Laryngoscope Investigative Otolaryngology

Human Primary Middle Ear Epithelial Cell Culture: A Novel in vitro Model to Study Otitis Media

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Objectives: Otitis media (OM) is a ubiquitous pediatric disease leading to a significant health care burden. There is no medication beneficial to resolving COM fluid, highlighting the need for research in the field. Crucially, current human middle ear epithelial cell models are transformed cells not recapitulating physiological functions. Herein, we describe a new method to proliferate and differentiate pediatric primary middle ear epithelial cells (pMEEC) from patients as a physiological model for the study of OM.

Methods: We adapted a cell reprogramming protocol using irradiated fibroblast feeder medium in addition to Rho kinase inhibitor to proliferate pMEEC collected during cochlear implant surgery. Cells were plated on transwell membranes, proliferated with conditionally reprogrammed culture medium, and transferred to air–liquid interface (ALI). Cultures were maintained for 4 weeks at ALI, photos were taken and cell lysates and secretions were collected over time for characterization analysis using quantitative polymerase chain reaction, Western bolt, and proteomics. Keratins, MUC5B and MUC5AC mucins, and beta tubulin (TUBB) were analyzed at the mRNA and protein level.

Results: Cultures took a mean of 2 weeks to proliferate before transwell plating and forming a tight epithelium at ALI from 2 to 4 weeks. Although mRNA expression of MUC5B, MUC5AC, TUBB, and keratin 5 (KRT5) were variable depending on the differentiation stage and the patient, both TUBB and KRT5 proteins were detected until week 2.

Conclusion: We demonstrate a novel method to proliferate and differentiate pMEECs that express epithelial markers and that are able to secrete mucins for the study of OM.

Key Words: Differentiation, otitis media, primary middle ear cell culture, reprogramming.

Level of Evidence: NA

INTRODUCTION

Otitis media (OM) is an infection of the middle ear with accumulation of fluid in the middle ear cavity. It is responsible for an estimated health care cost of 2.4 billion dollars and 15 million pediatrician visits every year in the United States.[1] Acute OM (AOM) is characterized by the early response of the middle ear epithelium to infection, and is usually successfully treated with antibiotics. If several AOM episodes reoccur the middle ear epithelium may remodel, promoting its capability to secrete mucins. This results in a highly viscous mucoid fluid, characteristic of chronic OM (COM). Mechanisms responsible for middle ear epithelium remodeling leading to COM remain mostly unknown, limiting treatment strategies for patients. In fact, there is no medication beneficial to resolve COM which leading to frequent surgery in order to remove accumulated fluid.

Research on OM relies on in vivo and in vitro models that both have limitations. in vivo models, mostly developed in mice and chinchilla, offer the benefits of whole organisms comprising different cell types and involve the immune system. However, due to a small middle ear cavity, in vivo experiments can be challenging, especially in small animals. Another limitation includes that some species have spontaneous OM or are not commonly exposed to human pathogens. On the other hand, in vitro cell lines are transformed by viral genes and allow unlimited access to biological material for experiments. Middle ear epithelial cell lines are widely used for OM research: human middle ear epithelial cell line HMEEC-1 transformed by E6/E7 of the human papilloma virus,[2] mouse middle ear epithelial cell line mMEEC transformed by the SV40 gene,[3] and chinchilla middle ear epithelial cells transformed by SV40.[4]

Several primary cell cultures of middle ear epithelium have been published but were shown to require the collection of middle ear tissue and to be limited to few passages due to the lack of proliferation of differentiated cells.[5–8] Our laboratory attempted to culture primary pediatric human middle ear epithelial cells (pMEECs) by brushing the middle ear of children undergoing cochlear implantation. Similar protocols were used, but the limited amount of cells in addition to their limited proliferation

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DOI: 10.1002/lio2.319

ability did not permit to generate enough biological material for experiments.

In order to improve the current in vitro models for OM research, we decided to pursue the culture of pMEECs using a conditional reprogramming cell (CRC) method. Schlegel et al.'s laboratory developed a protocol permissive of primary epithelial cells entering a stem-cell like state and proliferating by culturing these in irradiated fibroblast medium with Rho kinase inhibitor ROCKi.[9,10] After proliferation, reprogrammed epithelial cells are able to differentiate in their original phenotype in presence of their specific differentiation medium and do not exhibit any cancerous characteristic. Herein, we studied whether the CRC method allows for growth of pMEEC and the subsequent differentiation of these at air–liquid interface (ALI).

MATERIALS AND METHODS

pMEEC Collection

pMEECs were harvested during cochlear implant placement of children aged 1-10 years at Children's National Medical Health System, according to the approved protocol reviewed and approved by the Institutional Review Board. An informed consent was obtained from all subjects. For the two highlighted patients in the results section, the cause of deafness was isolated enlarged vestibular aqueduct in one and CMV related SNHL in the other. Two separate otologic micro-cup straight forceps bites over the middle ear mucosal sample overlying the planned cochleostomy was obtained and immediately placed in 6 mL of pre-chilled bronchial epithelial basal medium (BEBM, Lonza) with 1% penicillin and streptomycin (PS, ThermoFisher Scientific). Additionally, a 1-mm bronchial brush was passed twice over the middle ear mucosa. Samples were immediately brought to the laboratory and manipulated under sterile conditions.

CRC Medium Preparation

CRC medium was prepared by mixing equal volumes of proliferation medium and irradiated fibroblast medium supplemented with 10 μM ROCK inhibitor Y-27632 (Tocris Bioscience) as follows. Proliferation medium was composed of BEBM supplemented with Singlequots Kit (Lonza, Walkersville, MD) containing bovine pituitary extract, hydrocortisone, hEGF, epinephrine, transferrin, insulin, triiodothyronine, retinoic acid, gentamycin, and amphotericin-B. Irradiated fibroblast medium was prepared by culturing 3T3 NIH murine fibroblasts (ATCC) on 150 cm^2 dishes in DMEM supplemented with 4 nM L-Glutamine and 10% calf bovine serum (CBS). When reaching 70%–100% confluence, 3T3 were exposed to proliferation medium supplemented with 5% CBS and were irradiated at 3000 rad (30GY). Cells were then detached using trypsin and plated in other 150 cm^2 dishes for 72–96 hours. The medium collected (CRC medium) was filtered with 0.22 μm filter, aliquoted, and frozen at −80°C.

Culture Conditions

The middle ear mucosal sample was shaken vigorously several times in the medium and the tube was centrifuged at 1000*g* for 5 minutes. The supernatant was removed and the pellet was reconstituted in 100 μL of trypsin EDTA 0.05% (ThermoFisher Scientific) for 7 minutes at 37°C. A 2 mL of CRC medium was added to the tube and the sample was transferred to a human Collagen IV (Sigma) coated 25 cm^2 flask (Corning) containing 3 mL of warm CRC medium. Cells were left in an incubator at 37°C and 5% CO_2 for a week without changing the medium and monitored for cell attachment and proliferation. If cells were observed, CRC medium was changed more frequently and pMEEC were left proliferating until 70%–90% confluence. Cells were then detached with trypsin EDTA 0.05% 2–5 minutes, collected, pelleted and plated in several 75 cm^2 flasks. When pMEEC were 70%–90% confluent, they were detached with trypsin and plated in 12-well or 6-well transwell insert plates (0.4 μm pores, polyesther membrane, Corning) precoated with human Collagen IV. The CRC medium was changed every other day in the basal and apical compartment until cells reached 90% confluence. The medium was then replaced with fully supplemented BEBM, that is, differentiation medium, on the basal side while the apical compartment was left without medium (ALI). pMEEC were cultured 1 to 4 weeks at ALI and secretions, total RNAs, cell lysates, and paraformaldehyde-fixed wells were collected. Pictures were taken at ALI day 1, week 1, 2, 3, and 4.

REAL-TIME POLYMERASE CHAIN REACTION (PCR). Quantitative PCR (qPCR) was conducted for both MUC5B and MUC5AC expression. The qPCR method is as published elsewhere.[11] The following primers were employed MUC5B forward primer, 5′ CGATCCCAACAGTGCCTTCT 3′, MUC5B reverse primer, 5′ CCTCGCTCCGCTCACAGT 3′, MUC5AC forward primer, 5′ GGCAACACCCTCCTCTAGCA 3′, MUC5AC reverse primer, 5′ CCGTGGAAGGCTCTGTGAT 3′;. β-actin expression was used as an internal control. Real-time RT-PCR was performed on the generated cDNA products in the ABI Prism 7900 Fast RT sequence detection system (Applied Biosystems, Foster City, CA) as described previously. Quantification of the expression of the genes of interest relative to week 1 was obtained using the $\Delta\Delta Ct$ method.[12]

Immunofluoresence

pMEEC were cultured on collagen IV coated chamberslides until confluence in CRC medium, placed in differentiation medium for 48 hours and fixed in paraformaldehyde 3% for 20 minutes. After rinsing 3X with PBS 1X, cells were stored at 4°C until immunostaining was performed. Cells were permeabilized with PBS Tween 20 0.05% for 5 minutes; nonspecific sites were saturated using 0.01% PBS Tween-20 3% Bovine Serum Albumin for 30 minutes. This solution was also used to incubate primary anti-human antibodies cytokeratin 5 ab52635 (Abcam, Cambridge, MA), cytokeratin 14 LL001 (Santa Cruz, CA), cytokeratin 15 LHK15 (Abcam), junction plakoglobin SAB2500802 (Sigma Aldrich, St Louis, MI) at 1/500 dilution and the secondary antibodies anti-goat Alexa 488 A11055 anti-rabbit Alexa A10042 at 1/500 (Invitrogen, Carlsbad, CA). Three washes of 30 minutes were done in order to reduce the nonspecific signal of the antibodies. 4′,6-diamidino-2-phenylindole (DAPI) at 1:8000 in PBS was finally used to stain DNA. The chambers were removed and the slide was mounted using Permount mounting medium (Fisher Scientific, Suwanee, CA) with a coverslip. Slides were stored at 4°C in dark before immunofluorescent analysis using an Olympus FV1000 confocal microscope (Olympus, Rocklin, CA).

Western Blotting

Cells were lyzed with RIPA buffer with 1% protease inhibitor cocktail. Bicinchoninic acid assay (BCA) (Thermo Scientific, Belfonte, PA) was performed to assay the total quantity of proteins. A 50 μg of each sample was separated by electrophoresis on NuPAGE Novex 4%–12% Bis-Tris gels (Life technologies, Carlsbad, CA). The Kaleidoscope marker was used as a colored standard (Bio-Rad, Hercules, CA). Proteins were then transferred to a nitrocellulose membrane blocked with 5% non-fat dry milk in PBS

with 0.05% Tween-20 (PBST). The membrane was incubated with the primary antibodies: monoclonal anti human cytokeratin 5 ab52635 (Abcam), monoclonal anti human cytokeratin 14 LL001 (Santa Cruz, CA), monoclonal anti human cytokeratin 15 LHK15 (Abcam), monoclonal anti human beta tubulin D10 (Santa Cruz, CA) used at 1/1000 in 10 mL of 2.5% milk solution in PBST. Secondary anti-mouse (sc-2005) or anti-rabbit (sc-2004) antibodies coupled to HRP from Santa Cruz were used at 1/10 000 dilution. Detection was performed with a SuperSignal West Dura Extended Duration Substrate kit (Pierce) according to the manufacturer's instructions.

For mucins, western blot analysis was performed as previously described.[13] Briefly, samples containing 50 µg total proteins were separated by electrophoresis on a 1.0% agarose (Life technologies) gels. 1X Tris-acetate-EDTA (TAE) containing 0.1% SDS, 1 mM EDTA, and 40 mM Tris acetate (pH 8.0) was used as the electrophoresis buffer. Samples were solubilized in sample buffer containing urea, denatured at 95°C for 10 minutes and loaded into a horizontal gel apparatus. Electrophoresis was performed at 35 V for 2 hours, and at 15 V overnight. Proteins were transferred under positive pressure onto a polyvinylidine difluoride (PVDF) membrane (Millipore, Bedford, MA). It was then incubated for 1 hour at RT in 5% non-fat dry milk in PBST and then in 2.5% milk with a rabbit polyclonal anti-MUC5B antibody H-30 (Santa Cruz, CA) at 1:300 dilution. For MUC5AC immunostaining, the membrane was probed using LUM5-1, a rabbit polyclonal anti-MUC5AC antibody generously provided to us by Dr. Mehmet Kesimer, University of North Carolina at Chapel Hill at a 1:4000 dilution.[14] The secondary antibody goat-anti rabbit coupled to HRP at a 1:20 000 dilution (Santa Cruz, CA) was used for the immunodetection. As a control, 50 µg total protein from secretions of normal human bronchial epithelium (Lonza) grown at ALI for 2 weeks were used as a positive control for protein detection.

Histopathological Evaluation of pMEEC Grown at ALI

pMEEC cells were cultured on transwells at ALI and fixed with 10% formalin for 24 hours, embedded in paraffin and processed for H&E (hematoxylin and eosin) and periodic acid Schiff staining (PAS). The slides were observed with a BX51 Olympus microscope (Olympus) with the objective 40×.

In Solution Preparation of Secretions for Mass Spectrometry

pMEEC secretions were collected in BEBM on the apical and basal sides of inserts for 4 hours when collecting samples at week 1, 2, 3, and 4 of ALI. Secretions from the same compartment were pooled, centrifuged at 4°C for 10 minutes at 10 000*g* to remove debris and frozen until further use. After collecting all time points, samples were thawed and concentrated with Amicon 3K columns (Millipore) until having maximum 200 µL. A Bradford assay was performed to determine protein concentration (Biorad, Hercules, CA). Then, secretions were processed by in solution digestion prior to doing liquid chromatography with tandem mass spectrometry (LC MS/MS) as follows. In all, 50 µg of proteins were diluted with Ultrapure water (Sigma Aldrich) to

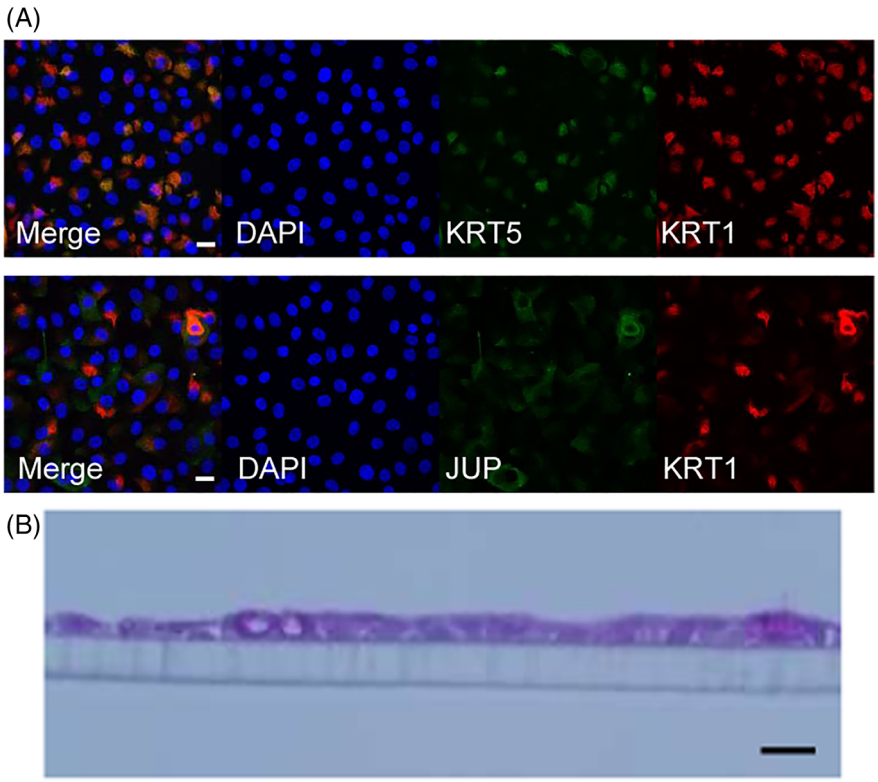

Fig. 1. Epithelial markers in submerged cultures and histologic picture of differentiated pediatric primary middle ear epithelial cells (pMEECs). (A) pMEECs were cultured in conditional reprogramming cell (CRC) medium on collagen IV coated chamberslides. When reaching confluence, pMEEC were placed in differentiation medium for 48 hours. Cells were then rinsed with PBS 1X and fixed with PFA. Immunofluorescence staining was performed and samples were observed with an Olympus FV1000 confocal microscope. DAPI: DNA dye; KRT5: cytokeratin 5; KRT14: cytokeratin 14; KRT15: cytokeratin 15; JUP: junction plakoglobin. (B) pMEECs were cultured on collagen IV coated transwells and cultured for 2 weeks of ALI. Cells were fixed in formalin 10%, dehydrated with ethanol baths, embedded in paraffin, cut and stained for H&E and PAS. Pictures of the slides were taken with an Olympus BX50 microscope. For both images: scale bar = 20 µm.

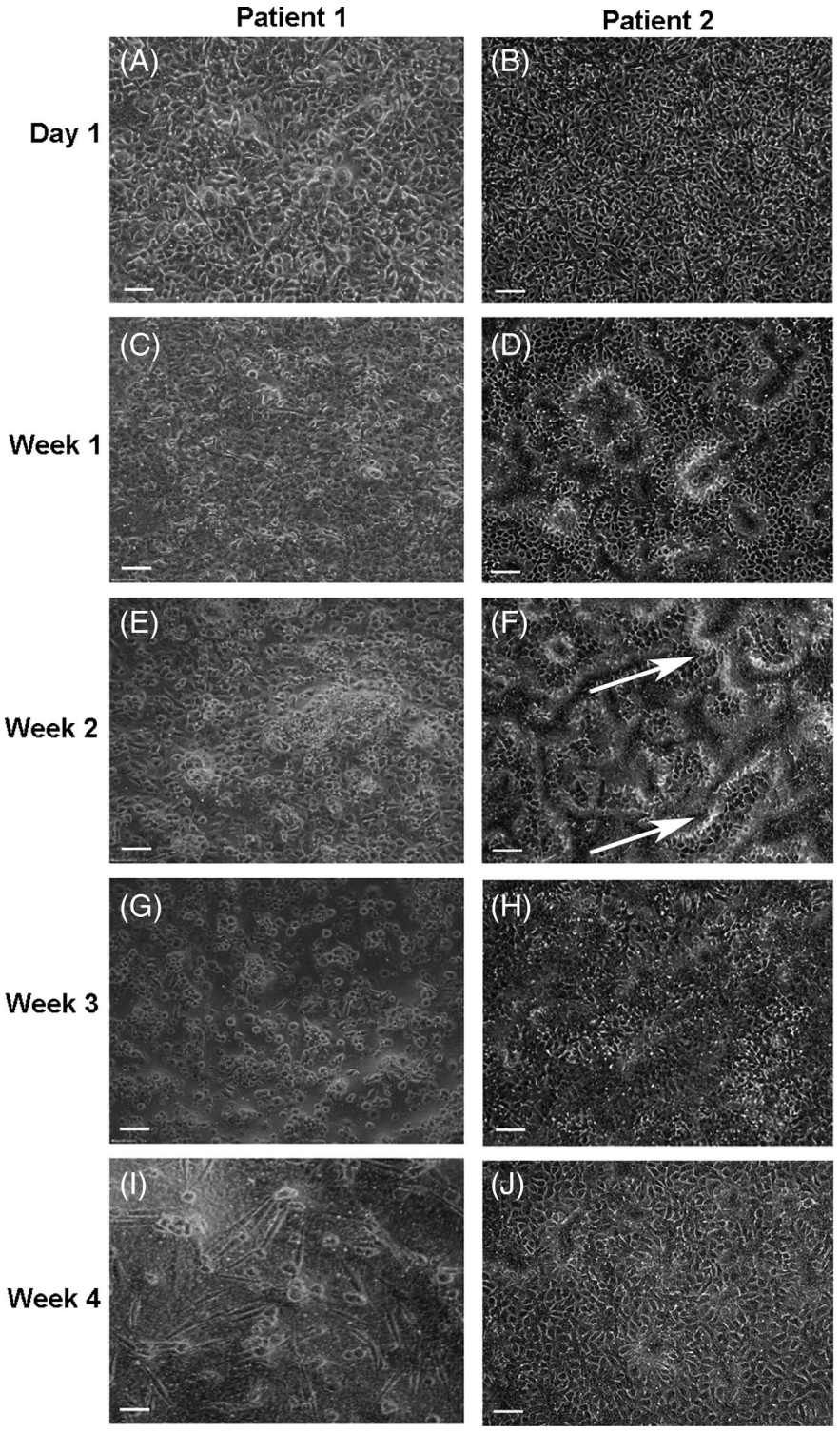

Fig. 2. Phenotype of pMEEC cultures at air–liquid interface (ALI) from 1 day to 4 weeks of differentiation. pediatric primary middle ear epithelial cells (pMEECs) were cultured as described in the Materials and Methods section and plated on transwell membranes with medium in the apical and basal compartments. When reaching confluence, the apical medium was removed and the basal medium was replaced by differentiation medium (ALI). Pictures of pMEEC from two patients were taken with a bright field microscope with the 20× objective after 1 day to 4 weeks of ALI. Scale bar = 100 μm. Arrows represent thicker layers, representing area of cellular overgrowth/thickening.

reach 50 μL of volume and proteins were precipitated with prechilled acetone at −20°C for 30 minutes. Samples were then centrifuged 30 minutes at 16 000*g* and the pellet was reconstituted in acetone and centrifuged again. The pellet was dissolved in 8 M urea and 45 mM dithiothreitol was then used to reduce proteins, following alkylation by adding 100 mM iodoacetamide.

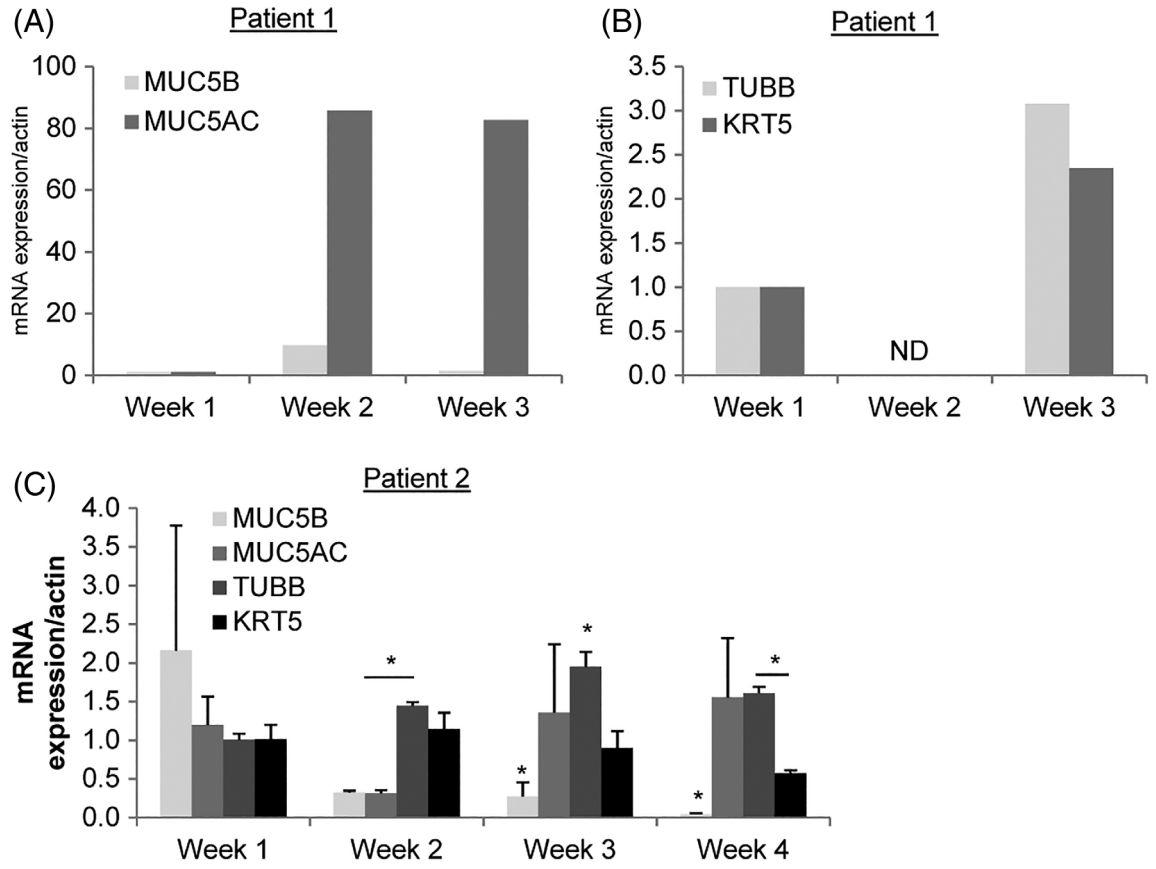

Fig. 3. mRNA expression of markers of epithelial differentiation in pediatric primary middle ear epithelial cell (pMEEC) cultures at ALI. pMEECs were cultured and differentiated at air–liquid interface (ALI) up to 4 weeks. After every week of ALI, cell lysates were recovered with the PureLink RNA kit lysis buffer and stored at −80°C until further use. RNA isolation, reverse transcription and polymerase chain reaction (PCR) were then performed to analyze MUC5B and MUC5AC (A and C), beta-tubulin (TUBB) and cytokeratin 5 (KRT5) (B and C) mRNA expression. * statistically significant $P < .05$ compared to week 1 expression. Patient 1: $n = 2$ replicate experiments; Patient 2: $n = 4$ replicate experiments.

Proteins were finally diluted in 100 mM ammonium bicarbone, digested with 0.1 µg/µL trypsin-gold (Biorad) for 16 hours and desalted with C18 ZipTip (Millipore).

MS and Protein Identification

MS analysis and data processing were carried out as previously reported by our group[13,15] but with a different platform. Briefly, the peptide mixtures from each sample were sequentially analyzed by LC MS/MS using nano-LC system (Easy nLC1000) connected to Q Exactive HF mass spectrometer (Thermo Scientific). The platform is configured with nano-electrospray ion source (Easy-Spray, Thermo Scientific), Acclaim PepMap 100 C18 nanoViper trap column (3 µm particle size, 75 µm ID × 20 mm length), EASY-Spray C18 analytical column (2 µm particle size, 75 µm ID × 500 mm length). The peptides were eluted at a flow rate of 300 nL/min using linear gradients of 5%–25% Acetonitrile (in aqueous phase and 0.1% Formic Acid) for 40 minutes, followed by 25%–45% for 10 minutes, and static flow at 90% for 10 minutes. The mass spectrometry data was collected in data-dependent manner switching between one full scan MS mode (m/z 380–1400, resolution 60 000, AGC $3e^6$) and 10 MS/MS mode (resolution 15 000); where MS/MS analysis of the top 10 target ions were performed once and dynamically excluded from the list for 20 seconds. The MS raw data sets were searched against UniProt human database that also included common contaminants using MaxQuant software (version 1.5.5.1).[16] We used default parameters for the searches, first search peptide tolerance 20 ppm, main search peptide tolerance 4.5 ppm, maximum two missed cleavage; and the peptide and resulting protein assignments were allowed at 0.01 FDR (thus 99% confidence level).

Statistical Analysis

The statistical difference between experimental and control groups was determined by two-tailed Student *t* tests for pairwise comparisons of numerical data, and ANOVA test followed by Dunnet test or Wilcoxon tests for multiple group comparisons of numerical data. Significance level was set at $P < .05$.

RESULTS

pMEEC Proliferate in Presence of CRC Medium and Show Epithelial Cell Markers in Submerged Conditions

pMEEC were collected from the middle ear of patients undergoing cochlear implant surgery where there was no active OM based on preoperative and intraoperative observation. They were then placed in CRC medium in a 25 cm^2 flask until confluence (1 to 2 weeks of culture), passaged into 2 75 cm^2 flasks with CRC medium (2 weeks of culture) until confluence before being plated on collagen coated

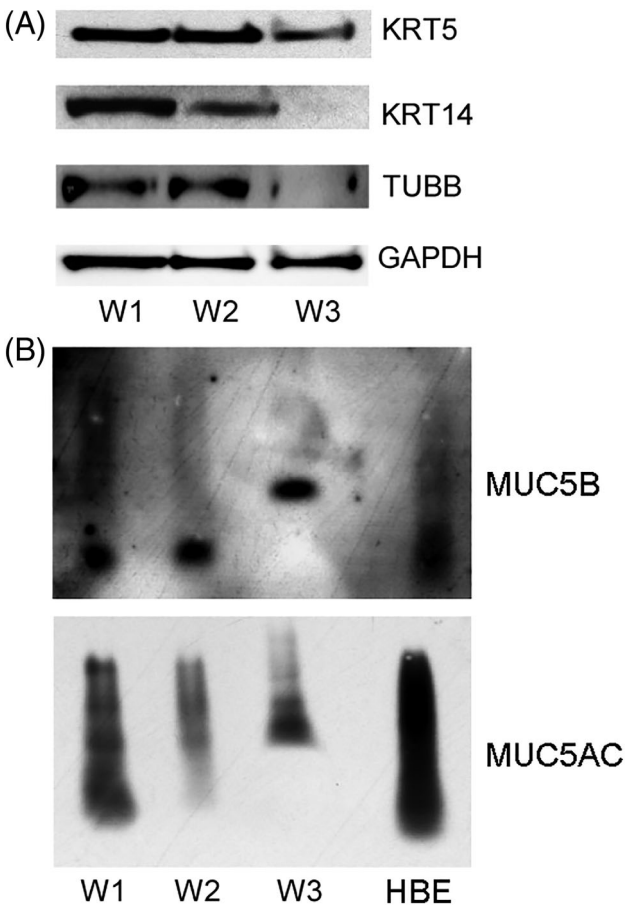

Fig. 4. Protein expression of differentiation markers in pediatric primary middle ear epithelial cell (pMEEC) at air–liquid interface (ALI). pMEEC were cultured and differentiated at ALI for up to 3 weeks. After every week at ALI, cell lysates were recovered in RIPA buffer with anti-protease inhibitor cocktail, and stored at −80°C until further use. 50 μg of proteins were used to detect cytokeratin 5 (KRT5), cytokeratin 14 (KRT14), beta-tubulin (TUBB) (A); MUC5B and MUC5AC (B). Human bronchial epithelial cell (HBE) secretions were used as positive controls for MUC5B and MUC5AC analysis.

transwell membranes or chamberslides. The mean doubling time for the cells was found to be 48 hours. After initial expansion, the density of cell plating into the 75 cm^2 flask was 1X104. Cells were harvested by trypsin and were counted. Mean cell counts were recorded. Initial attempts at initial expansion directly into a 75 cm^2 flask showed that the growth of the cells was too slow, >72 hour doubling time. To date, of the 10 patients we have consented for this method, we have successfully been able to expand MEECs in 6 of them. A critical component of getting cell growth yield is to actually visualize epithelial tissue in the micro-cup forcep bite (albeit a microscopic amount). The following characterization data shown come from the first two patients.

Primary MEEC plated on chamberslides were then placed in differentiation medium for 48 hours and were fixed to perform immunostaining (Fig. 1A). Markers of epithelial differentiation KRT5, KRT14, KRT15, and plakoglobin (JUP) were detected in pMEEC confirming that the cultures are epithelial cells. In addition, pMEEC were cultured in transwells for a week and placed at ALI for an additional 2 weeks to differentiate. Histologic sections were performed and then colored with H&E and PAS as shown in Figure 1B. After 2 weeks of differentiation, pMEEC formed a junctive epithelium with PAS staining, consistent with the formation of mucin producing goblet cells in culture.

pMEEC Exhibit a Tight Epithelium Phenotype at ALI

As in Figure 1B, pMEEC were plated on transwell membranes and cultured in CRC medium until confluence. Subsequently, cells were cultured in differentiation medium only in the basal compartment, at ALI. pMEEC were observed at day 1 and then every week following the first day of ALI with a bright field microscope (Figure 2). pMEEC covered the whole surface of the transwell at day 1. Cells showed islets on top of a lower layer of epithelium at week 1 and week 2 that disappeared at later time points. Although in some cases pMEEC maintained a tight epithelium until week 4 of ALI, in other cases the epithelial layer lost cells over time, with only a few remaining at week 4, displaying an atypical phenotype (long and thin).

pMEEC at ALI Express Mucosal Epithelial Markers at the Level of RNA and Protein

pMEEC RNAs and proteins were collected during differentiation to analyze the presence of markers described above. In one patient, only very low amount of RNAs and proteins recovered at week 4, indicating decreased cell stability after 3 weeks of ALI. Cells consistently expressed KRT5 and TUBB mRNA. Although TUBB mRNA increased from week 1 through 4, KRT5 mRNA expression remained stable (Fig. 3A). MUC5B and MUC5AC were both expressed at all times of differentiation, with variable expressivity over time (Fig. 3B), and MUC5B trending to decrease into weeks 3 and 4 of ALI.

Protein expression of these markers in cell lysates showed some differences relative to the noted mRNA expression (Fig. 4). KRT5 was expressed similarly at weeks 1 and 2 but decreased slightly at week 3. KRT14 was not evaluated at RNA level, but showed a strong expression at week 1, decreased at week 2 and was not detected at week 3 (Fig. 4A). MUC5B and MUC5AC were detected at all differentiation times and their expression decreased over time, displaying different gel-migration patterns, suggesting a modification in glycosylation between samples. For a separate patient, we found consistent KRT14 expression (Fig. 4B). For these experiments, secretions from normal human bronchial epithelial cells were used as a control for mucin protein detection.

pMEEC Secretome Analysis Reveals a Different Profile of Apical vs Basal Proteins, with Mucins only Being Secreted Apically

Mass spectrometry was performed with concentrated apical pMEEC washes and basal secretions over time. In order to compare samples, we normalized PC values to

TABLE I.
Twenty Most Abundant Proteins in Apical Secretions of Pediatric Primary Middle Ear Epithelial Cell (pMEEC) at Different Stages of Differentiation.

Gene Names	Protein Names	% Apical D1	% Apical W1	% Apical W2	% Apical W3	% Basal D1	% Basal W1	% Basal W2	% Basal W3	Total % Apical	Total % basal	Protein IDs
FLNB	Filamin-B	1.834	1.822	1.228	1.065	0.431	0.000	0.000	0.291	5.949	0.722	O75369
C3	Complement C3	1.146	1.524	1.456	1.279	2.526	3.032	1.109	2.574	5.405	9.241	P01024
HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein;Endorepellin	0.535	1.766	1.592	1.446	0.370	2.369	1.330	0.851	5.339	4.920	P98160
FLNA	Filamin-A	1.360	1.041	0.804	0.807	0.493	0.142	0.000	0.374	4.012	1.009	P21333
FN1	Fibronectin	0.810	1.134	1.122	0.944	2.218	2.132	3.104	2.657	4.010	10.111	P02751
MYH9	Myosin-9	0.902	1.171	0.576	0.685	0.000	0.000	0.000	0.021	3.334	0.021	P35579
ACTN4	Alpha-actinin-4	0.886	0.911	0.728	0.807	1.232	0.663	0.000	0.830	3.332	2.726	O43707
ACTN1	Alpha-actinin-1	0.932	0.874	0.713	0.761	1.232	0.616	0.000	0.706	3.280	2.554	P12814
AGRN	Agrin	0.489	1.004	0.880	0.883	0.678	2.321	2.217	1.432	3.255	6.649	O00468
LAMA3	Laminin subunit alpha-3	0.642	0.948	0.955	0.609	0.678	1.516	0.222	0.976	3.154	3.391	Q16787
PLEC	Plectin	1.498	0.353	0.713	0.472	0.123	0.000	0.000	0.083	3.035	0.206	Q15149
LAMC2	Laminin subunit gamma-2	0.535	0.855	0.698	0.472	1.171	1.800	2.217	1.204	2.559	6.392	Q13753
LAMB3	Laminin subunit beta-3	0.428	0.855	0.743	0.502	0.739	1.563	1.330	1.183	2.528	4.816	Q13751
THBS1	Thrombospondin-1	0.550	0.744	0.637	0.594	0.801	1.326	0.665	0.602	2.524	3.395	P07996
TNC	Tenascin	0.382	0.799	0.698	0.624	0.246	0.426	0.000	0.540	2.503	1.213	P24821
LAMA5	Laminin subunit alpha-5	0.306	0.930	1.062	0.198	0.000	0.379	0.000	0.021	2.495	0.400	O15230
TF	Serotransferrin	0.458	0.725	0.561	0.670	2.218	2.842	10.865	2.263	2.414	18.188	P02787
MSN	Moesin	0.642	0.595	0.516	0.548	0.924	0.947	0.000	0.830	2.300	2.702	P26038
IQGAP1	Ras GTPase-activating-like protein IQGAP1	0.458	0.409	0.667	0.746	0.000	0.000	0.000	0.062	2.281	0.062	P46940
MUC5B	Mucin-5B	0.000	0.353	0.819	1.050	0.062	0.000	0.000	0.000	2.222	0.062	Q9HC84

TABLE II.
Twenty Most Abundant Proteins in Basal Secretions of Pediatric Primary Middle Ear Epithelial Cell (pMEEC) at Different Stages of Differentiation.

Gene Names	Protein Names	% Apical D1	% Apical W1	% Apical W2	% Apical W3	% Basal D1	% Basal W1	% Basal W2	% Basal W3	Total % Apical	Total % basal	Protein IDs
TF	Serotransferrin	0.458	0.725	0.561	0.670	2.218	2.842	10.865	2.263	2.414	18.188	P02787
FN1	Fibronectin	0.810	1.134	1.122	0.944	2.218	2.132	3.104	2.657	4.010	10.111	P02751
C3	Complement C3	1.146	1.524	1.456	1.279	2.526	3.032	1.109	2.574	5.405	9.241	P01024
ANXA2	Annexin A2	0.489	0.576	0.470	0.472	1.787	1.611	3.991	1.225	2.007	8.613	P07355
KRT1	Keratin, type II cytoskeletal 1	0.229	0.353	0.197	0.411	1.540	0.805	4.656	1.142	1.191	8.144	P04264
CFB	Complement factor B	0.397	0.558	0.637	0.533	1.047	1.421	3.104	1.183	2.125	6.756	P00751
AGRN	Agrin	0.489	1.004	0.880	0.883	0.678	2.321	2.217	1.432	3.255	6.649	O00468
LAMC2	Laminin subunit gamma-2	0.535	0.855	0.698	0.472	1.171	1.800	2.217	1.204	2.559	6.392	Q13753
ENO1	Alpha-enolase	0.581	0.595	0.349	0.472	2.218	1.137	1.552	0.872	1.996	5.779	P06733
TPI1	Triosephosphate isomerase	0.321	0.353	0.273	0.274	1.294	0.947	2.661	0.581	1.221	5.483	P60174
HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein;Endorepellin	0.535	1.766	1.592	1.446	0.370	2.369	1.330	0.851	5.339	4.920	P98160
LAMB3	Laminin subunit beta-3	0.428	0.855	0.743	0.502	0.739	1.563	1.330	1.183	2.528	4.816	Q13751
ANXA5	Annexin A5	0.290	0.390	0.288	0.320	1.047	0.947	1.774	0.851	1.289	4.620	P08758
ANXA1	Annexin A1	0.413	0.483	0.394	0.411	1.171	1.232	1.109	1.080	1.701	4.590	P04083
PGK1	Phosphoglycerate kinase 1	0.382	0.465	0.288	0.396	1.602	1.137	1.330	0.436	1.531	4.505	P00558
ANXA3	Annexin A3	0.306	0.260	0.212	0.274	1.171	0.947	1.552	0.747	1.052	4.418	P12429
LCN2	Neutrophil gelatinase-associated lipocalin	0.183	0.223	0.182	0.228	0.616	0.426	2.882	0.477	0.817	4.402	P80188
GPI	Glucose-6-phosphate isomerase	0.367	0.372	0.227	0.259	1.479	0.853	1.330	0.581	1.225	4.243	P06744
CTSB	Cathepsin B	0.275	0.297	0.212	0.228	0.924	0.805	1.552	0.664	1.013	3.946	P07858
ALDOA	Fructose-bisphosphate aldolase A	0.413	0.483	0.394	0.396	1.294	0.995	0.665	0.934	1.686	3.888	P04075

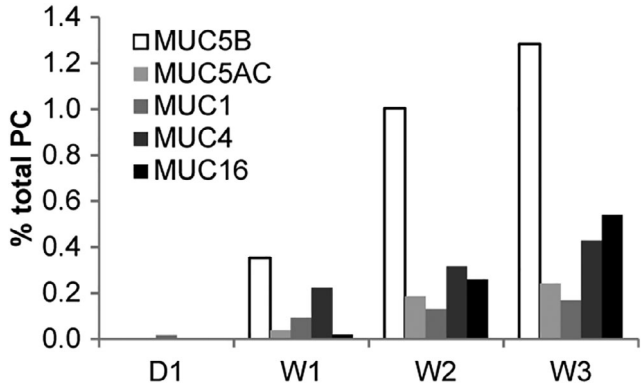

Fig. 5. Mucin proteins in apical secretions of pediatric primary middle ear epithelial cell (pMEEC) detected by mass spectrometry. pMEECs were cultured and differentiated at air–liquid interface (ALI) for up to 3 weeks. Apical secretions were collected 4 hours in serum free medium and same conditions were pooled. Then, proteins were concentrated with Amicon 3K columns and prepared for mass spectrometry analysis by performing in solution digestion with trypsin. Results are expressed as mucin protein peptide percentage.

the total PC of each sample to express results as percentages. The whole dataset of proteins for two patients are available in the Supplemental Tables S1 and S2 and the 20 most abundant proteins in apical and basal secretions are listed in Tables I and II. Overall, filamin B (FLNB), complement C3 (C3) and basement membrane-specific heparin sulfate proteoglycan core protein (HSPG2) were the three most abundant proteins in apical secretions. Basal secretions were comprised primarily of extracellular matrix and immunity mediated proteins such as serotransferrin, fibronectin, C3, and Annexins. Notably, mass spectrometry identified multiple mucins only in the apical secretions; including MUC5B, MUC5AC, MUC1, MUC4, and MUC16 (Fig. 5). MUC5B was among the 20 most abundant proteins in apical secretions.

DISCUSSION

The unlimited propagation for mammalian cells offers the opportunity to conduct research on the same cells in a reproducible manner. The main strategies to obtain immortalized cells have been to use viral oncogenes, usually SV40 large T antigen or the E6/E7 proteins of the oncogenic human papillomavirus, resulting in the deregulation of p53 and Rb pathways that can impact experimental results.[17,18]

A new culture method has shown promising results for the sustained culture of primary cells. Without viral transformation, primary cells undergo senescence after a determined number of mitoses and cease to proliferate. The CRC method using irradiated feeder cell medium supplemented with ROCK inhibitor allows for primary epithelial cells enter a stem cell like state and proliferate very efficiently.[19] When placed in a differentiation medium specific to the origin of the epithelial cells, they were able to differentiate in their original phenotype, without any karyotype or cancerous abnormality, and without selecting a subpopulation of cells but rather the entire population.[10]

The mechanisms by which irradiated fibroblasts stimulate the proliferation of target cells are only partially known. Gamma irradiated feeder cells are metabolically active cells unable to multiply and that stably secrete growth factors that support the expansion of hard to culture target cells. Some of the factors involved in this process have been shown to be leukemia inducible factor (LIF) that activates the JAK-STAT pathway, fibroblast growth factor 2 (FBF-2), and some extracellular matrix proteins.[20] The combination with ROCKi has been shown to induce hTERT, limiting cell senescence, a mechanism similar to immortalization using human papillomavirus.[20]

ROCKi itself was shown to alter actin and myosin activity in cell culture, allowing for cell proliferation.[21,22] Overall, this culture strategy permits to temporarily induce similar pathways than virus immortalization (telomerase induction and cytoskeleton remodeling) but later on return to a differentiated phenotype when the CRC medium is removed.

This study demonstrated the utility of the CRC culture method with pediatric human epithelial middle ear epithelial cells to study OM in vitro with a more physiological model expressing and secreting mucins. This is of significant importance in OM research as although existing transformed middle ear epithelial cell lines are able to express mucin mRNA, they do not reliably secrete mucin glycoproteins. Collectively, our extensive characterization of middle ear primary cell batches from different patients showed: 1) augmented proliferative phenoptype without senescence while submerged in CRC media allowing for multiple passages, 2) confirmation of epithelial lineage of these cells, 3) the ability for these cells to form a differentiated epithelium at ALI expressing tight junctions, cytoskeletal markers (TUBB) along with epithelial keratins, 4) PAS positivity, along with the consistent secretion of mucin glycoproteins, and 5) a natural variability in gene and protein expression of some of these markers potentially representing a underlying differential regulatory process of these markers across cells. Given how many cells seem to die after 4 weeks of ALI, we feel 2–3 weeks of ALI is the best time point to use the cells for further experiments.

Our current study has several key limitations. Given that the main focus was to describe the methodology, feasibility, and potential use of CRC methods to allow for the culture middle ear epithelial cells from children, we used a small sample size that did not allow us to correlate data with clinical or demographic subgroups. Moreover, we did not measure the conditional response of these cells to OM pathologically relevant pro-inflammatory stimulation. Accordingly, further studies are still needed to define the precise differences and similarities in the inflammatory responses of CRC derived from primary middle ear cells.

In conclusion, we have established and characterized a model that allows for the proliferation, passaging, and differentiation of pediatric primary middle ear epithelial cells. This in vitro culture system should greatly enhance the study of middle ear epithelial responses, including the overproduction of mucin glycoproteins, in OM research.

CONFLICT OF INTEREST

None declared.

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