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Porcine Nasal Epithelial Cultures for Studies of Cystic Fibrosis Sinusitis

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Abstract

Background—Transgenic cystic fibrosis (CF) murine models do not develop spontaneous lung or sinus disease, two major causes of morbidity in human CF patients. Because of these limitations, transgenic CFTR^{-/-} pigs have been developed and are currently being characterized. These CF animal models have phenotypes closely resembling that of human CF subjects. The objectives of the current study were to develop primary porcine nasal epithelial (PNE) cultures and evaluate their usefulness as a means to investigate sinonasal transepithelial transport and CFTR function.

Methods—PNE derived from the septum or turbinates of CFTR^{+/+} and CFTR^{-/-} pigs were cultured at an air-liquid interface to confluence and full differentiation. Epithelial monolayers were mounted in Ussing chambers to investigate pharmacologic manipulation of ion transport. Ciliary beat frequency (CBF) and scanning electron microscopy of monolayers were used to indicate degree of ciliation and cell differentiation.

Results—Stimulation of CFTR-mediated anion transport (I_{sc} in $\mu\text{A}/\text{cm}^2$) was significantly greater in epithelia derived from the septum when compared to turbinates (33.04 $\pm$ 1.17 vs. 18.9 $\pm$ 0.73; $p < 0.05$). cAMP-activated Cl⁻ secretion was absent in CFTR^{-/-} and present in CFTR^{+/+} epithelia. Calcium-activated Cl⁻ (CaCC) secretion was increased in CF, however, overall Cl⁻

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transport through CaCCs was very low. Degree of ciliation (90%) and CBF were similar between groups.

Discussion—Septal PNE exhibit a robust ion transport phenotype and indicate CFTR^{-/-} sinus disease could be attributable to diminished alternative pathways for Cl⁻ transport. Overall, PNE have similarities to human respiratory epithelia not demonstrated in murine cells and represent useful *in vitro* models for studying CF sinus disease.

Keywords

Transepithelial Ion Transport; CFTR; Sinusitis; Chloride Secretion; Porcine Nasal Culture; Mucociliary Clearance

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease caused by genetic mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel.¹ CF patients develop severe chronic rhinosinusitis (CRS) as well as lung, pancreatic, intestinal, and hepatic disease. Transgenic cystic fibrosis (CF) murine models have facilitated studies of CF pathogenesis and treatment due to the difficulty studying human CF pathogenesis.² We recently described and characterized a primary cell culture model of murine nasal septal epithelium (MNSE) grown at an air-liquid interface (ALI) that demonstrates excellent differentiation, confluence, and a robust, physiologic ciliary beat frequency (CBF) stimulated by ATP and other agonists.³ Unfortunately, small rodents such as mice do not reproduce several key aspects of human physiology. They are phenotypically different from humans with CF with regards to many organ systems, including the respiratory system, thereby limiting their ability to model human disease. Mice with CF do not acquire spontaneous bacterial airway infections⁴, which represent the major cause of morbidity and mortality in CF patients. In addition, male mice are generally fertile,⁴ develop postnatal intestinal obstruction⁵ (as opposed to meconium ileus), lack significant hepatobiliary disease,⁴ and have less severe pancreatic complications.⁶

Despite the physiologic differences, murine respiratory epithelial cultures are in widespread use for studies of CF airway disease because transgenic CFTR^{-/-} mice are readily available to most research centers. Limitations of the murine CF model has led to the recent development and ongoing characterization of pig^{7,8} and ferret⁹ transgenic CFTR^{-/-} animal models. These larger animal models are thought to more closely resemble human airway pathophysiology.^{9,10} Transgenic CFTR^{-/-} pigs in particular have been shown to develop spontaneous airway disease, meconium ileus, biliary cirrhosis, and degenerated vas deferens.^{8,11} Importantly, they also develop sinonasal abnormalities and spontaneously exhibit CF sinus disease similar to that of humans.¹² Considering the similarities in pathophysiology between CFTR deficient pigs and humans, developing a well-differentiated porcine airway culture model is critical for *in vitro* experimentation. Moreover, characterization of the bioelectric phenotype in CF porcine airway epithelium may offer new insight regarding the mechanisms that underlie CF sinus disease.

The objectives of the current study were to develop a straightforward method to cultivate and characterize primary porcine nasal epithelial (PNE) cultures and evaluate their usefulness as a model for transepithelial ion transport and cystic fibrosis transmembrane conductance regulator (CFTR) function.

MATERIALS AND METHODS

Institutional Animal Care and Use Committee approval were obtained prior to the initiation of the study.

Tissue Culture

Several techniques for culturing differentiated PNE were investigated, including previously published methods utilized by Chen et al.^{13,14} Confluence of cultured primary porcine nasal epithelia at an air liquid interface was attained more readily by adapting previously published methods from our laboratory.^{3,15–20} Neonatal pigs were obtained from Exemplar Genetics (Des Moines, Iowa). The nasal septa or inferior turbinates of five wild type (CFTR^{+/+}) and two CFTR^{-/-} neonatal pigs were harvested for the purpose of this study. Tissues were transferred into 20-mL volumes of dissociation media containing minimal essential medium (MEM; Invitrogen), penicillin (60 IU/mL), streptomycin (60 µg/mL), 1.4 mg/mL Pronase (Roche Applied Science, Indianapolis, IN, USA) and 0.1 µg/mL DNase (Roche Applied Science), and incubated in a 5% CO₂ chamber at 37°C for 1 hour. Enzymatic dissociation was quenched with 5 ml of sterile 5% fetal bovine serum (FBS; Sigma Aldrich). Tubes were inverted 12 times to facilitate dissociation of epithelial cells. The tissue was removed and the cell suspension centrifuged, resuspended in culture media, and incubated at 37°C for 2 hours in 100 mm Primaria™ culture dishes (BD Biosciences) to remove fibroblast contamination. Suspensions were collected, centrifuged, resuspended and cell yield determined using a hemocytometer.

Cells were seeded at a density of 4×10^5 cells on semipermeable support membranes (Costar® Transwell® clear 24-well plate inserts, 0.4-µm pore; Corning Life Sciences, Lowell, MA, USA) previously coated with 50 µg/mL human placental collagen (type VI; Sigma Aldrich, St. Louis, MO, USA). The cells were bathed in 200 µL culture media on the apical surface and 600 µL in the basal compartment. Cells were incubated at 37°C in 5% CO₂ in a humidified incubator for 4 days. On the fourth day, the apical culture medium was removed along with any non-adherent cellular debris. Basal culture medium was replaced with 600 µL of differentiation media consisting of a 1:1 mixture of DMEM and Nutrient Mixture Ham's F-12 containing 100 IU/mL of penicillin, 100 µg/mL streptomycin, and 2% NuSerum™ (BD Biosciences). Basal medium was replaced twice weekly. Ussing Chamber, SEM, and CBF analysis were performed when cell monolayers were fully differentiated with widespread ciliogenesis and transepithelial resistances $> 350 \Omega \cdot \text{cm}^2$ (n = 5 per condition). Due to the rarity of CFTR^{-/-} PNE, cells were reserved for Ussing chamber analysis.

Scanning Electron Microscopy (SEM)

SEM was performed on porcine septal and turbinate ALI monolayers following two weeks. After rinsing with PBS, filters were fixed with Karnovsky's fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.2M cacodylate buffer; pH 7.4). Monolayers were progressively dehydrated in ethanol (up to 100%) with subsequent HMDS treatment, and air-dried overnight in a fume hood. Specimens were then mounted on SEM stubs and the mucosal surface was sputter-coated with gold palladium to a depth of 12 nm. Mucosal membranes of ALI monolayers were examined with an AMR-1400 scanning electron microscope at an accelerating voltage of 20 kV. Photomicrographs of ALI monolayers were evaluated for percentage of ciliated epithelium.

Electrophysiology

Solutions and Chemicals—Chemicals were obtained from Sigma Aldrich. The bath solution contained (in mM): 120 NaCl, 25 NaHCO₃, 3.3 KH₂PO₄, 0.8 K₂HPO₄, 1.2 MgCl₂, 1.2 CaCl₂, and 10 glucose, with pH 7.3–7.4 gassed in a mixture of 95% O₂-5% CO₂ at 37°C. All experiments were conducted with low Cl⁻ (6 mM) in the mucosal bath. Pharmacologic preparations were applied as follows: amiloride (100 μM), forskolin (20 μM), CFTR_{inh}-172 (10 μM), and UTP (150 μM). Amiloride blocks epithelial sodium channels (ENaC), ensuring that observed changes in short-circuit current (I_{SC}) are independent of effects on epithelial sodium channel activity. Forskolin activates CFTR via a cAMP-mediated mechanism, and CFTR_{inh}-172 is a very specific inhibitor of CFTR. UTP treatment leads to increased cytosolic Ca²⁺, stimulates calcium-activated chloride channels (e.g. TMEM16A²¹) and promotes calcium-mediated CBF activity.

Short Circuit Current and Conductance Measurements—Transwell inserts were mounted in Ussing chambers to monitor ion transport and pharmacologic blockade. Monolayers were studied under short-circuit conditions after fluid resistance compensation using automatic VCC 600 voltage clamps (Physiologic Instruments, San Diego, CA). Transwell inserts were mounted in the 37°C bath solution, gassed with a 95% O₂-5% CO₂ mixture. I_{SC} was measured at 1 sample/second, with the conventions that a positive deflection represents the net movement of anion in the serosal to mucosal direction. Conductance (*G*_{*t*}) was determined as the reciprocal value of the resistance. Resistance, in turn, was calculated according to Ohm's law.

Ciliary Beat Frequency (CBF)

Images were visualized with 20X objective on an inverted scope (Fisher Scientific, Pittsburgh, Pa.). Data was captured using a Model A602f-2 Basler area scan high-speed monochromatic digital video camera (Basler AG, Ahrensburg, Germany) at a sampling rate of 100 frames per second. Images were analyzed using the Sisson-Ammons Video Analysis (SAVA) system version 2.1.6.²² For each experiment, a large area of beating cilia within air-liquid interface cultures was identified with the inverted microscope. A baseline recording for CBF was conducted for each cell monolayer prior to administration of the following test solutions: forskolin, UTP, and PBS (vehicle control). Increased fluid depth on the apical surface stimulates CBF by virtue of improved hydration and optimized fluid dynamics.

Whole field analysis was performed with each point representing an individual cilium. The reported frequencies represent arithmetic means of all data points. Each analysis was normalized to fold-change over baseline. Experiments were performed at ambient temperature (23°C).

Statistical Analysis

Statistics were performed using two-tailed unpaired t-tests with $n = 5$ per condition for all Ussing chamber data and analysis of variance with post hoc Tukey-Kramer for CBF.

RESULTS

Ciliary differentiation visualized with SEM was similar among septal and turbinate PNE

Septal and turbinate CFTR^{+/+} PNE cultured ALI monolayers exhibited full differentiation and confluence with resistance $> 350 \text{ } \Omega/\text{cm}^2$ by 14 days. Scanning electron micrographs of all PNE cultured ALI monolayers at 14 days had approximately 80–90% differentiated cilia throughout the apical monolayer surface, with similar morphology of turbinate and septal epithelial cells (Figure 1).³

Forskolin and UTP stimulate CFTR^{+/+} PNE CBF from septal and turbinate tissues in a similar fashion

Strong CBF was demonstrated in all PNE groups indicating well-differentiated cells with mature cilia. CBF (fold-change/baseline) was significantly increased in PNE derived from both septum and turbinate tissue following administration of forskolin or UTP ($p < 0.05$) (Figure 2). CBF stimulation was similarly augmented in both cell culture models (septum; 2.32 ± 0.18 and 2.4 ± 0.24 vs. turbinate 2.38 ± 0.12 and 2.61 ± 0.17 (forskolin and UTP, respectively) when compared to PBS (septum; 1.82 ± 0.26 vs. turbinate; 1.74 ± 0.24).

Septal PNE exhibit greater transepithelial ion transport than turbinate PNE

Ussing chamber analysis was performed on CFTR^{+/+} from septal and turbinate derived tissues. Transepithelial ion transport (change in short-circuit current = I_{SC} in $\mu\text{A}/\text{cm}^2 \pm \text{S.D.}$) was significantly more robust in cell monolayers derived from septal tissue when compared to turbinates (Figure 3). For CFTR^{+/+} cells, septal PNE had increased amiloride-sensitive (-7.0 ± 0.42 vs. -3.99 ± 0.54 ; $p < 0.05$) and CFTR-mediated I_{SC} (forskolin, 33.04 ± 2.03 vs. 18.88 ± 1.26 ; $p < 0.05$), which was abrogated by CFTR_{inh}-172 (-8.95 ± 2.0 vs. -5.63 ± 0.94 ; $p < 0.05$). Calcium-activated Cl^- secretion was also increased when compared to turbinate PNE (2.3 ± 0.14 vs. 0.76 ± 0.28 ; $p < 0.05$) (Figure 3).

Based on a robust technique for procuring and differentiating sinus PNE cells, we focused our next comparison between CFTR^{-/-} and CFTR^{+/+} epithelia. As expected, CFTR^{-/-} PNE demonstrated no CFTR-dependent anion transport. Amiloride-sensitive (6.16 ± 0.25 vs. 4.98 ± 0.62 ; $p < 0.05$) and UTP-stimulated I_{SC} (3.02 ± 0.85 vs. 1.31 ± 0.57 ; $p < 0.05$) were also enhanced in CFTR^{-/-} septal PNE. The G_t were reflective of the overall greater degree of ion transport present in septal PNE, although UTP-dependent changes were not statistically significant in septal versus turbinate CFTR^{-/-} PNE (Figure 4).

DISCUSSION

The lack of a suitable animal model has hindered studies of CF pathophysiology and treatment. While mice with targeted CFTR gene alterations are readily available for *in vitro* and *in vivo* investigation, they do not develop the clinical manifestations typical of human CF.⁴ The development and characterization of the CF porcine model represents a major advance that will allow studies of CF airway pathophysiology. At birth, CFTR^{-/-} pigs display exocrine pancreatic destruction, gallbladder abnormalities, intestinal lesions (meconium ileus and microcolon), and focal biliary cirrhosis similar to humans.^{7,10} CF pigs spontaneously develop hallmark features of CF lung and sinus disease including inflammation, remodeling, mucus accumulation, and infection.²³ This animal model is therefore ideal for investigating CRS pathogenesis associated with genetic and acquired defects of CFTR,²⁴ evaluating CFTR restoration via gene therapy,²⁵ testing the activity of CFTR correctors and potentiators, and determining whether such agents reserve normal ion transport by potentiating the CFTR activation pathway.^{24,26–29}

The present study described the standardization and characterization of primary porcine nasal epithelial cell cultures developed at an air-liquid interface for studies of CF (and non-CF) sinusitis using a technique that differs from previously published protocols.¹³ Our data indicate that Primary PNE cell cultures provide an accurate and robust experimental means to approximate airway cellular physiology *in vivo* and allow direct comparisons of otherwise genetically similar animals. Nasal primary cells also provide mechanistic comparisons with the nasal potential difference assay, a mainstay of CFTR detection *in vivo*.

Cell culture conditions for PNE derived from septum or turbinate tissues were optimized to obtain 80–90% ciliated cell surface as demonstrated by SEM. PNE have robust CBF that responds well to forskolin and UTP. The overall phenotype of ion transport in PNE cell cultures established here was consistent with an electrically active, mucociliary airway epithelium containing both Na⁺-absorptive and Cl⁻-secretory pathways. However, primary septal PNE cultures demonstrated a more robust ion transport phenotype with greater changes in I_{SC} and conductance compared to turbinate cultures derived from turbinate tissue. Septal CFTR^{-/-} PNE also had greater amiloride and UTP-sensitive I_{SC} when matched to turbinate CFTR^{-/-} cultures. Thus, the nasal septum is the preferable source of epithelium for the expansion of primary PNE cell cultures when used for evaluating pharmacologic manipulation of ion transport, as they may be more sensitive to modulators of ion transport.

Interestingly, all PNE preparations had diminished CaCC-mediated Cl⁻ secretion as measured by UTP activation. Murine CFTR^{-/-} airway epithelium has very robust CaCC transport and does not develop spontaneous airway disease.³⁰ Therefore, we speculate that the development of porcine CFTR^{-/-} sinus disease could be attributable in part to diminished alternative pathways for Cl⁻ transport. This assertion will require further evaluation. In any case, the data presented here indicate that PNE has similarities in ion transport phenotype to human nasal epithelia which are quite different than murine nasal cells not exhibited with murine nasal cells and represent useful models for studying CFTR activity and disease pathogenesis in the future.

CONCLUSION

For studies of airway ion transport, the porcine nasal septum provides an advantageous source of primary nasal epithelia when compared to turbinate tissues, since septal cells exhibit a more robust ion transport phenotype. Porcine CFTR^{-/-} sinus disease may be attributable to diminished alternative (non-CFTR) pathways for Cl⁻ transport, since UTP-mediated changes in short-circuit current and conductance were observed to be minimal. Primary PNE cultures could provide a physiologically relevant means to investigate sinonasal disease *in vitro*, and allow for the evaluation of emerging therapies directed towards CF and non-CF CRS pathogenesis.

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Figure 1. Scanning electron microscopy of primary CFTR^{+/+} porcine nasal epithelial cell cultures derived from septum (A and B) and turbinate (C and D) tissues. Ciliogenesis (approximately 80 to 90%) and morphology were very similar in both tissue sources.

Figure 2.

Ciliary beat frequency analysis of CFTR^{+/+} porcine nasal epithelial cultures. Forskolin (activates protein kinase A dependent pathways) and UTP (activates intracellular calcium signaling) significantly increase ciliary beat in septal and turbinate nasal epithelial cells.

Figure 3.

Representative Ussing chamber tracings of $CFTR^{+/+}$ and $CFTR^{-/-}$ porcine nasal epithelial cultures with sequential administration of amiloride, forskolin, $CFTR_{inh}$ -172, and UTP. Note the robust $CFTR$ -dependent I_{SC} following administration of forskolin in $CFTR^{+/+}$ with no change in $CFTR^{-/-}$ cultures after the same treatment protocol. By convention, a positive deflection in the tracing is the movement of anion in the serosal to mucosal direction (A). Ussing chamber summary data demonstrating increased transepithelial ion transport (Na^+ and Cl^-) for $CFTR^{+/+}$ septal epithelium when compared to turbinate monolayers (B). Summary data for $CFTR^{-/-}$ epithelium indicate higher amiloride- and UTP-sensitive I_{SC} in septal cultures (C). Calcium-activated Cl^- secretion was minimal in $CFTR^{+/+}$ and $CFTR^{-/-}$ monolayers.

Figure 4.

Change in Gt induced with pharmacologic manipulation demonstrates significantly greater change with all drugs tested in CFTR^{+/+} epithelial cultures (A). Amiloride-sensitive changes in conductance were greater in CFTR^{-/-} PNE, UTP-mediated Gt were not significantly different between the two tissue sources (B).