

Functional Characterization of a Novel 3D Model of the Epithelial-Mesenchymal Trophic Unit

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Take home message: A functionally robust model of the bronchial mucosa for drug discovery and preclinical testing.

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ABSTRACT

Background: Epithelial-mesenchymal communication plays a key role in tissue homeostasis and abnormal signalling contributes to chronic airways disease such as COPD. Most *in vitro* models are limited in complexity and poorly represent this epithelial-mesenchymal trophic unit (EMTU). We postulated that cellular outgrowth from bronchial tissue would enable development of a mucosal structure that recapitulates better *in vivo* tissue architecture.

Methods: Bronchial tissue was embedded in Matrigel[®] and outgrowth cultures monitored using time-lapse microscopy, electrical resistance, light and electron microscopy. Cultures were challenged repetitively with cigarette smoke extract (CSE).

Results: The outgrowths formed as a multicellular sheet with motile cilia becoming evident as the Matrigel[®] was remodeled to provide an air-interface; cultures were viable for more than one year. Immunofluorescence and electron microscopy (EM) identified an upper layer of mucociliary epithelium and a lower layer of highly organized extracellular matrix (ECM) interspersed with fibroblastic cells separated by a basement membrane. EM analysis of the mucosal construct after repetitive exposure of to CSE revealed epithelial damage, loss of cilia, and ECM remodeling, as occurs *in vivo*.

Conclusions: We have developed a robust bronchial mucosal model. The structural changes observed following CSE exposure suggest the model should have utility for drug discovery and preclinical testing, especially those targeting airway remodeling.

INTRODUCTION

Human airway diseases, such as asthma and chronic obstructive pulmonary disease (COPD) are complex multi-factorial diseases; both genetic and environmental components contribute to their pathology. While limited investigations can be undertaken in humans following *in vivo* challenge with agents like allergens, respiratory viruses, ozone or diesel exhaust fumes, these approaches are not amenable to detailed mechanistic studies requiring interventions that cannot be used ethically in people (1). As an alternative, small animals, such as rodents, are widely used to create disease models as an integrated physiological system for studying pathological mechanisms (2;3). These models benefit from transgenic and gene knockout approaches, allowing investigation of the precise function of specific molecules in a pathologic process (4). However, animal models fail to reproduce all features of human asthma or COPD, especially the complex interplay between genetic and environmental stimuli that together drive the disease (5;6). Thus, there is an urgent need to develop human cell-based models of the airway which more accurately recapitulate aspects the human airways.

Several human tissue/cell-based models have been developed, including *in vivo* heterotypic xenografts (7), *ex vivo* tissue explants (8), lung slices (9) or *in vitro* cultures of primary cells derived from airway disease patients (10). Such models have the advantage of using cells containing relevant genetic and epigenetic information that contribute to underlying pathogenetic processes, increasing confidence in results from mechanistic studies, including target identification and validation, as well as toxicological testing. Tissue explants offer the advantage of retaining the *in vivo* architecture, but they are of limited life-span *ex vivo* and do not preserve an intact epithelial barrier which compromises studies involving challenge with environmental

stimuli. As an alternative, polarised, differentiated epithelial cultures are now widely employed for *in vitro* epithelial response studies. These models have allowed identification of differences in epithelial function in asthma and COPD (10;11), consistent with the epithelium playing a key role in translating gene-environment interactions in airway disease. However, even these models have limitations, as they are a single cell-type and do not recapitulate the complex cell-cell and cell-matrix interactions that occur *in vivo*. Such interactions are critical for control of tissue homeostasis and repair after injury (12).

The airway epithelial-mesenchymal trophic unit (EMTU) was originally proposed by Evans and colleagues (13) to highlight the importance of airway structural cells in controlling tissue homeostasis. According to this model, the epithelium and underlying mesenchymal cells communicate in a bi-directional way to control responses to environmental stimuli. We have postulated previously that abnormal epithelial responses to environmental stimuli are propagated and amplified within the EMTU, leading to augmented inflammatory and remodeling responses in asthma and other airway diseases (12). Evidence of cellular cross-talk has already been demonstrated in simple experiments using epithelial-derived conditioned media or epithelial-fibroblast co-cultures, where fibroblasts respond to epithelial-derived signals to drive inflammatory or remodeling responses (14). However, no studies have been performed in which an intact epithelial cell layer and fibroblasts are in intimate contact, surrounded by their own matrix. This is a severe deficiency in current models, since abnormalities in matrix organisation and mechanical properties are now recognised as contributing to disease pathogenesis (15;16). Thus, the availability of an integrated model of human bronchial mucosa is paramount. For this reason, our aim was to develop a 3D outgrowth model of the human bronchial mucosa, starting

from bronchial biopsies acquired during bronchoscopy. We postulated that outgrowth from bronchial tissue would enable the structural cells to develop a mucosal structure that recapitulated *in vivo* tissue architecture. Here we describe the model and its morphological, structural and ultra-structural characteristics, as well as its functional properties relevant to epithelial barrier function and extracellular matrix remodeling independent of inflammatory cells.

METHODS

Patient Characterization and Fiberoptic Bronchoscopy

Subjects without lung disease were recruited at Southampton General Hospital, Southampton, UK or the University of Palermo and La Maddalena clinic, Palermo, Italy following ethical approval from the relevant Ethics Committee and informed consent. Bronchial biopsies were obtained by fiberoptic bronchoscopy (FB-20D; Olympus, Japan) in accordance with standard published guidelines (17) and placed in ice-cold DMEM containing 20% fetal bovine serum (FBS), 50IU/ml penicillin, 50µg/ml streptomycin, 30ng/ml amphotericin B and 1mM glutamine (transportation medium). Tissue was transferred into 5cm² Petri dish with fresh medium and incubated at 37°C for 24hrs prior to use in the EMTU model. This identified infected samples not suitable for long term culture and thus reduced wastage of Matrigel®.

The 3D ‘EMTU’ Model.

Bronchial biopsies were cut into approximately 0.5mm³ fragments using a scalpel, placed onto 6.5mm Transwells® (BD Biosciences, Belgium, PET, area = 0.33cm², pore size 0.45µm) and embedded in 45µl of growth factor reduced Matrigel® (Corning, USA). The Transwell® support

was placed into a cell culture dish containing 330µl of 1:1 mixture of Bronchial Epithelial Growth Medium (BEGM, Clonetics™, Scientific Laboratory Supplies, UK) and DMEM/FBS growth medium (DMEM containing 10% FBS, 50IU/ml penicillin, 50ug/ml streptomycin and 1mM glutamine) for provision of nutrients via the basal surface of the membrane support (Figure 1A). During the first week, the dose of amphotericin B in the BEGM was doubled; thereafter, the normal dose was used. The growth medium was replaced every 48 hours during the culture period.

The culture develops as an outgrowth of a coherent sheet of cells from the biopsy to form a multi-layered structure; the outgrowth was monitored using contrast phase microscopy and when the cell layer was confluent (around 30 days), the biopsy was removed to allow the well to be completely filled with a cell layer; if sufficient tissue remained, this was used to develop a new outgrowth culture. Transepithelial Electrical Resistance (TEER) was measured using chopstick electrodes (EVOM voltohmmeter, World Precision Instruments, UK). CSE was prepared as previously described (18). After completion of differentiation (around day 28), mature cultures were exposed to 10, 15 or 30% (v/v) CSE in medium; exposures were made every 48 hours for up to 21 days, as indicated in the results section. Basolateral medium was replaced every 48 hours. For measurement of IL-8 release, IL-8 released between days 12 and 14 was measured by ELISA (Luminex, MOSS S.p.A, Italy).

Methylene blue staining of semi thin sections

At Days 4, 8, and 12, cultures were fixed, post-fixed and dehydrated before embedding in Epon resin. Semithin (2µm) sections were then cut and placed on glass slides. The specimens were

stained with methylene blue solution (Sigma-Aldrich, UK) and observed with light microscope (LEICA DM4000, Leica Microsystems, Germany).

Immunofluorescence staining

At Day 12, cultures were washed and methanol-fixed for immunostaining, as previously described (10). The following primary Abs were used: anti-cytokeratin13 (CK13) (monoclonal IgG1 Ab, clone 1C7, Abnova, USA) and anti-alpha smooth muscle actin (aSMA) (monoclonal IgG Ab, clone 1A4, Sigma, UK). Cells were observed using a LEICA inverted fluorescent microscope.

Scanning Electron Microscopy (SEM)

At Days 10, 20 and 30, cultures were fixed in 2% paraformaldehyde and 0.5% glutaraldehyde, washed and post-fixed with 1% osmium tetroxide. Samples were washed with Millonig buffer and dehydrated in an ascending ethanol series (30, 50, 70, 95 and 100%). The samples were critical point-dried using CO₂, attached to specimen stubs with silver tape, metallized with gold and observed using an electron microscope (JSM-6301F; JEOL Ltd, Japan).

Transmission Electron Microscopy (TEM)

At Days 10, 20 and 30, cultures were fixed, post-fixed and dehydrated before embedding in Epon resin, as previously described (19). Ultrathin (50nm) sections were then cut and placed on Cu/Rh grids. The specimens were contrasted with 7% uranyl-acetate in methanol and Reynold's lead citrate buffer before imaging with transmission electron microscope (JEM-1220; JEOL, Japan).

RESULTS

Morphological and functional characterization of the EMTU model derived from human bronchial biopsies.

Bronchial biopsies were embedded in growth factor-reduced Matrigel[®] in Transwell[®] supports using BEGM/DMEM growth medium (see below) in the basolateral compartment (Figure 1A). The culture developed as an outgrowth of cells from the biopsy to form a multi-layered structure. Expansion of the cultures was monitored by contrast phase microscopy (Figure 1B). Time-lapse phase contrast microscopy revealed that the cultures developed as an outgrowth from the biopsy, with cells migrating as a coherent sheet (Suppl. Figure 1).

Initial experiments evaluated the media combinations for optimal growth of both epithelial cells and fibroblasts from the biopsy. This involved testing various ratios of BEGM (B) and DMEM/FBS (D). In 4B:1D medium, little fibroblast growth was obtained, whereas 1B:4D resulted in predominant growth of stellate fibroblasts that invaded the Matrigel[®] with few epithelial cells. Use of 1B:1D supported growth of both cell types and was used for all the cultures described below. We also tested embedding the biopsies in gels cast from collagen I instead of Matrigel[®]; however the collagen gels either failed to support development of the cultures or the cultures rapidly developed necrotic centres.

Immunofluorescent staining of the outgrowth using cytokeratin 13 and alpha-smooth muscle actin antibodies showed that the leading edge of cells was epithelial with a second front of α -SMA positive cells, approximately 400-700 μ m from the leading edge (Figure 2). The morphology of the cultures was further studied by histological staining (Figure 3). After 10-12

days, the nylon membrane covering the bottom of the insert was completely covered with newly grown tissue, and from that time, the culture had a three-dimensional structure. At this stage, the cells had a flattened appearance and the culture was still covered with Matrigel[®], but after 20 culture days the matrix was remodeled to spontaneously form an air-interface (ALI). This resulted in development of a multicellular layer with surface microvilli and some ciliated cells, and was accompanied by an increase in TEER which plateaued after around 7 days post-ALI (Fig 3B). By 30 days of culture, many goblet cells and ciliated cells were evident and the morphological features of the outgrowths did not change significantly after this point. Video microscopy of the live cultures demonstrated that the cilia were functionally active (Suppl. Figure 2). In our experience, unless specific damage occurred during the culture period (eg. contamination with infectious organisms), and proper culture conditions were maintained, these 3D outgrowths could be maintained for over a year.

Ultrastructural characterization of the EMTU model derived from human bronchial tissue.

Electron microscopy was used to study the ultrastructural features of the culture models. As shown in Figure 4, the cultures showed a progressive development and differentiation of their apical surface. After 10 days, this surface was completely covered with microvilli of epithelial cells (Figure 4 A-C). At around 20 days, the apical surface started to present a few ciliated elements (Figure 4 D-F) which spread to around 40% of the total surface area after 30 days (Figure 4 G-I). This ratio between ciliated cells and cells with microvilli (40:60) did not change significantly after this time point. The cilia measured around 8 μm , which is comparable to the ciliary length in the normal bronchial epithelium. Further ultrastructural analysis of the cilia showed that they had the typical 9+2 axoneme structure of motile cilia (Figure 5A and inset) and

well-formed basal bodies (Figure 5B). The basal epithelial cells were separated from the mesenchymal layer by a well-developed basement membrane to which basal epithelial cells were attached via hemidesmosomes (Figure 5C). The latero-lateral surfaces presented adhesive junctions, such as tight junctions (Figure 5D) and desmosomes (Figure 5E).

Application of freeze-fracture SEM to the cultured cell layer enabled the observation of the inner components of the culture in more detail. Figure 6A shows a 32-day culture with a complex network of elongated cells underneath the epithelial cell layer. The elongated, spindly elements rested on the porous support of the culture insert and their cytoplasmic membranes formed long stellate processes that interacted with other cells (Figure 6B, C). The appearance and location of the elongated cells suggest a mesenchymal/fibroblastic phenotype; this organization is reminiscent of the attenuated fibroblast sheath of the EMTU, as described by Evans et al (13). This possibility was further explored using TEM on 30-day-old cultures, where it was possible to identify two distinct strata: the top one formed of a layer of pseudostratified epithelial cells and the bottom one formed by ECM interspersed with isolated fibroblast-like cells (Figure 7A). In the lower mesenchymal cell layer, there was a well-defined ECM and the fibroblasts possessed lipid droplets similar to those found in hepatic stellate cells (20) (Figure 7B). They also exhibited cytoplasmic processes with caveolae-like structures (Supplemental Figure 3A), extruding proteic-like material into the extracellular space to eventually form the structured ECM surrounding the cells. The fibrillar material surrounding the fibroblasts exhibited the characteristic D-banding of collagen fibrils, suggesting production of mature ECM (Figure 7C and Supplemental Figure 3B). In addition to epithelial cells and fibroblasts, EM did not identify other cell types, as confirmed by immunofluorescence staining: the cultures did not express CD3,

CD4, CD8, CD18, CD28, CD45, CD64, CD68 or MPO (markers of leukocytes, lymphocytes, macrophages and neutrophils) (data not shown).

Response of the EMTU Model to CSE exposure

Once mature cultures had formed, their response to CSE was evaluated. Fresh medium containing 10 or 30% CSE was added to the cultures every 48 hours for 14 days, with control cells being exposed to medium alone. While the cultures tolerated repetitive challenges using 10% CSE with no loss of barrier function (Figure 8A), 30% CSE eventually caused disruption of the barrier after around 5-7 days of continuous CSE exposure (Figure 8A). Nonetheless, even after 14 days of repeated exposure to 30% CSE, the cultures retained the ability to secrete significantly ($p < 0.05$) more IL-8 than control cultures (mean (\pm SD) = 8137 ± 138 versus 5585 ± 4 pg/ml IL-8 released between days 12 and 14 of the challenge using cultures from 2 independent donors). A concentration of 15% CSE was used to evaluate the effect of repetitive smoke challenge on the ultrastructure of the cultures after 21 days of CSE exposure. TEM showed that CSE caused a partial remodeling of the architecture of the cultures. In particular, the apical epithelial cells completely lost their cilia which were replaced by thick microvilli, entirely covered by mucus (Figure 8B). Moreover, collagen production by the fibroblasts increased, resulting in a marked thickening of the basement membrane and disarray of the fibroblast layer with increased ECM production (Figure 8C and inset).

DISCUSSION

We established a novel *in vitro* model mimicking the EMTU of the bronchial mucosa that could be useful in the study of the pathogenesis of lung diseases such as asthma and COPD. This

model offers the possibility to conduct long term experiments, evaluate interactions between different cell populations in a three-dimensional environment and administer exogenous molecules. The mature EMTU model had an ultrastructure that was similar to that observed in electron micrographs of *ex vivo* bronchial tissue (21), it was functionally intact as determined by TEER measurements, and able to respond to repetitive challenge with CSE. Using this novel model, we showed that chronic exposure to CSE caused morphological changes including thickening of the basement membrane, loss of cilia, increased mucus production and disarray of the *lamina propria*. These features of remodeling of the bronchial mucosa are typical of those observed *in vivo* following chronic cigarette smoke exposure (22-24) and occurred without immune cell involvement.

Primary bronchial epithelial cultures or cell lines still represent the most commonly used *in vitro* human models to study airway responses to specific stimuli (25). However, one of the main limitations of these models is the general absence of ECM components and the loss of proper cell-cell interactions that arises from the former. In recent years, the aspiration to recapitulate a 3D tissue micro-architecture has prompted the development of more innovative, integrated models using tissue engineering (25;26) or xenograft models (7). While co-culture models have been developed (27;28), they utilize bronchial epithelial cells and fibroblasts that have initially been cultured separately and co-cultured in a range of formats including Transwell[®] systems (or similar synthetic polymer supports (28)), where the epithelial cells and fibroblasts are physically separated by porous supports. Probably the most ‘natural’ system to date involves placing epithelial cells onto the surface of collagen gels containing embedded fibroblasts (29). However, even though collagen is a natural product, it is artificially cast and the fibroblasts are already

phenotypically modified by culture on plastic. Furthermore, these models have a relatively short life, so it is not possible to undertake long-term experiments. Nonetheless, such a model has been used to investigate the effects whole smoke exposure with clear evidence of decreased cilia and increased goblet cell differentiation, but ECM remodeling was not explored (30). The alternative xenograft approach involves seeding human airway epithelial cells into decellularized rat tracheas and culturing in the flanks of nude mice (7). While grafts seeded with epithelial cells from asthmatic donors had three times more fibrillar collagen compared to non-asthmatic grafts, this presumably reflects migration of rodent mesenchymal cells into the graft. In the current EMTU model, both epithelial cells and fibroblasts grow out autonomously from the bronchial biopsy into a 3D gel, whose composition is similar to that of normal ECM. After the initial expansion phase and turnover of the Matrigel[®] to produce an ALI, fibroblasts start to produce newly formed ECM, structurally distinct from the Matrigel[®], with clear evidence of fibrillar collagen production. In parallel, the epithelial cells start to differentiate, and after around 28-30 days of culture, a fully differentiated bronchial epithelium forms. This is separated by a functional basement membrane from a newly constituted *lamina propria*, where fibroblasts lay down ECM. Remarkably, epithelial differentiation takes place without the need to modify the culture medium with additional retinoic acid. This could be explained by the provision of differentiation factors by the supporting fibroblasts, which we found to contain lipid droplets on electron microscopy. Hepatic stellate cells contain lipid droplets which are a source of retinoids, but these are rapidly lost in hepatic fibrosis when the cells become matrix-producing myofibroblasts (20).

Epithelial-mesenchymal signalling plays a key role in embryonic development and tissue repair responses following injury or epithelial activation. While many *in vivo* animal models of chronic lung disease involve induction of a large inflammatory response followed by structural remodeling, recent studies have implied a role for mechanical forces in driving inflammation-independent remodeling (27;31;32). For example, application of lateral compressive strain to a tissue-engineered human airway wall model resulted in increased deposition of collagen as well as secretion of matrix metalloproteinases, leading to the conclusion that mechanical forces alone were sufficient to induce tissue remodeling (32). Consistently with this, recent *in vivo* studies have demonstrated that bronchoconstriction can induce remodeling responses in the bronchial mucosa of mild asthmatic subjects without inflammation changes (33). Our studies support the concept that epithelial-mesenchymal signalling can drive remodeling responses. However, in our case, the stimulus was chemical rather than mechanical. Since we observed that CSE exposure caused loss of cilia, it is noteworthy that gene deletion of the intraflagellar transport molecule, IFT88, caused loss of motile cilia in murine airways, resulting in airway remodeling without any evidence of inflammation (34). Separation of the structural cell responses from those driven by inflammatory cells may provide new insights into disease pathogenesis and enable identification of novel targets for therapy, especially targeting those features of the disease that are corticosteroid-unresponsive. The discovery that many asthma (35) and COPD (36) susceptibility genes are expressed by epithelial cells and bronchial fibroblasts adds further weight to our paradigm that assigns to the EMTU a central role in the pathogenesis of lung diseases (37).

Although we believe the EMTU model is a significant advance over current models, there are prospects for further development. Most notably, its current form lacks a circulation or the

dynamic effect of interstitial flow. With the development of tissue engineering approaches, this could be addressed in the future through the employment of microfluidics (38). This would provide the opportunity to selectively add environmental challenges or (inhaled) drugs to the epithelial compartment and cytokines or (systemic) drugs to the mesenchymal compartment. Furthermore, it may be possible to combine a microfluidic system with the delivery of immune or inflammatory cells into the tissue construct to produce a truly dynamic model of the human bronchial mucosa.

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DECLARATION OF INTERESTS

Drs. Davies and Holgate report being co-founders and share-holders in the University spin-out company Synairgen. The other authors have no relevant competing interests to declare.

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Figure legends

Figure 1 Schematic representation of the configuration of the EMTU model (A) and phase contrast images (B-D) taken 4 (B), 8 (C) and 12 (D) days after establishment of the outgrowth culture. The biopsy tissue can be seen as the dark shadow at the top of each photomicrograph. Scale bar = 1mm.

Figure 2 Immunofluorescent confocal image of a day 12 outgrowth culture stained for cytokeratin 13 (green) and α -smooth muscle actin (red). The image shows the 3D maximum projection and the orthogonal slices indicated by the dotted lines. The dark area on the left of the image is where the biopsy was removed before fixing and immunostaining. Scale bar = 287 μ m.

Figure 3 Representative images of formalin-fixed, paraffin embedded sections of EMTU outgrowth cultures harvested after 10, 20 and 30 days and stained histochemically with methylene blue (A). Scale bar = 20 μ m. TEER measurements of the cultures at the times indicated (B). N = 8-15.

Figure 4 Representative SEM images of EMTU outgrowth cultures showing the ultrastructural morphology of the apical surface after 10 (A-C), 20 (D-F) or 30 (G- I) days of culture. Each row shows 3 magnifications of the same culture. Cell-cell junctions, microvilli and cilia can be observed. Scale bars are as indicated.

Figure 5 Representative TEM images of EMTU outgrowth cultures showing the ultrastructural morphology of the epithelial cells after 30 days of culture. Microvilli and cilia with a 9+2

axoneme structure (inset) can be observed (A) together with well-formed basal bodies (B). Adhesive junctions are also evident with formation of hemidesmosomes (C), tight junctions (D) and desmosomes attached to keratin filaments (E). Scale bars = A: 1 μ m, B: 200nm, C and D: 100nm, E: 200nm.

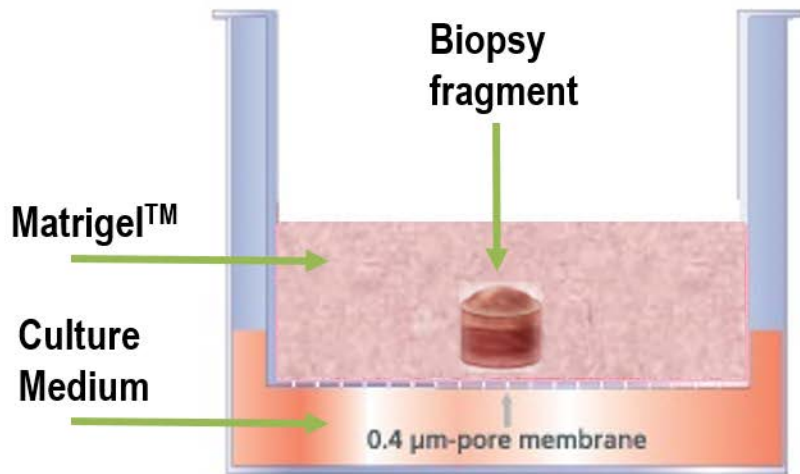
Figure 6 Representative freeze-fracture SEM images of a 32 day old culture. Low (A) and high (B) power magnification of the entire thickness of the culture showing the upper epithelial layer with the surface covered by areas of cilia surrounded by microvilli and the lower layer of dispersed and flattened fibroblastic cells. High power image of the porous support showing the lower fibroblastic cell layer, highlighting their stellate nature (C). Scale bars are as indicated.

Figure 7 Representative TEM images of 30 day old EMTU outgrowth cultures showing the ultrastructural features of the entire thickness of the culture (A) and the subepithelial layer showing a section through a fibroblast with electron lucent lipid droplets within a well-defined fibrillar ECM (B) which has characteristic D-banding of mature collagen fibrils (C). Scale bars = A: 3 μ m, B: 1 μ m, C: 200nm.

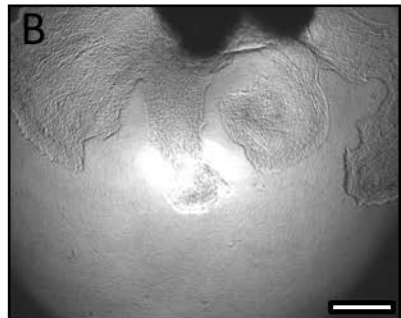
Figure 8 The effect of 10 (●) or 30% (■) CSE on ionic permeability of mature EMTU cultures (A). Exposure started on day 28 and cultures were re-challenged with CSE every 48 hours for 14 days. Grey circles (●) show the electrical resistance of untreated cultures (either pre-CSE exposure or without treatment post 28 days).

Representative TEM images of mature EMTU outgrowth cultures after 21 days of exposure to 15% CSE. This treatment caused loss of cilia and thickened microvilli surrounded entirely by a dense layer of mucus (B) and thickening of the subepithelial basement membrane and disorganisation of the collagen layer (C and inset). Scale bars = A: 200nm, B: 1 μ m.

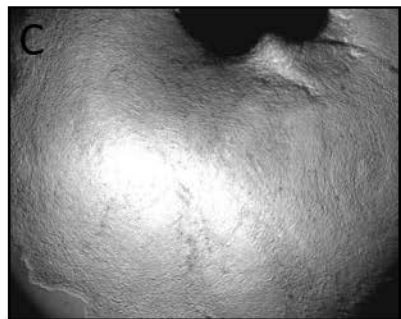
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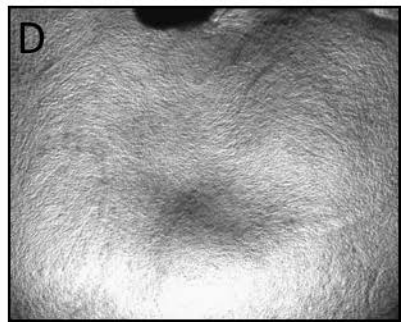
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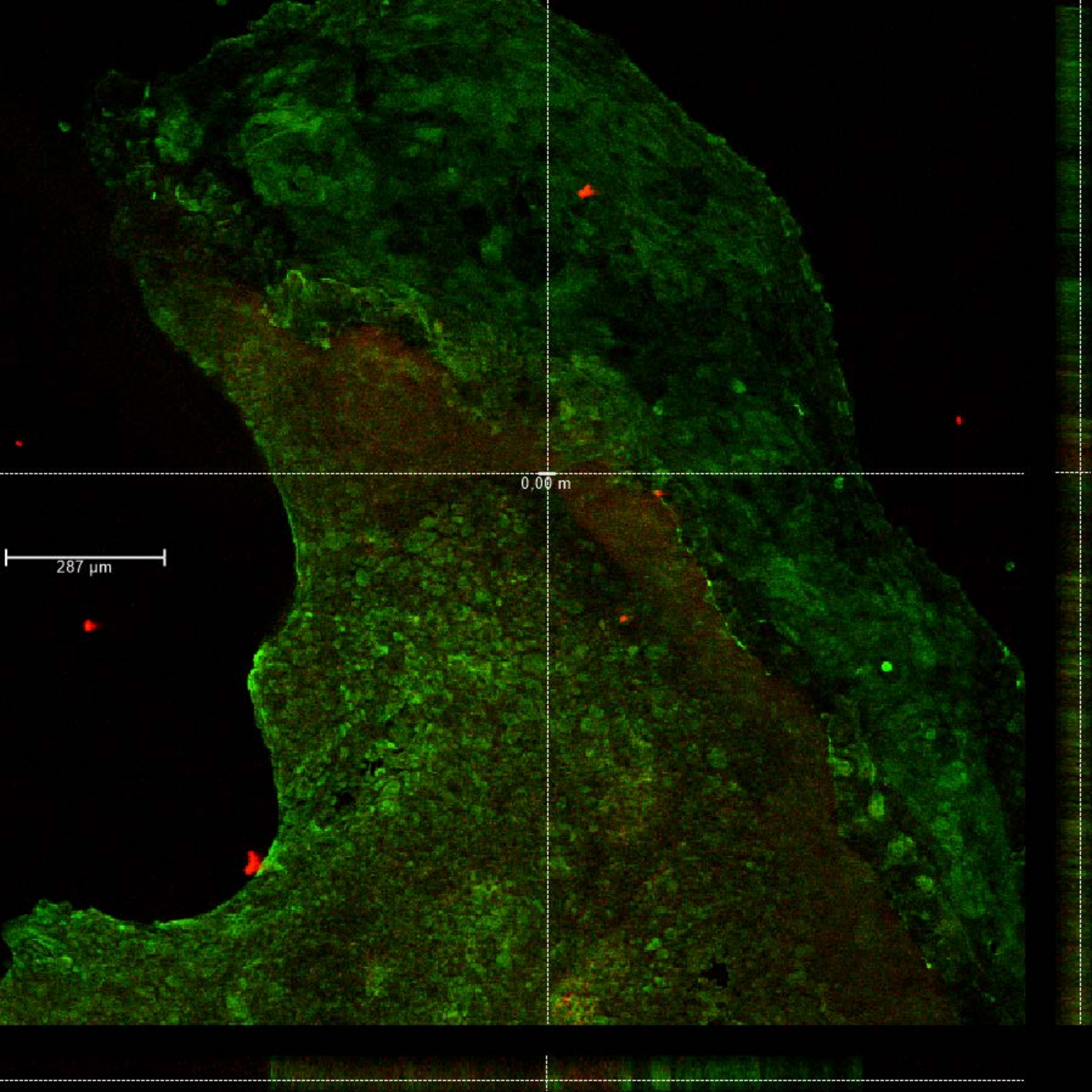


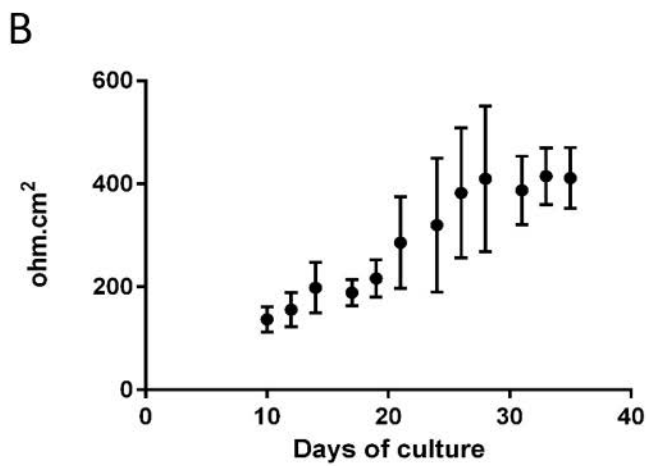
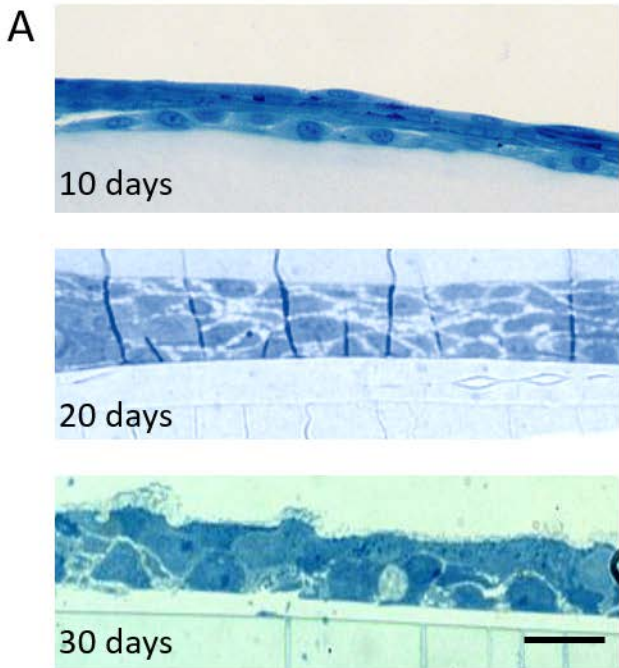
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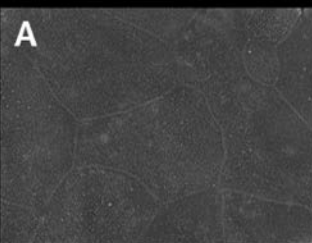


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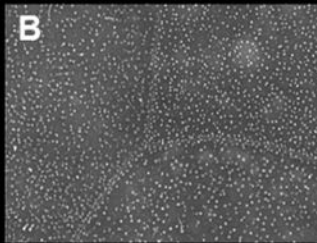




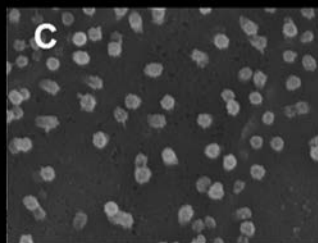




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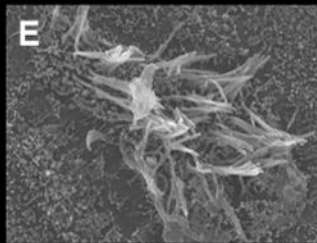
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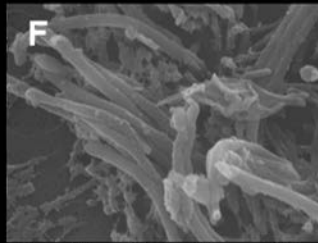
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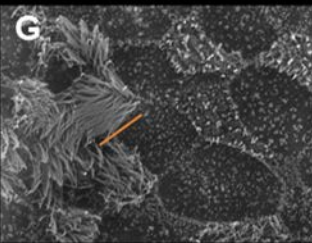
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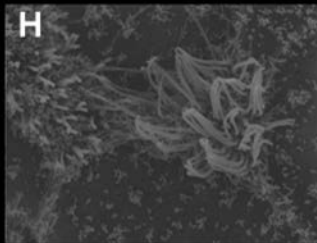
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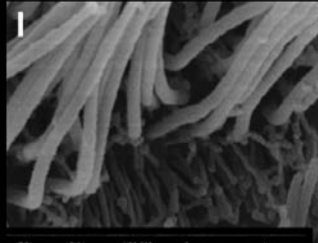
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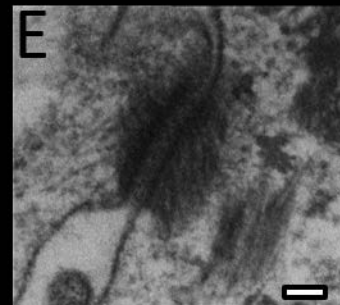
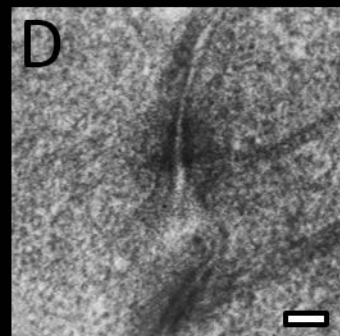
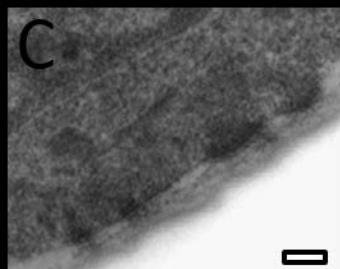
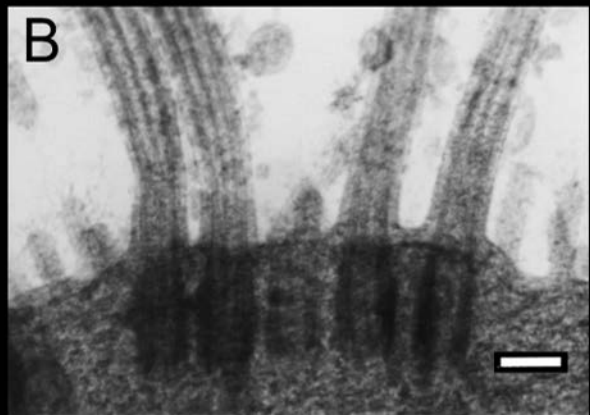
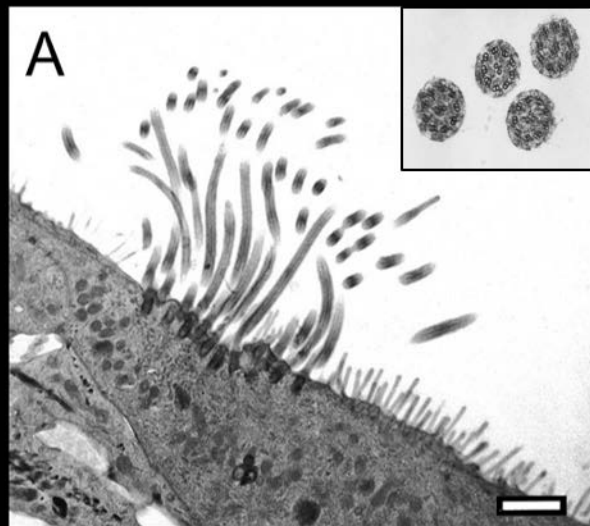
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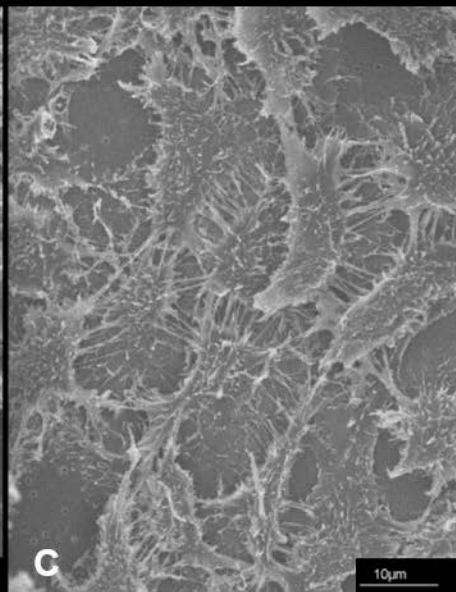
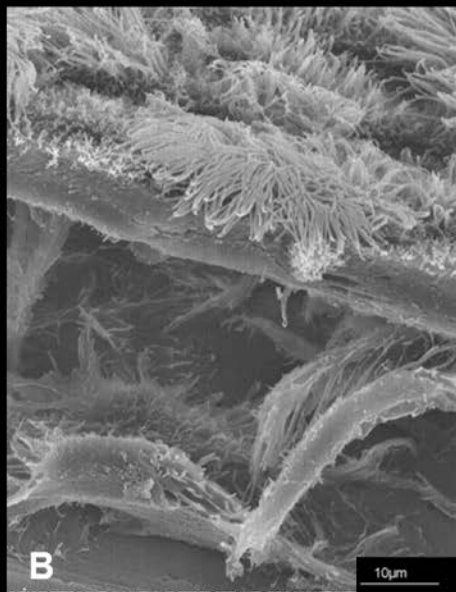
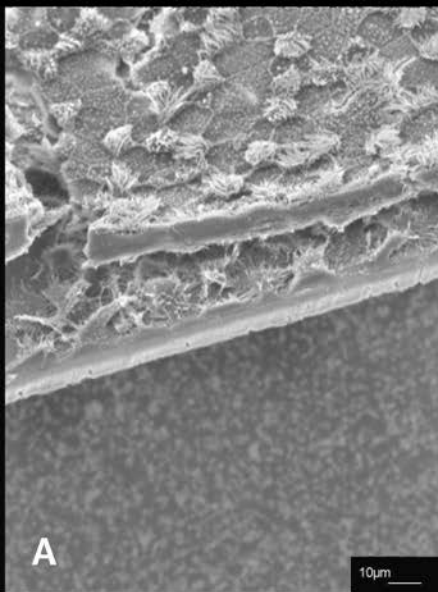


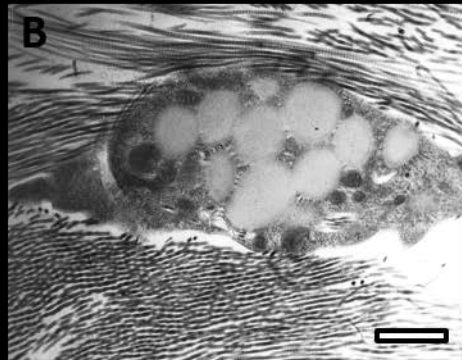
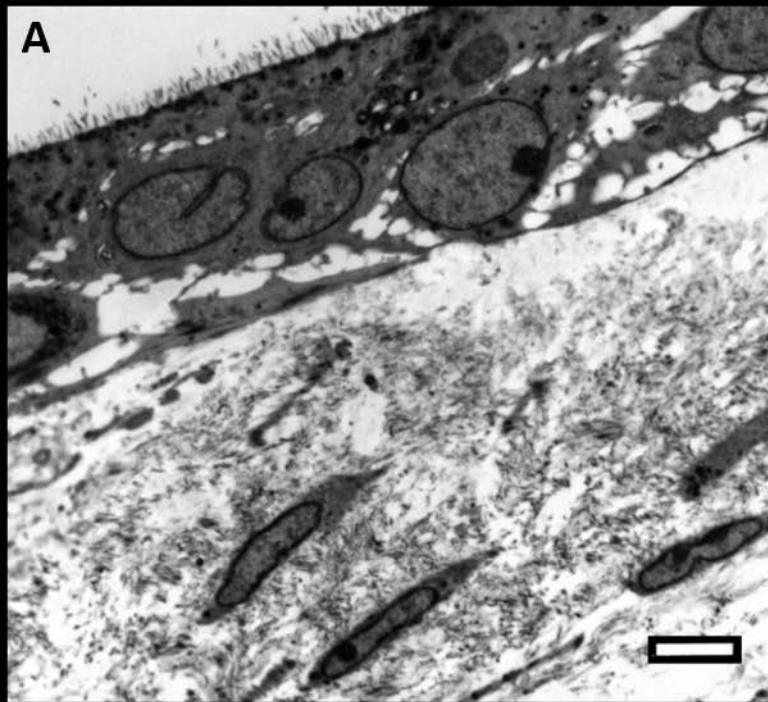
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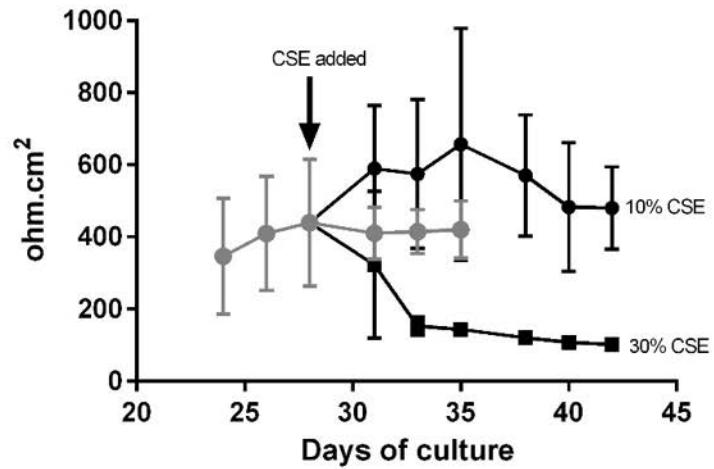
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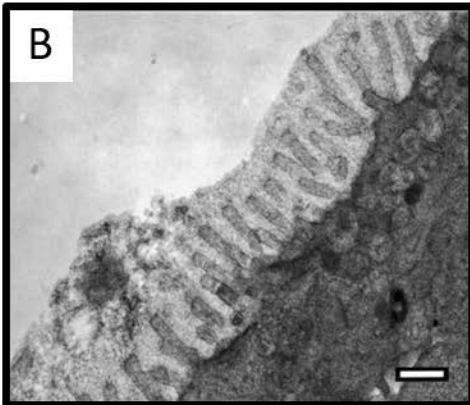




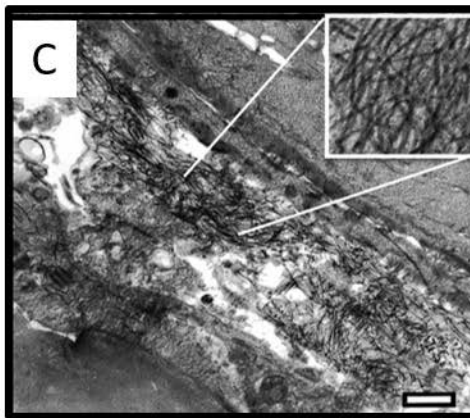
A



B



C



Supplemental Information

Functional Characterization of a Novel 3D Model of the Epithelial-Mesenchymal Trophic Unit

Fabio Bucchieri^{1,3,4,6}, Alessandro Pitruzzella^{3,4}, Alberto Fucarino^{3,4}, Antonella Marino Gammazza^{3,4}, Celeste Caruso Bavisotto^{3,4}, Vito Marcianò³, Massimo Cajozzo⁵, Giorgio Lo Iacono⁵, Roberto Marchese⁷, Giovanni Zummo³, Stephen T Holgate^{1,2}, Donna E Davies^{1,2*}.

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Supplementary Figure legends

Suppl Figure 1 Time-lapse phase contrast microscopy (frames taken every 10 minutes) from a representative 6 day-old culture showing how the cultures developed as an outgrowth of cells from the biopsy to form a multi-layered structure , with cells migrating as a coherent sheet. The video shows the last 18 hours in culture with the frames compressed into 10 sec.

Suppl Figure 2 Live video microscopy of a representative 32 day old culture showing beating cilia.

Suppl Figure 3 Representative TEM images of a 52 day-old culture illustrating cytoplasmic processes with caveolae-like structures as observed in fibroblastic cells (A), and high magnification image of fibrillar material surrounding the fibroblasts showing the characteristic D-banding of collagen fibrils (B).

