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TITLE:
Production, characterization and potential uses of a 3D tissue-engineered human oesophageal mucosal model

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KEYWORDS:
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SHORT ABSTRACT:
This manuscript describes the production, characterization and potential uses of a tissue engineered 3D oesophageal construct prepared from normal primary human oesophageal fibroblast and squamous epithelial cells seeded within a de-cellularised porcine scaffold. The results demonstrate the formation of a mature stratified epithelium similar to the normal human oesophagus.

LONG ABSTRACT:
The incidence of both oesophageal adenocarcinoma and its precursor, Barrett's Metaplasia, are rising rapidly in the western world. Furthermore oesophageal adenocarcinoma generally has a poor prognosis, with little improvement in survival rates in recent years. These are difficult conditions to study and there has been a lack of suitable experimental platforms to investigate disorders of the oesophageal mucosa.

A model of the human oesophageal mucosa has been developed in the MacNeil laboratory which, unlike conventional 2D cell culture systems, recapitulates the cell-cell and cell-matrix interactions present *in vivo* and produces a mature, stratified epithelium similar to that of the normal human oesophagus. Briefly, the model utilises non transformed normal primary human oesophageal fibroblasts and epithelial cells grown within a porcine-derived acellular oesophageal scaffold. Immunohistochemical characterization of this model by CK4, CK14, Ki67 and involucrin staining demonstrates appropriate recapitulation of the histology of the normal human oesophageal mucosa.

This model provides a robust, biologically relevant experimental model of the human oesophageal mucosa. It can easily be manipulated to investigate a number of research questions including the effectiveness of pharmacological agents and the impact of exposure to environmental factors such as alcohol, toxins, high temperature or gastro-oesophageal refluxate components. The model also facilitates extended culture periods not achievable with conventional 2D cell culture, enabling, *inter alia*, the study of the impact of repeated exposure of a mature epithelium to the agent of interest for up to 20 days. Furthermore, a variety of cell lines, such as those derived from oesophageal tumours or Barrett's Metaplasia, can be incorporated into the model to investigate processes such as tumour invasion and drug responsiveness in a more biologically relevant environment.

INTRODUCTION:
The oesophageal mucosa comprises a stratified, squamous epithelium above a layer of connective tissue, the lamina propria, and is one of the first sites to encounter ingested environmental stressors. Exposure to dietary toxins is implicated in the development of oesophageal squamous carcinoma, while duodenogastro-oesophageal reflux is a critical factor in the pathogenesis of Barrett's Metaplasia, which is associated with increased risk of progression to oesophageal adenocarcinoma. Oesophageal carcinomas are the 8th most common malignant tumour in UK males and oesophageal adenocarcinoma is rapidly increasing in the Western world[1]. Furthermore, there has been little improvement in disease prognosis, with an overall 5 year survival rate of around 15%. Consequently there is a need for experimental platforms to investigate the impact of exposure to environmental stressors on this oesophageal epithelium and their potential involvement in the development of metaplasia or neoplasia.

Although immortalised or tumour cell lines allow researchers to study the response of epithelial cells to these stressors *in vitro*, they remain proliferative and fail to differentiate into the mature epithelial cells found on the uppermost layers of the oesophageal mucosa.

Furthermore, cells lines that have already undergone tumourigenesis may provide only limited information regarding the initial responses of normal cells within the epithelium to environmental factors; and this is the stage when the potential for therapeutic intervention may be highest. Finally, conventional cell culture systems fail to capture the potentially important interactions between epithelial and mesenchymal cells and between these cells and the surrounding matrix that occur within tissues *in vivo*.

Animal models provide a more realistic microenvironment for studying the responses of the oesophageal epithelium and can incorporate the artificial induction of gastro-oesophageal reflux disease[2]. However it can be more challenging to manipulate the environmental stressors in these models and they may not fully represent the response within the human oesophagus.

Other experimental human oesophageal models have been developed that utilise primary cells, immortalised cells or tumour cell lines on a collagen, or combined collagen/Matrigel, scaffold containing fibroblasts[3,4]. It is less labour intensive to generate these scaffolds than the acellular oesophageal scaffold described in this manuscript, and these organotypic models provide a useful tool, particularly in the study of tumour invasion[5,6], where tumour cell infiltration into the collagen gel can be readily observed. However these collagen gels have non-native mechanical properties and lack certain features of the original tissue, including a specific basement membrane and the appropriate surface topography. This can influence the behaviour of cells resulting in, for example, poorer adhesion between the epithelium and scaffold when using a collagen gel scaffold[7]. As a consequence the acellular porcine oesophageal scaffold was developed, with the advantage of being a more biologically realistic scaffold and thus more appropriate for use as an experimental platform. It has also been shown that it is better to incorporate primary cells into the oesophageal constructs than immortalised oesophageal epithelial cell lines, such as Het-1A, since these cells form a multi-layered epithelium but fail to stratify or differentiate[4,7,8].

Consequently, this protocol has been adapted from a method already in use in the MacNeil laboratory for making tissue engineered skin and oral mucosa[9,10] and incorporates a de-cellularised porcine oesophageal scaffold combined with primary human oesophageal epithelial cells and fibroblasts. This protocol produces a mature, stratified epithelium, similar to that of the normal human oesophagus as demonstrated by CK4, CK14, Ki67 and involucrin staining. The resulting model provides an experimental platform to study responses to environmental stressors, and has been used effectively to investigate changes in gene expression in the oesophageal epithelium in response to refluxate components[11].

PROTOCOL:

Human oesophageal cells are obtained from patients undergoing gastric or oesophageal surgery. Informed consent is obtained for the tissue to be used for research purposes, and the tissue used anonymously under the appropriate ethical approvals (SSREC 165/03, Human Research Tissue Bank Licence 12179).

1. Isolation of human oesophageal epithelial cells

1.1) Working in a laminar flow tissue culture hood and using sterile technique, prepare the epithelial culture medium by mixing Dulbecco's Modified Eagle's medium (DMEM) and Ham's F12 in a 3:1 ratio (v:v). Add 10% (v/v) FCS, 10 ng/ml epidermal growth factor, 0.4 μg/ml hydrocortisone, $1.8x10^{-4}$ M adenine, 5 μg/ml insulin, 5 μg/ml transferrin, $2x10^{-7}$ M triiodothyronine, $1x10^{-10}$ M cholera toxin, $2x10^{-3}$ M glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.625 μg/ml amphotericin B.

1.2) Using standard sterile techniques, put 11 ml of DMEM, 10% FCS, $2x10^{-3}$ M L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.625 μg/ml amphotericin B into a T75 tissue culture flask. Add $1x10^{6}$ lethally irradiated mouse 3T3 fibroblasts[12] in 1 ml of the same medium and incubate overnight at 37 ˚C in a 5% CO_2, humidified atmosphere. This will produce a feeder layer for the subsequent culture of epithelial cells.

1.3) Using a scalpel and following the recommendations of a histopathologist, dissect an approximately 2 cm^2 sample of tissue from the disease-free background region of oesophageal squamous mucosa obtained from patients undergoing gastric or oesophageal surgery. Transport to the laboratory in a 50 ml container of sterile transport medium (PBS 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.625 μg/ml amphotericin B) at room temperature.

1.4) At this point, store the tissue in the transport medium for up to 12 hours (overnight) at 4 ˚C, for convenience; however do not use longer storage periods as they result in reduced cell viability.

1.5) Working in a laminar flow tissue culture hood and using sterile technique, sterilise a scalpel blade by soaking in 70% ethanol for 5 minutes. Rinse in sterile PBS.

1.6) Place the tissue in a sterile petri-dish and use the scalpel to cut it into 0.5 cm strips. Add 5 ml of 0.1% (w/v) trypsin in PBS to the petri-dish, place the lid on the dish and incubate for 1 h at 37 ˚C.

1.7) Add 5 ml of FCS to the tissue in the petri-dish to inhibit the trypsin. Holding the strip of tissue with sterile forceps, gently scrape the epithelial surface with the scalpel blade for one to two minutes per strip to remove the epithelial cells into the surrounding medium. Remove the scraped tissue and set aside. Repeat the process for all the tissue strips.

1.8) Collect the medium containing the detached epithelial cells into a 15 ml centrifuge tube and centrifuge (5 minutes, 200 x g). Pour off the supernatant and resuspend the pellet in 12 ml of epithelial medium (described in step 1.1).

1.9) Pour off and discard the medium from the feeder cell layer in the T75 flask prepared in step 1.2. Use a sterile pipette to add the cell suspension containing the freshly isolated epithelial cells to the flask and incubate at 37 ˚C in a 5% CO_2, humidified atmosphere.

1.10) After 24 hours pour off and discard the culture medium, which will also contain cell debris, and replace with 12 ml of fresh epithelial medium. Continue incubating at 37 ˚C in a 5% CO_2, humidified atmosphere. Colonies will start to appear over the next 24 to 48 hours and can be viewed by placing the culture flask under a standard light microscope. Pour off the spent culture medium and replace with an equal volume of fresh medium every 2 to 3 days.

1.11) Once the flask has reached 80% confluency, passage the cells[13]. Split the cells in a ratio of 1:5 to increase cell numbers for use in the model. Follow step 1.2 to establish feeder layers of irradiated 3T3 cells in each flask that will be receiving epithelial cells 24 hours prior to passaging the cells.

Note: Use the epithelial cells in the oesophageal model described below between passages 1 and 4 only.

2. Isolation of human oesophageal fibroblasts

2.1) Working in a laminar flow cabinet and using sterile technique, take the tissue set aside in step 1.7. Place in a sterile petri-dish and mince finely by chopping with a sterile scalpel blade. Add 10 ml of 0.5% (w/v) collagenase A solution and place the lid on the petri dish and incubate at 37 ˚C overnight in a 5% CO_2, humidified atmosphere.

2.2) Transfer the digest to a 50 ml centrifuge tube and centrifuge (10 min, 200 x g), carefully pour off and discard the supernatant and resuspend the pellet in 10 ml of fibroblast culture medium (DMEM 10% FCS, $2x10^{-3}$ M L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.625 µg/ml amphotericin B).

2.3) Place the suspension into a T75 flask and incubate at 37 ˚C in a 5% CO_2, humidified atmosphere. After 24 hours pour off the medium, which will contain debris and replace with 12 ml of fresh fibroblast medium. Replace the culture medium every 2 to 3 days and passage cells once they have reached 80% confluency[13], splitting the cells in a 1:5 ratio to increase cell numbers.

Note: Use the fibroblast cells in the model described below between passages 4 and 10 only.

3. Preparation of the de-cellularised oesophageal scaffold

3.1) Obtain intact porcine oesophagi at an abattoir from freshly slaughtered Landrace pigs that are used for food production. One single oesophagus will provide sufficient scaffolds for approximately 30 separate constructs.

Note: Due to the extensive and time consuming sterilisation protocol required, it is normally worth obtaining and processing a minimum of 10 oesophagi at one time.

3.2) Immediately place the freshly excised oesophagus into a 180 ml sterile pot containing approximately 150 ml of 10% povidone-iodine solution for 5 min to reduce any contaminating microbial load. Transfer the oesophagus to a second pot containing approximately 100 ml of sterile PBS containing 200 IU/ml penicillin, 200 µg/ml streptomycin, and 1.25 µg/ml amphotericin B.

3.2.1) Replace the lid on the container and transport the oesophagi back to the laboratory at room temperature. Two or three oesophagi can be transported in each container, depending upon their size.

3.3) Once at the laboratory, handle the tissue in a laminar flow hood using aseptic technique to minimise microbial contamination. Sterilise tweezers, scissors and scalpel blades by soaking for 5 minutes in 70% ethanol, and rinsing in sterile PBS.

3.4) Handle the oesophagus using sterile tweezers. Using the scissors, cut open the oesophagus longitudinally. Rinse the tissue by placing the oesophagus into a 180 ml sterile pot containing approximately 100 ml of sterile PBS and shaking gently to remove any debris.

3.5) Clean a cork dissection board by spraying liberally with 70% ethanol and allow to dry.

3.6) Remove the oesophagus from the PBS, and using sterile needles, pin out the oesophagus onto the board, mucosal surface uppermost. Grasp the mucosal surface at one end of the oesophagus with sterile tweezers and pull away from the board. This will separate the mucosa from the underlying submucosa.

3.6.1) Then, use a sterile scalpel blade to dissect the oesophagus longitudinally along the lamina propria, removing pins as the dissection progresses to allow the mucosa to be lifted away.

3.7) Retain the dissected mucosa and discard the underlying submucosa.

3.8) Cut off and discard the most proximal and distal 2 cm of the mucosa as there is a greater number of submucosal glands and a thicker lamina propria in those areas, respectively[7].

3.9) Use a scalpel to cut the remaining tissue into 5 cm^2. Place all the cut tissue into a 180 ml sterile pot containing approximately 150 ml of sterile PBS and agitate gently to rinse the tissue. If processing more than five oesophagi at one time it may be necessary to use multiple pots for this and the three subsequent steps.

3.10) Using sterile tweezers, transfer the cut tissue into a 180 ml pot containing 150 ml of sterile 1 M NaCl, 200 IU/ml penicillin, 200 µg/ml streptomycin and 1.25 µg/ml amphotericin B. Incubate for 72 h at 37 ˚C.

3.11) Place the tissue into a fresh 180 ml pot containing 150 ml sterile PBS. The epithelium

will have begun to visibly separate from the underlying tissue. Gently peel the detaching epithelium from the pig oesophagus using sterile forceps and discard.

3.11.1) Place the remaining tissue into a fresh 180 ml pot and add 150 ml of sterile PBS. Agitate gently for 5 minutes to wash. Pour off the PBS and repeat a further two times with fresh PBS.

3.12) Place all the tissue into a 500 ml glass bottle containing 400 ml of sterile 80% (v/v) glycerol solution for 24 hours at room temperature. Transfer to 400 ml of sterile 90% (v/v) glycerol solution for a further 24 hours.

3.13) Store in 400 ml of sterile 100% glycerol solution at room temperature for a minimum of 4 months to dehydrate and sterilise the tissue while preserving the integrity of the basement membrane.

4. Production of the culture media

4.1) Prepare chelated newborn calf serum for use in the culture medium.

4.1.1) Pour 100 g of Chelex 100 into a glass 1 L bottle and add de-ionised water. The exact volume is not critical but sufficient water must be added to cover the Chelex powder. Add a magnetic stirrer and sterilise by autoclaving (121 ˚C for 15 minutes).

4.1.2) Allow the bottle to cool and the Chelex to settle. In a laminar flow cabinet pour off the excess water, add 500 ml of newborn calf serum (NCS) and leave to stir overnight at 4 ˚C.

4.1.3) Stop stirring and allow the Chelex to settle, this can take up to 30 minutes. In a laminar flow cabinet decant the chelated serum using a sterile pipette, taking care not to disturb the Chelex, and store in 10 ml aliquots at -20 ˚C.

4.2) Prepare three different media in a laminar flow cabinet, commencing with pro-proliferative and ending with pro-differentiative formulations.

4.2.1) Prepare Composite Medium I comprised of DMEM and Hams F12 in a 3:1 ratio (v:v), 4 $x10^{-3}$ M L-glutamine, 0.5 µg/ml hydrocortisone, $1x10^{-4}$ M O-phosphorylethanolamine, $2x10^{-12}$ M triiodothyronine, $1.8x10^{-4}$ M adenine, $1.88x10^{-3}$ M $CaCl_2$, $4x10^{-12}$ M progesterone, 10 µg/ml insulin, 10 µg/ml transferrin, 10 ng/ml selenium, $1x10^{-3}$ M ethanolamine and 0.1% (v/v) chelated NCS.

4.2.2) Prepare Composite Medium II as Composite Medium I except replace chelated serum with 0.1% (v/v) non-chelated NCS.

4.2.3) Prepare Composite Medium III as Composite Medium II except omit progesterone and increase serum to 2% (v/v) non-chelated NCS.

5. Production of the human oesophageal mucosa model

5.1) Perform all work in a laminar flow hood, using aseptic technique to maintain a sterile environment. Sterilise all tools by soaking in 70% ethanol for 5 minutes, before rinsing in sterile PBS.

5.2) The day before it is required, rehydrate the acellular porcine oesophageal scaffold. One piece of scaffold is required for each construct to be prepared.

5.2.1) To rehydrate the tissue place sufficient pieces of porcine scaffold into a pot containing 100 ml of sterile PBS, agitate and soak for 10 minutes. Decant the PBS and replace with fresh PBS. Repeat the washing process five times to ensure removal of all glycerol traces.

5.3) Test the scaffold sterility by incubating the rehydrated tissue overnight in 100 ml of DMEM at 37 ˚C. If there is no change to the colour or turbidity of the culture medium at the end of the incubation period the scaffold is assumed to be sterile. Once sterility has been confirmed, place the 5 cm^2 acellular scaffold into a 6 well plate, submucosal side uppermost.

5.4) Place a sterile medical grade stainless steel ring (internal diameter 10 mm, external diameter 20 mm) onto the centre of each scaffold and gently press down using sterile forceps, to ensure an adequate seal with the tissue.

5.5) Harvest the fibroblasts using Trypsin-EDTA[13] to produce a cell suspension. Count the cells using a haemocytometer[14]. Centrifuge the cell suspension (10 min, 200 x g). Resuspend the cell pellet into an appropriate volume of fibroblast medium to produce a final cell count of 2.5 x 10^6 cells per ml.

5.6) Add 0.2 ml of fibroblast medium, containing 5 x 10^5 human oesophageal fibroblasts, within each ring. Flood the area around the outside of the ring with approximately 2 ml of fibroblast medium. Incubate at 37 ˚C in a 5% CO_2, humidified atmosphere.

5.7) After 24 hours remove the rings and fibroblast medium and add at least 5 ml of fresh fibroblast medium to each well, ensuring that the scaffold is totally immersed in medium. Culture for 1 week, at 37 ˚C in a 5% CO_2, humidified atmosphere replacing the medium every 2 to 3 days.

5.8) After 1 week remove the 6 well plates containing the scaffolds to a laminar flood hood, remove the medium and invert each scaffold using sterile forceps, placing the mucosal surface uppermost. This time point is referred to as Day 0.

5.9) Place the steel rings on the mucosal surface in the centre of the tissue as in step 5.4.

5.10) Prepare the T75 flasks of epithelial cells for trypsinization[13]. After the PBS wash and prior to the addition of trypsin-EDTA solution, add 2 ml of sterile 0.02 % (w/v) EDTA solution to

each flask. Incubate for 2 minutes at 37 ˚C. Tap the flask gently to selectively detach the i3T3 feeder layer while leaving the epithelial cells attached.

Note: It is generally not practical or necessary to patient match the epithelial cells to the fibroblasts already incorporated in the model the previous week.

5.11) Pour off the EDTA solution containing the feeder layer and add trypsin-EDTA solution to harvest the epithelial cells[13]. Count the cells using a haemocytometer[14]. Centrifuge the cell suspension (10 min, 200 x g). Resuspend the cell pellet into an appropriate volume of Composite Medium 1 to produce a final cell count of 5 x 10^6 cells per ml.

5.12) Add 0.2 ml of Composite Medium I, containing 1 x 10^6 epithelial cells, within the ring. Flood the area around the outside of the ring with approximately 2 ml of Composite Medium I and place the constructs into the incubator at 37 ˚C in a 5% CO_2, humidified atmosphere.

5.13) After 24 hours (Day 1) remove the rings and medium and add at least 5 ml of fresh Composite Medium I, ensuring the scaffolds are fully submerged and return to the incubator. After a further 24 hours (Day 2) remove the medium and replaced with at least 5 ml of Composite Medium II, again ensuring the scaffolds are fully submerged and return to the incubator.

5.14) On Day 4 place sterile medical grade stainless steel mesh grids (2 cm wide x 2 cm long x 0.5 cm high), into fresh 6 well plates, one per construct. Use sterile tweezers to transfer the constructs onto the top of the steel grids, mucosal surface uppermost.

5.14.1) Add sufficient Composite Medium III so that the liquid reaches the underside of the composite, but the surface is exposed to the air, this ensures that the sample is maintained at an air-liquid interface. The exact volume of medium required will vary depending upon the thickness of the scaffold, the volume of the well and the precise height of the stainless steel grid.

5.15) Maintain the composites at an air-liquid interface for between 10 and 20 days (Day 14 to Day 24), depending upon the requirements of the experiment. Replace the Composite Medium III with fresh medium every 2 to 3 days (Monday, Wednesday, Friday is a user-friendly regime).

Note: After 5 days at an air-liquid interface (Day 9) the constructs have a sufficiently mature oesophageal epithelium to be used in experiments of the impact of environmental factors on the oesophageal epithelium.

5.16) At the end of the experiment, fix the constructs for histological analysis and immunohistochemical staining[7], or extract protein[15] or RNA[11,16] from the epithelium for further analysis. If required, retain the conditioned culture medium for analysis of extracellular signalling molecules[17].

REPRESENTATIVE RESULTS:

This manuscript describes the process required, shown in schematic form in **Figure 1**, to culture 3D models of the human oesophageal epithelium successfully. To confirm the suitability of the model as an experimental platform histological and immunohistochemical studies have been undertaken comparing the cultured tissues with normal human oesophageal squamous mucosa.

Histological assessment of the epithelium produced by the method described shows a mature, multi-layered, stratified squamous epithelium (**Figure 2B**) which is comparable to that observed with the normal human oesophagus (**Figure 2A**), albeit thinner (5 to 10 layers of cells compared with 10 to 20 for the normal oesophagus), with the cells becoming progressively flatter and ultimately anuclear as they migrate towards the surface.

Immunohistochemical characterization of key markers of proliferation and differentiation demonstrate that the microanatomy of the model epithelium is similar to the normal human oesophageal epithelium. Comparable Ki67 expression is observed in both the native oesophagus and the model epithelium, with staining restricted to a subset of cells within the basal and immediately suprabasal layers (**Figures 3A and 3B**). This is analogous to studies which report that less than 10% of cells generally show expression of the proliferation marker, Ki67, in the normal oesophageal epithelium[18]. CK4 is normally expressed in stratified and columnar epithelia but is generally absent from the basal layers while CK14 is only positive in the basal layer[19]. In both the normal and model oesophageal epithelia, CK14 is observed in all cells in the basal layer (**Figures 3D and 3E**) while CK4 is observed throughout the epithelium except for the two most basal layers (**Figures 3G and 3H**). Involucrin expression is an early marker of differentiation, expressed in the suprabasal layers of the epithelium[20]. Again both the normal human oesophageal tissue and the model epithelium show staining that reflects this (**Figures 3J and 3K**).

Attempts to replace the primary oesophageal epithelial cells with immortalised oesophageal epithelial cells, such as Het-1A, were less successful and did not produce a valid model of the normal oesophageal epithelium. A multi-layered epithelium was formed; however the proliferative marker Ki67 was detected throughout the epithelium (**Figure 3C**) with no expression detected for any markers of differentiation (**Figures 3F, 3I and 3L**), indicating that the Het-1A cells produced a hyperproliferative epithelium with no evidence of normal stratification or maturation.

However, the model has been successfully modified to incorporate tumour cells by the replacement of primary epithelial cells with either oesophageal adenocarcinoma (OE33) or squamous carcinoma (OE21) cells. This demonstrates the flexibility of the model, enabling its use in investigating a range of oesophageal disorders at different stages of progression through the inclusion of a number of different cell lines. It can be seen that there is a marked difference in the responses from these two cell lines. OE21 squamous carcinoma cells produces an epithelium visible on the construct as a defined yellow region (**Figure 4A**) with large clefts

within the epithelium (**Figure 4B**), likely to reflect dysfunctional cell adhesion molecules. Including OE33 adenocarcinoma cells within the model results in a large amount of scaffold degradation, visible by eye as a thinning of the scaffold after 2 weeks growth at the air/liquid interface (**Figure 4C**) and confirmed in H+E analysis as an obvious reduction in the thickness of the scaffold in the region below the cells (**Figure 4D**). This is a likely to be a result of an interaction between the tumour cells and fibroblasts, since the scaffold degeneration is not observed in the absence of fibroblasts (**Figures 4E and 4F**). We have observed similar impacts on tumour invasion in the presence and absence of fibroblasts in equivalent melanoma models[21].

Figure 1: Schematic diagram showing the production of the oesophageal mucosa model. Human oesophageal fibroblasts are seeded onto the submucosal surface of the scaffold and cultured for 7 days. The scaffold is inverted, human oesophageal epithelial cells added and cultured submerged for 4 days. The construct is raised to an air-liquid interface for between 10 and 20 days. At the end of the experiment the construct can undergo further analysis as required.

Figure 2: Comparison of epithelium produced in oesophageal model with normal human oesophageal epithelium. H+E analysis of (A) normal human oesophageal epithelium and (B) oesophageal epithelium formed in the model of the human oesophageal mucosa. Scale bar is 500 μm.

Figure 3: Immunohistochemical characterization of the oesophageal epithelium produced in the model and comparison with the normal human oesophagus. IHC analysis of normal oesophagus (column 1) and the model oesophagus produced using primary human oesophageal epithelial cells (column 2) or immortalised oesophageal epithelial cells (column 3). Characterisation was by Ki67 (A-C), CK14 (D, E), CK4 (G, H) and involucrin staining (J, K). Scale bar is 200 μm. (This figure has been modified[7]).

Figure 4: The inclusion of tumour cell lines within the oesophageal model. The primary epithelial cells were replaced by the squamous carcinoma cell line, OE21, or the adenocarcinoma cell line, OE33. Images show the construct with the OE21 cells (A) or OE33 cells either in conjunction with (C) or in the absence of (E) fibroblasts, prior to fixing at the end of the culture period. The epithelia including the OE21 (B) or OE33 cells with (D) or without (F) fibroblasts are visualised by H+E staining. Scale bar is 500 μm.

DISCUSSION:

This manuscript describes the production and characterization of a biologically relevant human oesophageal mucosal model suitable for use as an experimental platform to study the impact of exposure to environmental stressors upon the oesophageal epithelium.

The most critical steps for the successful production of a human oesophageal mucosal model are: ensuring that the majority of the epithelial cells remain proliferative and have not already begun to differentiate prior to seeding them on the scaffold; maintaining an air-liquid interface

for the composite to ensure correct maturation of the epithelium; maintaining sterility throughout the extended culture period.

Due to the limited amounts of human tissue available for cell isolation it is not generally possible to obtain sufficient cells from a single tissue sample to seed freshly isolated cells directly onto the scaffold and consequently a cell expansion phase is required where the cells are grown in standard 2D cell culture conditions. It is important to ensure that cells remain proliferative during this period. This is achieved by ensuring that the cultures are never allowed to reach full confluency, using cells in the model only up to passage 4 and carefully monitoring cell morphology. Thus cells should retain their distinctive proliferative polygonal morphology and the tight “cobblestone” pavement-like appearance characteristic of proliferative epithelial cells rather than the more diffuse appearance observed in cultures which have begun to differentiate. The second critical step is controlled by ensuring that the cell culture medium covers the top surface of the stainless steel grids used to lift the cultures to air-liquid interface but the uppermost surface of the construct itself is not submerged. The third step is dependent upon strict adherence to the extended sterilization protocol when producing the de-cellularised porcine scaffold and the use of good sterile technique throughout the process.

Our results show that primary human oesophageal cells are required to produce a normal, mature, stratified epithelium. If an immortalised cell line was used, albeit derived from the human oesophageal epithelium, the cells remained proliferative and failed to differentiate to form a mature stratified epithelium. The resulting epithelium could not be used as a satisfactory model for the normal epithelium, demonstrating the unsuitability of the immortalised cell line Het-1A for use in the model. However other cell lines such as those derived from oesophageal tumours or Barrett’s Metaplasia can be incorporated into the model either in place of or in addition to the primary epithelial cells to produce models for the study of tumour progression, invasion and the response to pharmacological agents.

Experiments can be performed using the oesophageal model to simulate the exposure of the oesophagus to discrete, pulsatile events during swallowing or reflux. This is achieved by repeatedly submerging the constructs in culture medium containing the compound(s) to be tested and rinsing in PBS before returning the construct to an air-liquid interface. The exposure times and frequency can be modified to reflect the process being simulated, ensuring that the epithelial layer is exposed to the environmental factor(s) while allowing the culture to continue at an air-liquid interface. This method has been used in our laboratory to investigate the impact of reflux on the normal oesophageal epithelium, where the model was exposed to specific gastro-oesophageal reflux components for 10 minutes twice a day for 11 days[11]. Alternatively the constructs can be exposed to continuous levels of environmental stressors by including these compounds in the culture medium. However, since the epithelium must be exposed to an air-liquid interface for the epithelium to mature and differentiate properly[7], it is not possible to continuously submerge the constructs in the culture medium. Consequently, exposure to the stressor will only be via the submucosal surface in these experiments, which may limit the relevance of results obtained.

The technique is relatively labour intensive and consequently is better suited to the screening of relatively limited numbers of compounds. As a result, when using the model as an experimental platform, it is appropriate to first perform preliminary studies using conventional cell culture techniques to identify a limited number of conditions prior to further analysis using the more physiologically relevant oesophageal model described here[11]. In addition to immunohistochemical analysis it has also been possible to examine changes in the epithelial gene expression profile following exposure to environmental stressors. This was achieved by manually stripping away the epithelium, extracting the RNA and converting it to cDNA to isolate the genes being expressed and analysing by microarray to obtain gene expression profiles[11]. In this way it has been possible to increase the information available from the model as an experimental platform. Early changes in the epithelial gene expression profile following simulated exposure to gastro-oesophageal reflux have been proposed and from these results new areas have been suggested for further investigation in the prevention of the development of Barrett's Metaplasia, the metaplastic precursor to OAC[11]. The model could also be used to study protein expression using methods such as Western blot analysis[15] and gene expression using Northern blot analysis[16]. Moreover studies of extracellular signalling molecules could be performed on the culture medium[17].

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DISCLOSURES:
The authors have nothing to disclose.

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