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Methodology for the Establishment of Primary Porcine Vocal Fold Epithelial Cell Cultures

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Abstract

Objective: A current lack of methods for epithelial cell culture significantly hinders our understanding of the role of the epithelial and mucus barriers in vocal fold health and disease. Our first objective was to establish reproducible techniques for the isolation and culture of primary porcine vocal fold epithelial cells. Our second objective was to evaluate the functional significance of cell cultures using an in vitro exposure to an inflammatory cytokine.

Methods: Epithelial cells were isolated from porcine vocal folds and expanded in culture. Characterization of cultures was completed by immunostaining with markers for pan-cytokeratin (epithelial cells), vimentin (stromal cells), von Willebrand factor (endothelial cell), and MUC1 and MUC4 (mucin) glycoproteins. Established epithelial cell cultures were then exposed to the inflammatory cytokine tumor necrosis factor alpha (TNF-α) for 24-hours, and transcript expression of MUC1 and MUC4 was evaluated.

Results: Reproducible, porcine vocal fold epithelial cell cultures, demonstrating cobblestone appearance characteristic of the typical morphology of epithelial cell cultures were created. Cells showed positive staining for pan-cytokeratin with limited expression of vimentin and von Willebrand factor. Epithelial cells also expressed MUC1 and MUC4. TNF-α significantly increased transcript expression of MUC4.

Conclusion: Here, we present the first report of successful culture of primary porcine vocal fold epithelial cells. Cultures will provide researchers with a valuable new in vitro tool to investigate vocal fold epithelium and mucus as well as the effects of common challenges, including inflammatory cytokines, on these barriers.

Keywords

Vocal fold; epithelium; mucin; porcine; cell culture

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INTRODUCTION

The vocal fold epithelium is the first layer of cellular interaction with inhaled and systemic irritants in the airway.[1] A thin layer of fluid, referred to as mucus, covers the epithelial surface and, together with epithelial cells, play a key role in the formation of biological barriers that protect the vocal folds from such irritants.[2] Vocal fold epithelial cells and mucus have been implicated in the development of voice disorders resulting from phonotrauma,[3] laryngopharyngeal reflux,[2,4] and laryngitis.[5] Alterations in the protective properties offered by epithelial cells and the mucus layer in these disorders not only promotes the accumulation and entry of damaging irritants into the vocal folds but also may compromise vocal fold vibration and voice quality. Yet, comprehensive study of vocal fold epithelial cells and the associated mucus layer and their contribution to the development of voice disorders is hindered by lack of epithelial cell culture techniques. Techniques for epithelial cell culture are required in order to facilitate the investigation of epithelial and mucus-specific contributions to vocal fold disease processes.

Cell cultures are a valuable tool for the controlled, in vitro study of cellular and molecular mechanisms underlying vocal fold function in health, injury, and disease. Cultures of vocal fold fibroblasts from a variety of species are currently applied in basic research.[6–8] Use of these cells has permitted significant advances in our understanding of vocal fold tissue engineering,[9–11] disease processes,[12,13] and potential therapeutics.[14] There have been reports on the culture of human epithelial cells from other locations in the larynx, including the supraglottic and posterior cricoid areas.[15–17] However, these cells are a different epithelial cell type; they are pseudostratified columnar, whereas those covering the membranous vocal folds are stratified squamous.[1] Cell culture techniques vary widely by tissue site; consequently, these existing culture techniques are not a viable alternative. To date, to the best of our knowledge, only two investigations have reported culture of human vocal fold epithelial cells.[18,19] However, the success rate of human vocal fold epithelial cultures has either not been stated or been reported at less than 50%.[18] Furthermore, studies of human epithelial culture from the vocal folds and other sites within the larynx typically do not test for contaminating cell lineages.[15,16,18] Responsiveness, or functionality of cultures to physiologically relevant external stimuli has also not been routinely tested.[18] Although human cultures are highly valuable, primary vocal fold epithelial cell cultures are rarely feasible with disease-free tissue from human donors. Furthermore, researchers are required to separate normal from diseased tissue prior to epithelial cell isolation,[18] increasing the likelihood of contamination of cultures with diseased cells. Epithelial cells are difficult to obtain from healthy human vocal folds without significant risk to vocal function. We are in critical need of alternative techniques for vocal fold epithelial cell cultures that are easily reproducible with continuously available sources.

Animal models are frequently used to study vocal fold function in vivo and in vitro. There has been a report of successful culture and characterization of rabbit vocal fold epithelial cells.[20] An ideal animal model of epithelium will demonstrate morphological and functional properties similar to those of humans. A porcine model was chosen for the current study because it has advantages over other animal models, such as rabbit. Specifically, the morphology of vocal fold epithelium of pigs, compared to that of other animals, is most

similar to that of humans[21] and has been used extensively in ex vivo models to investigate epithelial barrier function.[22–24] In addition, fresh porcine vocal fold tissue is readily and continuously available from animals intended for sacrifice. Finally, a porcine model provides for the large amount of tissue often required for successful epithelial cell culture.[18] Once established, cultures can be used to study cell behavior in health and disease.

Mucin production and regulation by epithelial cells is critical to the formation and protective properties offered by the vocal fold mucus barrier.[2] Specifically, highly hydrated mucin (MUC) glycoproteins are the primary protein constituent and building blocks of mucus and determine properties such as the amount and thickness of this barrier.[25] Currently, 20 mucin genes have been identified in airway epithelial cells and their encoded glycoproteins classified as secreted or membrane-associated. MUC1 and MUC4 are mucin glycoproteins that exist either in membrane-associated or secreted forms[26] and have been shown previously to be present in porcine vocal fold epithelial tissue.[27] These mucins are activated during inflammation in a variety of tissue types. Inflammation is a common clinical sign of patients with voice disorders.[28,29] For example, an immediate increase in inflammatory cytokines, including TNF-α is observed in response to vocal fold injury.[30] TNF-α upregulates mucin expression in epithelial cells derived from ocular,[31] airway,[32] and uterine tissues.[33] The effect of TNF-α on vocal fold epithelial MUC1 and MUC4 is currently unknown.

The primary purpose of this investigation was to establish a protocol for the isolation and culture of primary porcine vocal fold epithelial cells. Porcine epithelial cell cultures were expanded and then characterized using immunofluorescent staining for specific epithelial and nonepithelial markers, as well as MUC1 and MUC4. The secondary purpose of this study was to evaluate the functional significance of epithelial cell cultures by investigating whether the expression of MUC1 and MUC4 is influenced by the inflammatory cytokine TNF-α. This work expands upon previous studies by characterizing vocal fold cultures with both epithelial and nonepithelial markers, use of positive and negative controls, and demonstration of functionality of cultures with exposure to a physiologically relevant challenge. Porcine primary epithelial cell cultures will provide researchers with a valuable new in vitro tool to investigate vocal fold epithelium and mucus as well as the effect the effects of common challenges, including inflammatory cytokines, on these barriers.

MATERIALS AND METHODS

Vocal Fold Tissue Collection and Preparation

Porcine larynges were collected from local abattoirs from healthy, male and female 6-month-old animals, within 30 minutes of sacrifice. Given larynges were collected post-animal sacrifice, Institutional Animal Care and Use Committee approval was not required for this investigation. Larynges were transported to the laboratory in ice-cold, sterile phosphate-buffered saline (PBS) supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL). Immediately upon arrival to the laboratory, larynges were divided into two hemilarynges by dissection along the mid-sagittal plane to reveal the vocal folds. Vocal folds were resected to the thyroarytenoid muscle and removed from the larynx. The membranous vocal folds are covered by stratified squamous epithelium that transitions to pseudostratified

columnar epithelium at the anterior and posterior commissures, supraglottis, and subglottis.[1] To limit epithelial cultures to primarily squamous cells, resected vocal folds were trimmed to include only the membranous vocal fold and remove areas of transitional epithelium toward the anterior and posterior commissures and the supraglottis and subglottis. Trimmed membranous vocal folds, containing epithelium and superficial lamina propria, were transferred to a sterile laminar flow hood for epithelial cell isolation. Time between collection of larynges and beginning cell isolation was approximately 60 to 90 minutes.

Isolation and Culture of Vocal Fold Epithelial Cells

Vocal fold epithelial cells were isolated and cultured by modification of a cell culture technique described by Kalabis et al. for esophageal epithelial cells.[34] Starting amount of tissue for a single primary culture was both vocal folds from a single larynx. Resected vocal folds were gently washed two times in calcium and magnesium-free Hanks' balanced salt solution (HBSS) containing penicillin (100 U/mL) and streptomycin (100 μg/mL) (HBSS+). After washing, vocal folds were incubated overnight at 4°C with Dispase II (1 U/mL, Life Technologies Corp., Carlbad, CA) dissolved in HBSS+. Following overnight incubation, fine forceps were used to separate the epithelium as strips from the underlying superficial lamina propria. Epithelial strips were placed in fresh HBSS+. To disperse the tissue strips into a single-cell suspension, the strips were incubated in 1 mL trypsin-EDTA (Ethylenediaminetetraacetic acid) (0.05% (weight/volume) trypsin, 0.53 mM EDTA, Life Technologies Corp.) for 10 minutes at 37°C. The mixture was then vortexed for 10 seconds, and the trypsin-EDTA with floating cells were pipetted into a separate centrifuge tube containing 8 mL soybean trypsin inhibitor (0.25 mg/mL, Sigma Aldrich, St. Louis, MO). Remaining epithelial strips were incubated in an additional 1 mL of trypsin-EDTA for 5 minutes at 37°C and vortexed for 10 seconds. Trypsin-EDTA with floating cells and undigested strips were then transferred to the previous cell suspension in soybean trypsin inhibitor. The entire cell suspension was filtered through a 40-μm cell strainer and centrifuged at 188 g for 5 minutes at 4°C. Supernatant was discarded, and cells were resuspended in flavinoid adenine dinucleotide (FAD) media. FAD media was composed of Ham's F-12/DMEM (3:1 ratio), fetal bovine serum (2.5%), hydrocortisone (0.4 μg/mL), cholera toxin (8.4 ng/mL), insulin (5 μg/mL), adenine (24 μg/mL), epidermal growth factor (10 ng/mL), penicillin (100 U/mL), and streptomycin (0.01 mg/mL). Cells isolated from each larynx were plated in a single well of a six-well plate coated with collagen-IV (50 μg/mL, Sigma Aldrich). Cells were kept at 37°C in a cell culture incubator with 5% CO_2 to 95% air. Media was replaced every 48 hours. Cell morphology and growth was examined daily for 4 to 6 days using light microscopy.

Characterization of Vocal Fold Epithelial Cell Cultures

Vocal fold epithelial cell cultures were characterized using immunocytochemistry to confirm cell type and purity of culture. Cells were stained directly in plates following 4 to 6 days in cultures. To establish that cultures demonstrate primarily epithelial cell type; immunofluorescent staining was completed against pancytokeratin, which recognizes cytokeratins 5, 6, and 8. Expression of epithelial membrane-associated mucins was shown using antibodies against MUC1 and MUC4. To evaluate purity of the primary cultures, immunofluorescent staining was completed against vimentin and von Willebrand factor

(vWf) for detection of potentially contaminating stromal and endothelial cells, respectively. Staining for each marker was completed in three separate cultures. For primary antibody details see Table I.

Cell cultures were washed three times in PBS and fixed with 4% paraformaldehyde for 20 minutes. Cells were permeabilized with three washes in 0.1% Triton X-100 and blocked for 1 hour at room temperature in 10% normal goat serum. Cells were then incubated with primary antibodies diluted in 1% blocking solution at 4°C overnight. Incubation in 1% blocking solution served as a negative control. Cells were then washed three times in PBS and either AlexaFlour 488 goat anti-rabbit or anti-mouse IgG secondary antibody was applied to cells in the dark for 1 hour at room temperature (1:500, Life Technologies Corp.). Following three additional washes in PBS, VECTASHIELD HardSet Mounting Medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) was added to counterstain cell nuclei. Cells were immediately viewed and photographed using EVOS digital inverted fluorescent microscope (ThermoFisher Scientific, Waltham, MA).

Vocal folds dissected from porcine larynges were utilized as a positive control to assess the specificity of chosen markers for epithelial cells and contaminating cell lineages. Vocal folds were fixed in 10% neutral buffered formalin, dehydrated via an ethyl alcohol series, and embedded in paraffin. Tissue was sectioned (5 μm) and then characterized using immunofluorescence (pancytokeratin, vimentin, vWf) or immunohistochemistry (MUC1, MUC4). Sections were heat-retrieved using a citrate buffer (pH 6). Blocking was performed at room temperature by incubating sections in 10% goat serum for 60 minutes. Primary antibodies were diluted in 1% blocking solution and applied at 4°C overnight. For immunofluorescence, either AlexaFlour 488 goat anti-rabbit or anti-mouse IgG secondary antibody was applied to tissue sections in the dark for 1 hour at room temperature (1:500, Life Technologies Corp.). Sections were washed in PBS and VECTASHIELD HardSet Mounting Medium (Vector Laboratories) containing DAPI was added to counterstain cell nuclei. For immunohistochemistry, biotinylated secondary antibodies (ImmPRESS Polymer, Vector Laboratories) were added for 30 minutes; visualization was then achieved using DAB (3,3′-Diaminobenzidine) horseradish peroxidase substrate kit for immunohistochemistry for 3 to 5 minutes (BD Biosciences, San Diego, CA). Vocal fold sections were viewed and imaged using a Nikon Eclipse TE 2000-S (Tokyo, Japan).

In Vitro Exposure to an Inflammatory Cytokine

Vocal fold epithelial cells were grown to 70% to 90% confluence and then incubated in low serum FAD media (0.5% serum) for 24 hours. Cell cultures were challenged with 25 ng/mL TNF-α (N = 20) or a vehicle control (N = 22) for 24 hours. Total RNA from epithelial cells was harvested using RNeasy Mini Kit (Qiagen, Germantown, MD). RNA concentration and quality were assessed using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Rockford, IL) and stored at −80°C until use. Five hundred and fifty ng RNA were reverse-transcribed into complementary DNA using an Omniscript Reverse Transcription kit (Qiagen).

Porcine-specific primer sequences and accession numbers for MUC1 and MUC4 are displayed in Table II. Hypoxanthine phosphoribosyltransferase I (HPRT I) was selected as the endogenous reference gene. All primers were synthesized by Integrated DNA technologies (Coralville, IA). Specificity of these primer sequences has been assessed previously by DNA sequencing.[27] Quantitative polymerase chain reaction was performed using SYBR Select Master Mix (Life Technologies Corp.) in an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Amplification was completed under the following conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Relative quantitative analysis of MUC1 and MUC4 expression was completed using the standard comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$).[35] Raw Ct values were recording using ABI 7500 Real-Time PCR system software (version 2.3, Applied Biosystems). Normalized Ct values (ΔCt) were then obtained by subtracting the raw Ct value of the endogenous reference (HPRT) from the raw Ct value of MUC1 and MUC4. For comparison between TNF-α and vehicle-challenge cells, the difference between the average ΔCt values between groups was determined (ΔΔCt). Fold change in MUC1 and MUC4 expression was determined using the formula $2^{-\Delta\Delta Ct}$. Independent *t* tests ($P < 0.01$) were used to determine whether average ΔCt values were different between TNF-α and vehicle-challenge cells for MUC1 and MUC4.

RESULTS

Primary Vocal Fold Epithelial Cell Culture Morphology

Following 48 hours in culture, small clusters of cells were observed to attach to collagen-coated wells. Nonattached material, composed of isolated cells and detritus, was washed away during media change. Soon after, cell clusters assumed a flat, round shape and started to spread and migrate into small colonies (Fig. 1A). Discrete colonies continued to grow and coalesced into single cell monolayers. Monolayers were 70% to 90% confluent within 5 to 6 days (Fig. 1B). As cells expanded in culture, monolayers acquired cobblestone appearance characteristic of the typical morphology of epithelial cell cultures.

Characterization of Primary Vocal Fold Epithelial Cell Cultures

Characterization of the vocal fold epithelial cell cultures was performed by immunostaining. Vocal folds harvested from pig larynges were utilized as positive controls for the specificity of cell-type markers. Epithelial nature of the monolayers was confirmed by specific labeling of epithelial cells with pan-cytokeratin (Fig. 2A). In porcine vocal fold tissue, pan-cytokeratin expression was also isolated to the cells of the epithelium (Fig. 3C). In addition, porcine vocal fold fibroblasts (Fig. 3A) did not express pan-cytokeratin (Fig. 3B), further demonstrating the specificity of pan-cytokeratin as a marker of porcine vocal fold epithelial cells. To evaluate the purity of vocal fold epithelial cell cultures, immunofluorescence was further utilized to probe for vimentin, a stromal cell marker, and vWf, a common marker of endothelial cells. Isolated staining of vimentin and vWF was observed in vocal fold epithelial cultures. Using a combination of light microscopy and immunofluorescence, the proportion of vimentin positive cells did not exceed 5% (Fig. 4A), and vWF did not exceed

1% (Fig. 4B). In porcine vocal fold tissue, vimentin staining was mostly localized to the lamina propria, with a few isolated epithelial cells also staining positive (Fig. 4E). Cells in culture that were epithelial in appearance did not express vimentin (Fig. 4A). vWf factor was positively expressed in vocal fold tissue endothelial and glandular cells (Fig. 4F). Positive staining for MUC1 (Fig. 5A) and MUC4 (Fig. 5B) was also observed in epithelial cultures. Although MUC4 was present in the majority of cells, MUC1 only stained a portion of cells, and staining was less intense. In porcine vocal fold tissue, a similar staining pattern of MUC1 (Fig. 5E) and MUC4 (Fig. 5F) was observed.

In Vitro Exposure to an Inflammatory Cytokine

Having confirmed the presence of MUC1 and MUC4, porcine epithelial cells cultures were used to investigate whether the expression of MUC1 and MUC4 is influenced by the inflammatory cytokine TNF-α. Data are presented as the fold change in mucin gene expression in TNF-α challenged cells relative to the vehicle-challenged cells (Fig. 6). TNF-α significantly increased MUC4 expression by 2.5 fold relative to vehicle-challenged cells ($t(40) = 0.85$, $P < 0.001$). No significant changes in MUC1 expression in response to TNF-α were observed ($t(40) = 0.38$, $P = 0.07$).

DISCUSSION

Vocal fold epithelial and mucus barriers protect the vocal folds from insults, providing a physical barrier and lubricating vocal fold surface during vibration.[1] However, our understanding of the role of epithelium and mucus in vocal fold health in the pathogenesis of voice disorders is hindered by a lack of methods for epithelial cell culture. There has been a limited number of reports describing laryngeal epithelial cells cultures from humans[16,19] and rabitts.[20] However, epithelial cells from different tissues and species often differ substantially in cell isolation techniques and culture requirements.[36] Here, we established a method for porcine vocal fold epithelial cell culture, using readily accessible slaughterhouse material. Cells exhibited morphology and staining patterns consistent for epithelial cells with minimal contamination by other cell types. In addition, cultures demonstrated positive staining for MUC1 and MUC4 glycoproteins, which is important for maintenance of the mucus layer. In addition, MUC4 transcript expression increased following exposure to the inflammatory stimulus TNF-α.

In the current study, we created vocal fold epithelial cell cultures from porcine tissue. Although cultures of human epithelial cells are highly valuable, success in previous attempts to culture human vocal fold epithelial cells has been variable.[18] Furthermore, the limited availability of healthy, human vocal fold tissue results in a small number of biological replicates, thus reducing the usability of a human model. The porcine model is well established in vocal fold-related research, especially as pertains to study of the vocal fold epithelial barrier.[1,23,24,27] In addition, the large quantities of cells typically required for successful epithelial cell culture may preclude the use of human tissue specimens and smaller animal models. Porcine tissue is readily available from slaughterhouses, and animals are typically healthy and roughly the same age, thus providing consistency across replicates. There has been one additional report of success with culturing porcine laryngeal epithelial

cells.[37] However, because these cultures contained supraglottic cells, they were not a pure vocal fold epithelial cell population but do stress the usability of porcine tissue for a laryngeal cell culture.

Vocal fold epithelial cells were isolated and cultured by modification of a culture technique for esophageal epithelial cells described by Kalabis et al.[34] This technique was chosen because, like the vocal folds, the esophageal epithelium is stratified squamous. The first modification of the technique was use of a different culture medium. In the current study, we employed FAD medium, containing a small percentage of serum, whereas the original study utilized commercially available keratinocyte–serum-free medium (KSFM). FAD media has been utilized successfully previously to create stem cell-derived epithelial cells in a novel model of the vocal fold mucosa.[38] Qualitatively, preliminary studies also suggested more reliable survival and growth of porcine vocal fold epithelial cells with the FAD as compared to KSFM medium. The second modification was use of collagen-IV coated plate to facilitate early attachment and subsequent expansion of epithelial cells. Collage-IV is critical structural component of the basement membrane that anchors epithelial cells to the underlying lamina propria,[39] and in early experiments we observed greater attachment of cells to coated as compared to uncoated plates.

There are some key methodological differences between the cell culture technique described here and other laryngeal epithelial culture techniques described in the literature. One key difference relates to the method of epithelial cell isolation from the tissue of origin. For human vocal fold culture, epithelial cells were either cultured directly from tissue explants[18] or from pieces of vocal fold tissue that were dissociated using a combination of collagenase and DNase. In the latter technique, dissociated cells were plated; after 30-minutes, nonattached cells presumed to be epithelial were collected and further expanded.[19] Trypsin was utilized to isolate epithelial cells from human supraglottic and postcricoid tissue specimens.[15,16] The vocal fold epithelium is composed of multiple layers of closely packed cells, tightly attached to the basement membrane. Consequently, we chose to utilize a dispase solution, which digests collagenous extracellular matrix in order to separate the epithelium from the underlying lamina propria prior to the isolation of individual cells.[20] Such techniques are preferable because they limit the number of potentially contaminating cell lineages introduced to the culture. Mizuta et al.[20] used a similar technique to isolate rabbit vocal fold epithelial cells. The choice of culture medium also differed between studies, mostly as related to the presence or absence of serum. Human laryngeal epithelial cells have been cultured in serum-free[16,18] and low serum medium.[19] Rabbit vocal fold epithelial cells were also cultured used serum containing medium; however, the serum was at a higher concentration than the low serum medium used here. It is not surprising to observe these differences in protocols. Medium is a critical factor in cell culture technology, and selection of an appropriate medium varies widely based on cell type and species.[40] In many cases, researchers need to modify medium for their specific culture needs.

The cell culture technique utilized in this study was highly reproducible. We cultured porcine vocal fold epithelial cells from approximately seven larynges per week for 6 months. On average, five of these cultures were successful per week. Consequently, we estimate that successful epithelial growth occurred in 70% of samples. This is significantly greater than

the 40% success rate reported for the culture of human glottic epithelial cells.[18] Our greater success rate is likely in part due to the larger amount of starting tissue available from porcine as compared to human vocal folds. Positive staining for pan-cytokeratin revealed cultures were epithelial cells with some isolated staining for stromal (vimentin) and endothelial cells (vWf). In current study, we utilized porcine vocal fold tissue as a positive control and porcine vocal fold fibroblasts as a negative control for our chosen markers of epithelial cells and contaminating cell lineages. Pan-cytokeratin staining was isolated to the porcine vocal fold epithelium and did not stain porcine vocal fold fibroblasts, indicating the specificity of this marker for vocal fold epithelial cells. In porcine vocal fold tissue, vimentin primarily stained stromal cells; however, it did also demonstrate some isolated staining of epithelial cells. It has been shown previously that vimentin is expressed in both stromal and epithelial cells.[41] However, positive staining of vimentin in our vocal fold epithelial cultures was restricted to cells with spindle-shaped morphology, consistent with fibroblasts. Almost no staining of vWf was observed in epithelial cell cultures. In porcine vocal fold tissue, vWf stained endothelial cells as expected; however, it was also present in glandular cells and along the epithelial surface. vWf is a large glycoprotein highly related in structure to the mucin glycoprotein, MUC5B.[42] Positive staining in the glandular cells and along the epithelial surface was likely related to the vWf antibody recognizing MUC5B. The use of positive and negative controls helps confirm with confidence that our markers were specific for epithelial cells and contaminating cell lineages and strengthens our assertion of the primarily epithelial phenotype of porcine vocal fold cultures. Although contamination of epithelial cultures with other cell types could not be avoided, it was maintained at very low levels. Presences of contaminating cell lineages, especially stromal cells, is common in a variety of primary epithelial cell types—including those from cultured from the intestines,[43] gingiva,[44] and skin[45]—and does not restrict the usefulness of these models.

Subculture or passage of cells refers to the process of transferring cells from a previous culture to fresh grown medium. Using a variety of techniques, we did attempt to subculture our porcine vocal fold epithelial cell cultures. This included nonenzymatic (i.e., cell scraper) and enzymatic techniques (i.e., trypsin-EDTA, dispase) to remove primary cells from the originating plate. Plating densities of subcultured cells also was systematically varied. Within 1 to 2 days, we observed some cellular attachment and growth (Fig. 7A); however, cells often did not have the cobblestone appearance consistent with healthy epithelial cells. Typically, by day 5 (Fig. 7B) the majority of cells had died. Primary cells are useful experimental tools; however, they do have a finite life span with epithelial cells, often having a significantly shorter life span than stromal cells like fibroblasts.[46] Our findings indicate the porcine vocal fold epithelial cells may only successfully grow as primary cells and spontaneously cease growing with subculture attempts. We acknowledge this as a limitation of the current work. However, given the ready availability of tissue from pigs intended for sacrifice, this should not significantly reduce the usability of this model.

MUC1 and MUC4 were expressed in our culture, indicating that these cells produce glycoproteins critical to the formation of the protective vocal fold epithelial mucus layer. These are large molecular weight, membrane-bound glycoproteins found in the epithelium of a variety of tissue types including the vocal folds.[27,47] Although tethered to cells through a membrane-spanning domain, a prominent extracellular domain may also be proteolytically

cleaved and secreted into the mucus layer covering the vocal fold epithelial surface. Porcine vocal fold epithelial cell cultures stained positive for both MUC1 and MUC4. Whereas MUC4 was present in the majority of cells, MUC1 only stained a portion of cells, and staining was less intense. This is consistent with MUC1 and MUC4 staining patterns in porcine vocal fold epithelial tissue seen here and in previous studies.[27] We found MUC4 transcripts were significantly upregulated by TNF-α. This suggests that inflammation with associated release of inflammatory cytokines in vivo may also have the potential to influence mucin expression at the vocal fold epithelial surface. TNF-α is produced by a variety of cell types, including macrophages, lymphocytes, fibroblasts, and epithelial cells, and is significantly increased in the vocal folds in as little as 1 hour following injury.[30,32] TNF-α upregulates MUC1 expression in epithelial cells derived from ocular,[31] airway,[32] and uterine tissues.[33] Whereas MUC1 expression was increased following TNF-α exposure, this was not significant. This may be related to lower level of expression of MUC1 as compared MUC4 in porcine epithelial cells. The effect of TNF-α on MUC4 expression has not been well established. Other cytokines, including IL-4, significantly increase MUC4 transcription in respiratory epithelial cells.[48] The exact function of MUC4 in the vocal fold epithelium is currently not known. This glycoprotein consists of MUC4α and MUC4β subunits.[47,48] In the lung, it has been suggested MUC4 subunits together play a critical role in airway homeostasis and repair.[49] Specifically, the MUC4α subunit provides protective anti-adhesive properties, and the MUC4β subunit assists in the regulation of epithelial cell proliferation and differentiation.[47,48] Consequently, is it possible that increased MUC4 expression is part of a defensive response offered by the vocal folds in reaction to inflammatory stimuli. Future studies are needed that elucidate the functional significance of this finding. We demonstrated cultures are responsive to a common inflammatory stimulus experienced in the vocal folds and the utility of these cells for investigation of effect of cytokines or other physiological insults on vocal fold epithelium and associated-mucins.

CONCLUSION

By allowing significant control over environmental conditions, in vitro cell cultures are an excellent complement to in vivo work. Here, we present the first report of the successful primary culture of porcine vocal fold epithelial cells. Establishing reproducible methods for epithelial cell culture is a critical first step in elucidating the contributions of the epithelial and mucus barriers to vocal fold disease processes. Future applications of these cells are numerous. For example, cells can be used to investigate the epithelial and mucin response to a wide-range of irritants implicated in vocal fold injury. Cells can also be incorporated into a three-dimensional in vitro model of vocal fold mucosa. Previous success had been demonstrated incorporating human embryonic stem cell-derived epithelial cells[38] and rabbit vocal fold epithelial cells[20] in in vitro vocal fold mucosa models. Porcine epithelial cell cultures will provide researchers with a valuable new in vitro tool to investigate vocal fold epithelium and mucus barriers. Improved understanding of the role of the epithelial and mucus barriers in vocal fold disease development will likely contribute to future improve treatment outcomes.

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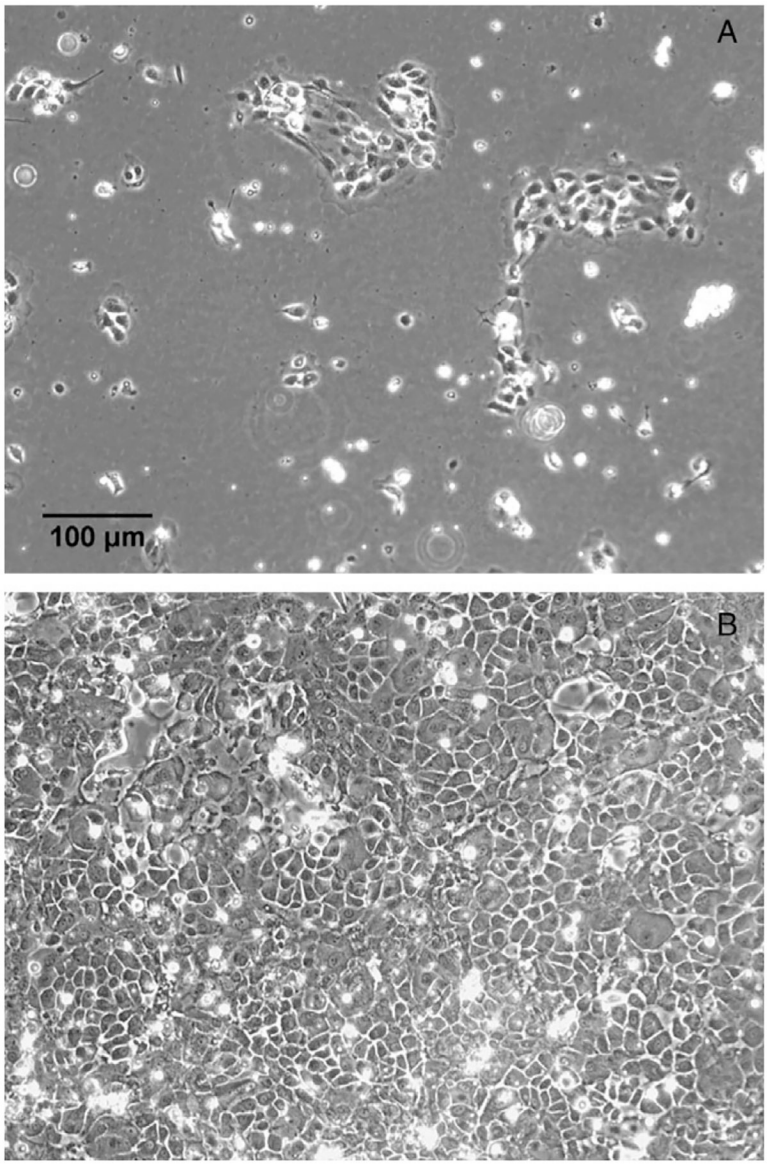

Fig. 1.

Porcine vocal fold epithelial cells following culture for 2 (A) and 5 (B) days demonstrate cobblestone appearance consistent with epithelial cells.

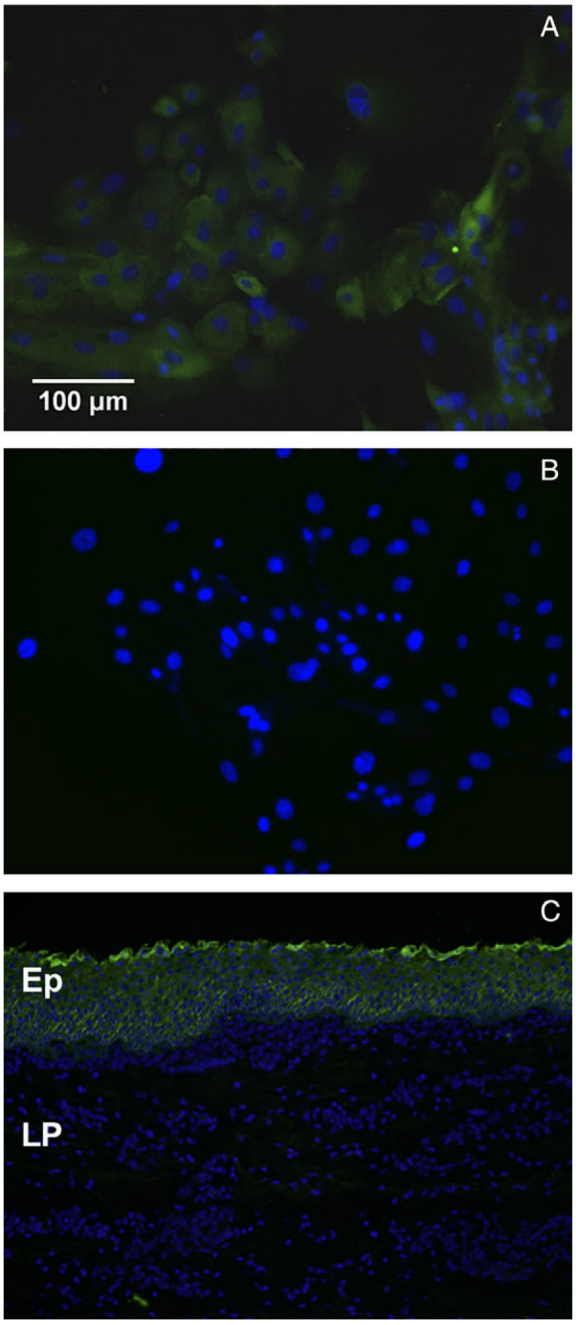

Fig. 2.

Immunofluorescence confirmed that vocal fold epithelial cells stained positive (green) for pan-cytokeratin (A). No staining was observed in cells treated with goat anti-mouse secondary antibody only (B). Porcine vocal fold tissue was utilized as a positive control for epithelial pan-cytokeratin expression. Tissue demonstrates positive staining (green) for pan-cytokeratin in the Ep, but not LP (C). DAPI (blue) was used as a nuclear stain. Ep = epithelium; LP = lamina propria.

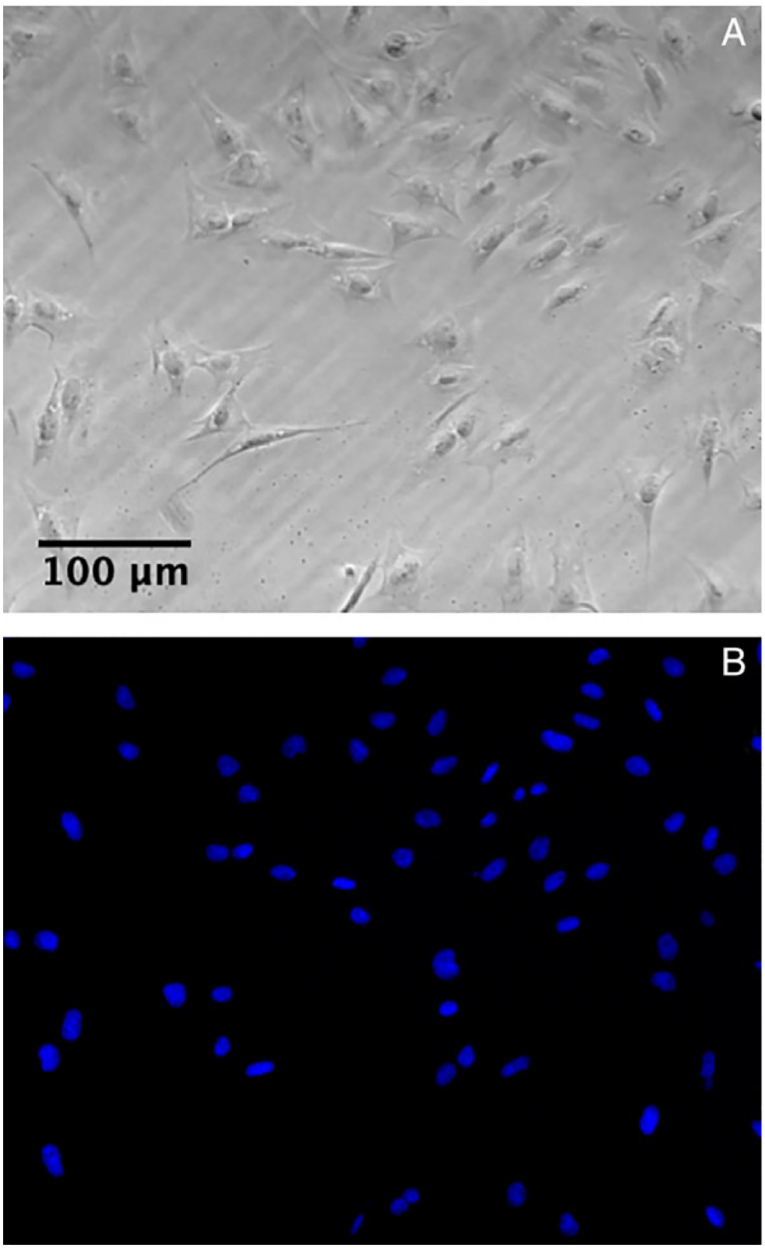

Fig. 3.

Porcine vocal fold fibroblasts demonstrated a spindle-shaped morphology (A). Immunofluorescence exhibited that porcine vocal fold fibroblasts were negative for pan-cytokeratin expression (B). DAPI (blue) was used as a nuclear stain.

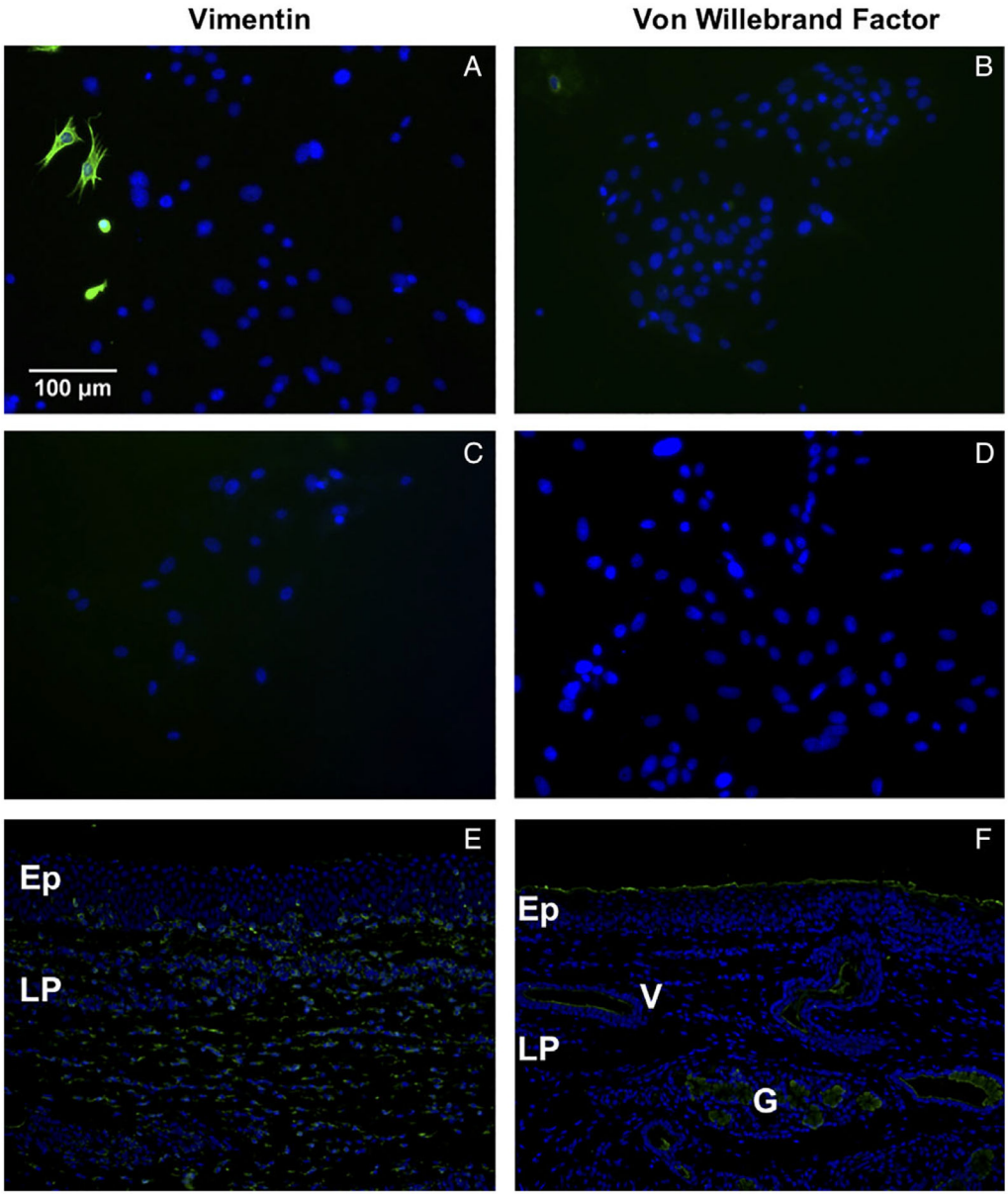

Fig. 4.

Immunofluorescence demonstrated some isolated positive staining (green) of cell cultures with the stromal cell marker vimentin (A) and endothelial cell marker vWf (B). No staining was observed in cells treated with goat anti-mouse (C) and goat anti-rabbit (D) secondary antibody only. Porcine vocal fold tissue was utilized as positive control for vimentin and vWf expression. Tissue demonstrates positive staining (green) for vimentin primarily in the LP (E). Tissue demonstrates positive staining (green) for vWf in V and G (F). DAPI (blue) was used as a nuclear stain. Ep = epithelium; G = mucus glands; LP = lamina propria; V = vessels; vWf = von Willebrand factor.

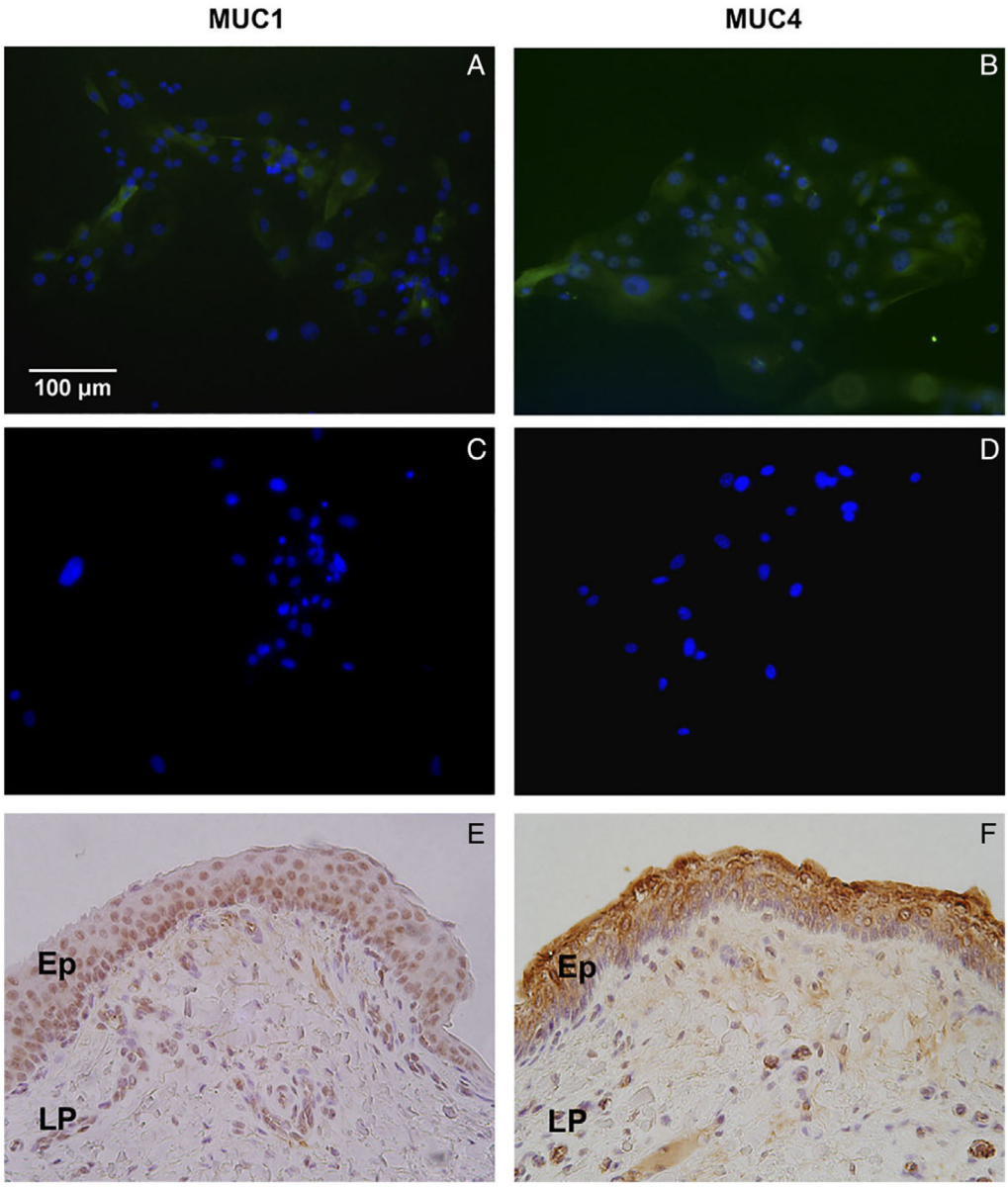

Fig. 5.

Immunofluorescence exhibited that vocal fold epithelial cells stained positive (green) for MUC1 (A) and MUC4 (B). No staining was observed in cells treated with goat anti-rabbit (C) and goat anti-mouse (D) secondary antibody only. DAPI (blue) was used as a nuclear stain. Porcine vocal fold tissue was utilized as positive control for epithelial MUC1 and MUC4 expression. Tissue demonstrates isolated positive staining (brown) in the Ep for MUC1 (E) and diffuse positive staining (brown) in the Ep for MUC4 (F). Hematoxylin (purple) was used as a nuclear stain. Ep = epithelium; LP = lamina propria; MUC = mucin.

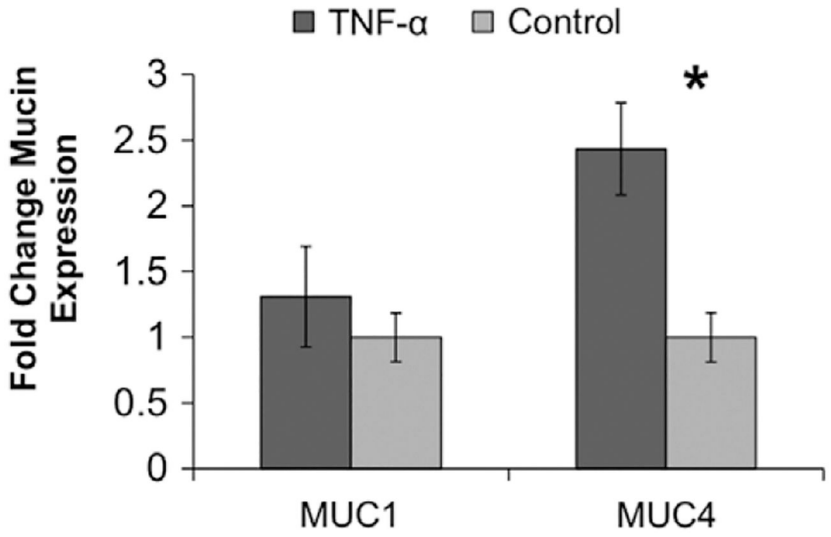

Fig. 6.

Fold change in MUC1 and MUC4 gene expression in TNF-α relative to vehicle-challenged cell cultures. Positive fold-change indicates an increase in gene expression. Expression of MUC4 significantly (*) increased following the TNF-α challenge. Error bars represent standard error. MUC = mucin; TNF-α = tumor necrosis factor alpha.

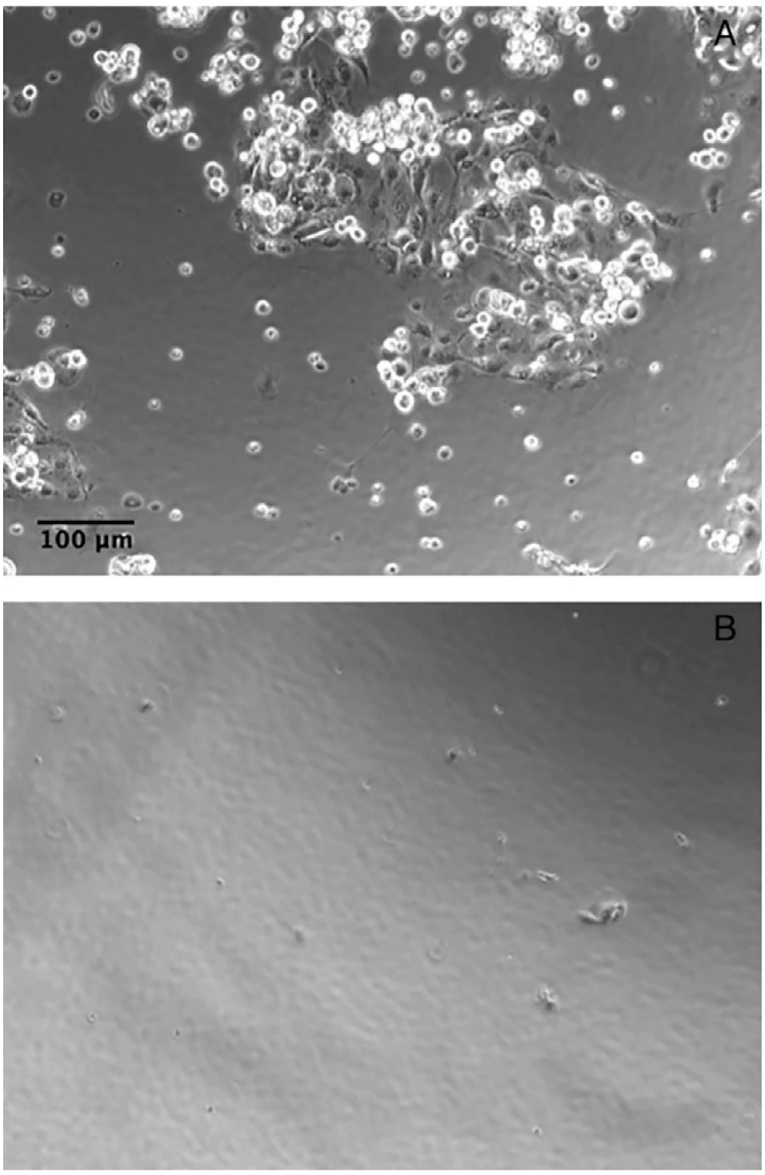

Fig. 7.

Porcine vocal fold epithelial subcultures following 2 (A) and 5 (B) days. Cellular growth was observed at day 2, but not at day 5.

TABLE I.

Immunocytochemistry Antibodies.

Protein	Species	Concentration	Catalog Number
Pan-cytokeratin	Mouse monoclonal	1:250	Abcam ab6401
Vimentin	Mouse monoclonal	1:500	Abcam ab8069
von Willebrand factor	Rabbit polyclonal	1:400	Dako A0082
MUC1	Rabbit polyclonal	1:100	Abcam ab15481
MUC4	Mouse monoclonal	1:100	Abcam ab60720

MUC = mucin.

TABLE II.

qPCR Primer Sequences.

Gene	Forward	Reverse	Accession Number
MUC1	agtgccgacgaaagaactgt	gctgccaggttcgagtaaga	AY243508
MUC4	ctgcaatgtcagttggctgt	acctaccaggccatcctttc	NM_001206344
HPRT I	aaggacccctcgaagtgttg	cacaaacatgattcaagtccctg	DQ845175

HPRT I = Hypoxanthine phosphoribosyltransferase I; MUC = mucin; qPCR = quantitative polymerase chain reaction.