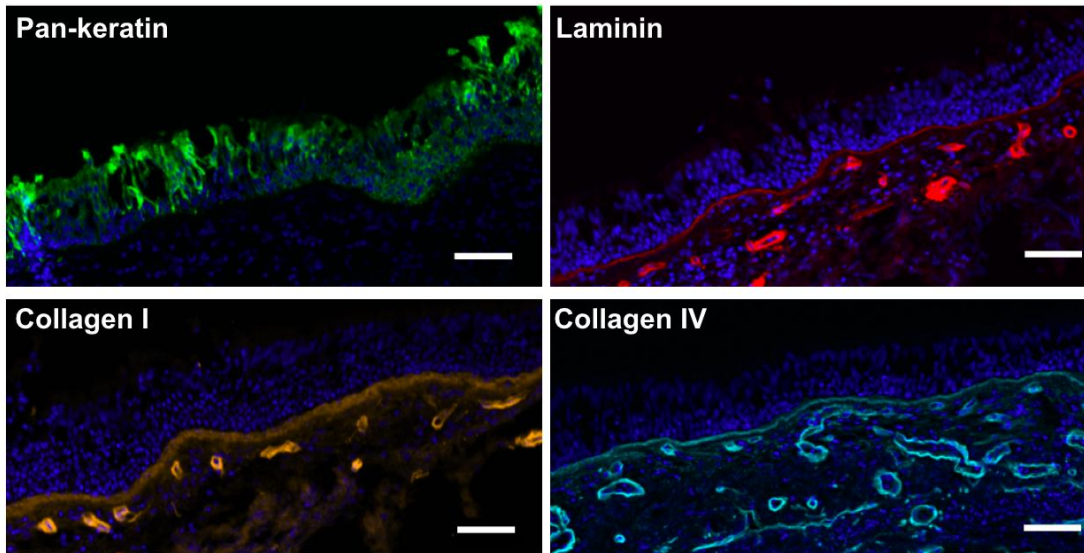
Donor ID	Donor Type	Donor Site	Culture Method
1	Human adult	Bronchus	BEGM
2	Human adult	Bronchus	BEGM
3	Human adult	Bronchus	BEGM
4	Human adult	Bronchus	BEGM
5	Human adult	Small airway	BEGM
6	Human adult	Trachea	BEGM
7	Human adult	Bronchus	BEGM
8	Human adult	Bronchus	BEGM
9	Human adult	Bronchus	BEGM
10	Human adult	Bronchus	3T3+Y
11	Human adult	Bronchus	3T3+Y
12	Human adult	Bronchus	3T3+Y
13	Human adult	Bronchus	3T3+Y
14	Human adult	Bronchus	3T3+Y

Supplementary Table 1: Human bronchial epithelial cell donors.

Respiratory epithelial cells were isolated from the upper airways of human adult patients. Some donor cultures were isolated and expanded in bronchial epithelial growth medium (BEGM), while others were expanded in 3T3-J2 mouse embryonic feeder cell co-culture in the presence of Y-27632, a Rho-associated protein kinase (ROCK) inhibitor. For experiments involving in vitro attachment and proliferation, cells grown in BEGM were used, while for experiments requiring differentiation towards a ciliated epithelium, basal cells cultured in 3T3+Y were used due to their superior differentiation at late passage.

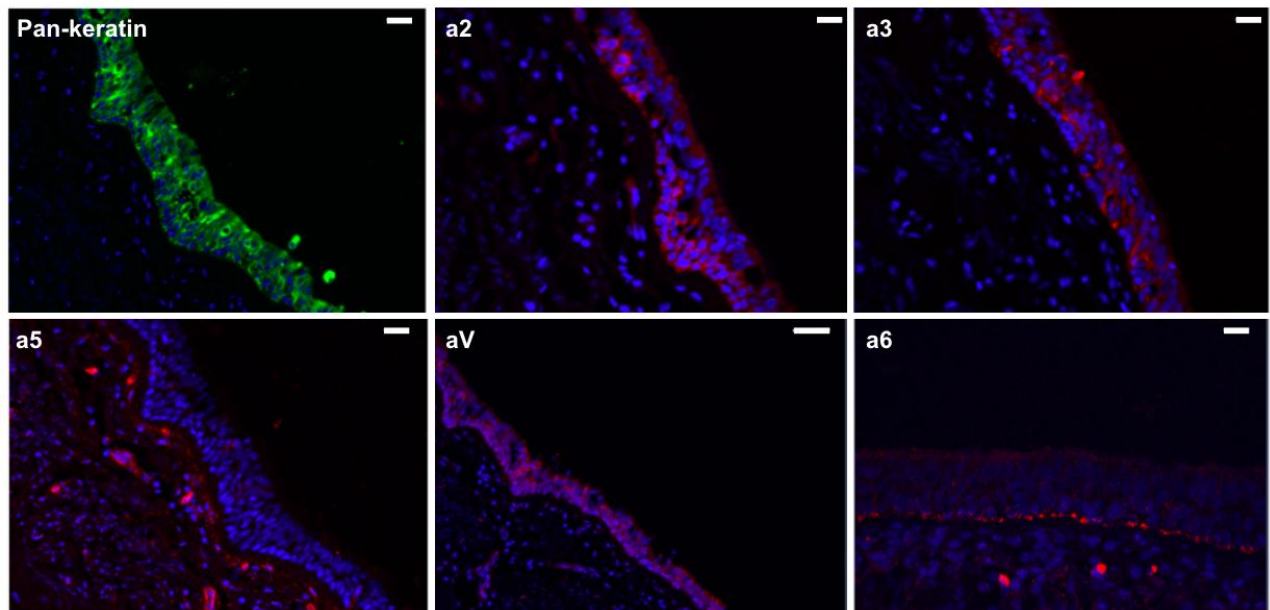
ID	1st Operation	2nd operation	Termination following grafting	Stenting	Immunosuppressive drugs	Outcome
1	Left sided thoracic flap raised with decellularized trachea wrapped in muscle	Graft TERM	7 days	Silicone Stent	Tacrolimus	No epithelial layer retained. Intense inflammatory infiltrate into the dermis layer.
2	Left sided thoracic flap raised with decellularized trachea wrapped in muscle	Graft TERM	7 days	Silicone Stent	Tacrolimus	
3	Left sided thoracic flap raised with decellularized trachea wrapped in muscle	Graft TERM	7 days	Silicone Stent	Tacrolimus	Died before endpoint due to wound dehiscence
4	Left sided thoracic flap raised with decellularized trachea wrapped in muscle	Graft TERM	7 days	Silicone Stent	Tacrolimus & dexamethasone	Minimal retention of epithelial cells, less intense inflammatory infiltrate
5	Left sided thoracic flap raised with decellularized trachea wrapped in muscle	Graft TERM	7 days	Silicone Stent	Tacrolimus & dexamethasone	
6	Left sided thoracic flap raised with decellularized trachea wrapped in muscle	Graft TERM	7 days	Silicone Stent	Tacrolimus & dexamethasone	
7	Left sided thoracic flap raised with decellularized trachea wrapped in muscle	Graft TERM	24 hours	Alginate Dressing	Tacrolimus & dexamethasone	Poor integration into underlying trachea
8	Left sided thoracic flap raised with decellularized trachea wrapped in muscle	Graft TERM	24 hours	Alginate Dressing	Tacrolimus & dexamethasone	Retention of epithelial layer, poor integration into underlying trachea
9	Left sided thoracic flap raised with decellularized trachea wrapped in muscle	Graft TERM	5 days	Alginate Dressing	Tacrolimus & dexamethasone	Loss of epithelial layer but integration of dermis with underlying trachea and evidence of vascular in growth
10	Left sided thoracic flap raised with decellularized trachea wrapped in muscle	Graft TERM	5 days	Alginate Dressing	Tacrolimus & dexamethasone	

Supplementary Table 2: *In vivo* rabbit protocols.



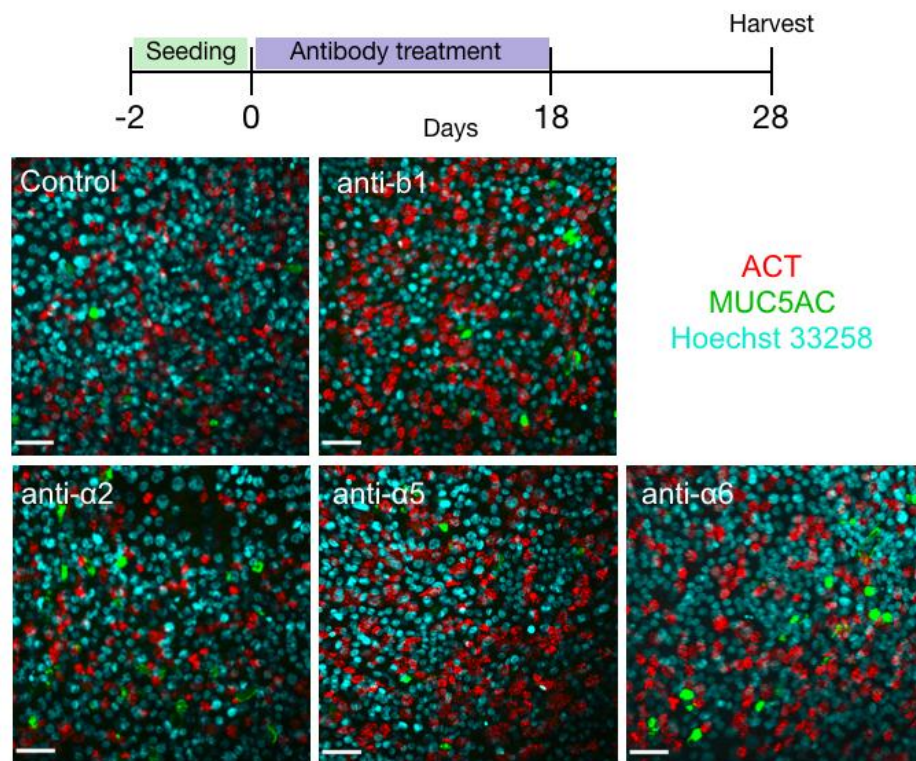
Supplementary Figure 1: ECM protein expression in the human tracheal mucosa.

Airway extracellular matrix (ECM) proteins were visualised in sections of formalin-fixed, paraffin-embedded human trachea. A pan-keratin antibody revealed epithelial cells (green; top left), while antibodies against the ECM proteins pan-laminin (red; top right), collagen I (orange; bottom left), and collagen IV (cyan; bottom right) established the localisation of these proteins in the basement membrane. Scale bars = 50 μm.



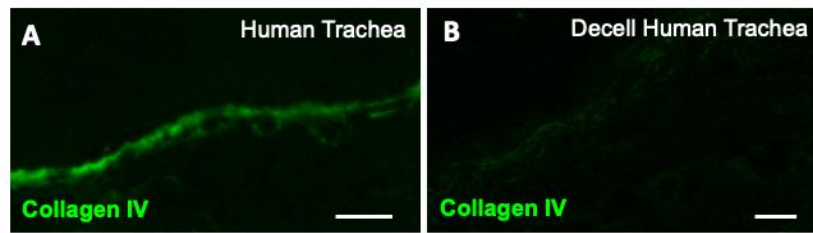
Supplementary Figure 2: Integrin subunit expression in human tracheal mucosa.

Expression of integrin subunits was assessed using immunofluorescence staining of sections of formalin-fixed, paraffin-embedded human trachea. A pan-keratin antibody revealed epithelial cells (green; top left), while antibodies against integrin $\alpha 2$ (red; top centre), $\alpha 3$ (red; top right), $\alpha 5$ (red; bottom left), αV (red; bottom centre) and $\alpha 6$ (red; bottom right) established the localisation of those proteins. Integrins $\alpha 2$, $\alpha 3$ and $\alpha 6$ were observed predominantly within the basal layer of the epithelium, while integrin αV was present throughout the epithelium. Integrin $\alpha 5$ was seen only within the lamina propria. Scale bars = 50 μm .

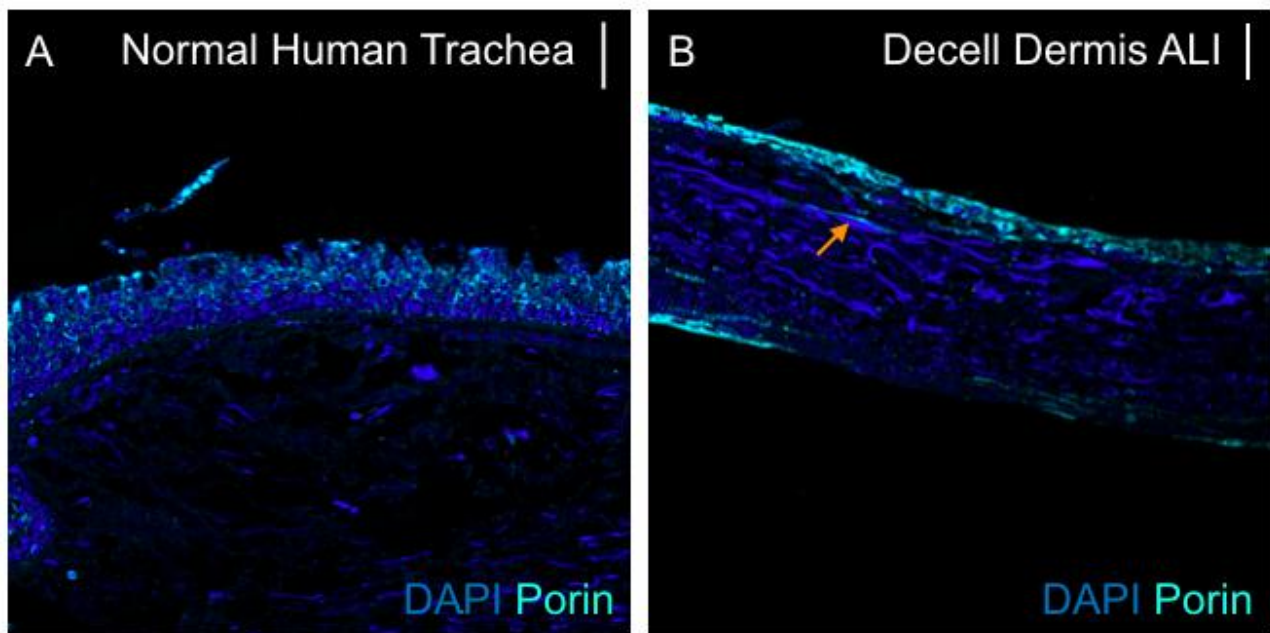


Supplementary Figure 3: Airway epithelial cell differentiation in ALI cultures incubated with blocking antibodies against integrin subunits $\alpha 2$, $\alpha 5$, $\alpha 6$ and $\beta 1$.

ALI cultures were fixed and processed for immunofluorescence staining at Day 28, having been antibody-treated at each feed (three times per week) between Day 0 and Day 18. Cells were stained for acetylated tubulin (ACT; red), MUC5AC (green) and counterstained with Hoechst 33258 (nuclei; cyan). Blocking antibodies against integrins $\alpha 1$ or $\alpha 3$ were not tested here given the failure of epithelial cells treated with those antibodies to form intact epithelia at ALI (Figure 1E). The data shown are representative images from one donor cell culture; comparable results were observed in an independent donor cell culture treated similarly. Scale bars = 37 μm .



Supplementary Figure 4: Immunofluorescence staining of sections of native human airway (A) and decellularized trachea (B). Collagen IV (green) was observed in native human airway. However, collagen IV reactivity is lost following decellularization, indicating a disruption of the basement membrane during this process. Scale bars = 50 μ m.



Supplementary Figure 5: Porin immunofluorescence staining (cyan) in a decellularized dermis-based scaffold seeded with airway epithelial cells and lung fibroblasts after 21 days at ALI.

Sections were counterstained using DAPI (nuclei; blue). Scale bars = 50 μ m.