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ORIGINAL RESEARCH

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Biobanked tracheal basal cells retain the capacity to differentiate

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Abstract

Objective: While airway epithelial biorepositories have established roles in the study of bronchial progenitor stem (basal) cells, the utility of a bank of tracheal basal cells from pediatric patients, who have or are suspected of having an airway disease, has not been established. *In vitro* study of these cells can enhance options for tracheal restoration, graft design, and disease modeling. Development of a functional epithelium in these settings is a key measure. The aim of this study was the creation a tracheal basal cell biorepository and assessment of recovered cells.

Methods: Pediatric patients undergoing bronchoscopy were identified and endotracheal brush ($N = 29$) biopsies were collected. Cells were cultured using the modified conditional reprogramming culture (mCRC) method. Samples producing colonies by day 14 were passaged and cryopreserved. To explore differentiation potential, cells were thawed and differentiated using the air–liquid interface (ALI) method.

Results: No adverse events were associated with biopsy collection. Of 29 brush biopsies, 16 (55%) were successfully cultured to passage 1/cryopreserved. Samples with higher initial cell yields were more likely to achieve this benchmark. Ten unique donors were then thawed for analysis of differentiation. The average age was 2.2 ± 2.2 years with five donors (50%) having laryngotracheal pathology. Nine donors (90%) demonstrated differentiation capacity at 21 days of culture, as indicated by detection of ciliated cells (ACT+) and mucous cells (MUC5B+).

Conclusion: Pediatric tracheal basal cells can be successfully collected and cryopreserved. Recovered cells retain the ability to differentiate into epithelial cell types *in vitro*.

Level of Evidence: Level 3.

KEYWORDS
biorepository, pediatrics, trachea basal cell collection, trachea brush biopsy

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1 | INTRODUCTION

One of the main functions of the trachea is mucociliary clearance of debris away from the lungs.[1] In addition, understanding the composition and function of the tracheal epithelium will provide perspective for the role of this tissue in healthy individuals, as well as those with known or suspected airway disease. For those with an airway injury, the epithelium moves toward the injury[2] and basal epithelial cells are required to repair the damaged region. It has been shown that the basal cell is a progenitor for ciliated and goblet cells.[3–12] Therefore, basal cells maintain healthy airway epithelium and repair damage through generating differentiated cells that restore epithelial function.[13]

While airway epithelial biorepositories have established roles in the study of bronchial progenitor stem (basal) cells,[14] a bank of tracheal basal cells from pediatric patients, who have or are suspected of having an airway disease, has not been established. Advancements in regenerative medicine and tissue engineering are working toward utilizing cells, a scaffold, and cell signaling to promote new tissue formation.[15] *In vitro* study of these cells can enhance cellular therapy options for tracheal restoration, graft design, and disease modeling. The ability to support the restoration of a functional epithelium is a key measure in each of these.

With utilization of the air–liquid interface (ALI) culture method, it allows airway epithelial cells to differentiate into ciliated cells, goblet/secretory cells, and basal cells. In addition, the epithelium has a structure and function that is representative of the epithelium in vivo.[16] The utilization of ALI culturing of tracheal cells has been reported in the literature with a variety of animal and human models.[17–27] While utilization of pediatric cells in ALI culturing has been minimally studied when researching lung disorders such as cystic fibrosis and asthma,[28] pediatric patients with laryngotracheal pathology has not been studied in this way. This culture process enables in vitro study of airway epithelial features, cell differentiation, and an evaluation of pathological changes in an injured epithelial layer.[29,30] Enhanced knowledge of these mechanisms can lead to improved treatment options for individuals who live with airway disease. Additionally, epithelial cultures have been grown at ALI and have served as a translational role in testing the responsiveness to modulators. The data have been used to request insurance coverage for modulator therapy.[31] Human ALI cultures have also been used in experiments where multiple cultures were treated with varying doses of agonists or antagonists.[32] However, in all instances care needs to be taken when expanding cells recovered from brush biopsies, so as not to exhaust their mitotic potential.[33]

With this gap in knowledge relating to laryngotracheal pathology in the pediatric population, through development of these standard operating procedures, we are preparing for future translational studies. Clonal isolates of human airway epithelial cells have been utilized for differentiation assays,[33] and they can again be utilized in this way in the future. Establishing this model system accomplishes our immediate goal of successfully culturing and assessing differentiation of the biobanked pediatric cells. The long-term goal is to ask additional questions related to our donor population. These questions will include assessing if cells maintain their disease phenotype and whether this is an intrinsic or extrinsic property. In addition, we could potentially identify those that would benefit from a graft intervention or medication.

In this study, we aimed to create a tracheal basal cell biorepository and then determined if the banked cells could be used to model the normal and diseased pediatric epithelium. Cells recovered by endotracheal brush biopsy, from healthy pediatric participants and those who have underlying medical conditions, were investigated in our laboratory.

2 | MATERIALS AND METHODS

2.1 | Participants

Pediatric patients undergoing scheduled direct laryngoscopy and bronchoscopy were identified at a tertiary-level pediatric hospital and were recruited voluntarily. Consent from legal guardians and assent was obtained from 32 patients, with an average age of 4.1 ± 4.6 years. In one instance, a consented case was not collected due to a change in bronchoscopy method (flexible instead of rigid), therefore specimens were collected from 31 patients. This study was approved by the institutional review board.

2.2 | Cell collection, processing, and culture

Endotracheal brush ($N = 29$) biopsies were collected according to previously published brushing methods[34] and used a cytology brush (Medical Packaging Corporation, Camarillo, CA). The cytology brush was placed under direct visualization with the assistance of the Hopkins rod telescope (KARL STROZ Endoscopy-America, El Segundo, CA). The brush was then spun in a focal region of the trachea five times for a total of approximately 10 s. Cells were liberated from the brushes using mechanical agitation in phosphate buffered saline (PBS) supplemented with β-mercaptoethanol (Acros Organics, NJ), then pelleted using centrifugation (2500 rpm × 5 min). The pellet was resuspended in 1 ml of red blood cell lysis buffer (Invitrogen/Thermo Scientific, Waltham, MA) and incubated on ice for 5 min. Lysis activity was inactivated by dilution with PBS (1:10) and centrifuged again. The cell pellet was resuspended in culture media for quantification and subsequent plating. Cells were evaluated for initial yield and viability by diluting an aliquot with Trypan Blue and a manual count using a hemacytometer.

Cells were cultured using the modified conditional reprogramming culture (mCRC) method. This method co-cultures the collected cells on a feeder layer of irradiated NIH-3T3 fibroblasts (ATCC #CRL-1658) with F-medium (F_{med}) supplemented with the Rho-kinase inhibitor, Y-27632.[35] At initial plating, culture medium ($F_{med} + Y$) is also supplemented with an antibiotic/antifungal cocktail containing Amphotericin B (Sigma, St. Louis, MO), Fluconazole (Gallipot, Rotterdam, the Netherlands), and Gentamicin Reagent Solution (Gibco/Thermo Fisher, Waltham, MA) at 100%. The cocktail is reduced to 50% concentration at day 2 of culture and eliminated at day 4 of

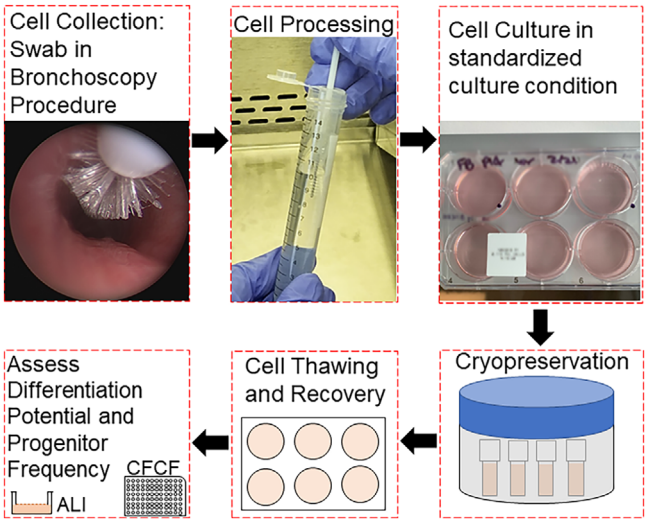

FIGURE 1 Flowchart for process of specimen collection

culture. Culture medium is changed on a Monday–Wednesday–Friday schedule.

Cultures were monitored for development of basal cell colonies and discarded if there was no colony development after 14 days of culture and did not reach 70% confluence. Visual inspection of the colony size was completed to ensure that the p0 cultures did not overgrow, which could lead to unsuccessful passage and depletion of mitotic potential. Cultures were expanded at passage 1 and cryopreserved as frozen stocks (up to 5×10^5–1×10^6 cells per vial) at passage 2 (Figure 1).

2.3 | Cryopreservation method

At passage 2, cells were suspended in $F_{med} + Y$ with 10% dimethyl sulfoxide (DMSO) and cryopreserved as frozen stocks (up to 5×10^5–1×10^6 cells per 1 ml vial). These vials were placed into a quick-freeze container and frozen according to the manufacturer's instructions, then maintained in liquid nitrogen for long-term storage.

2.4 | Thawing

Ten cultures, that had the greatest volume of frozen stocks, were thawed for cell recovery and preliminary analysis. These vials were retrieved from liquid nitrogen and immersed in a 37°C water bath for 1–2 min. Cells were not centrifuged following thaw and were added directly to irradiated NIH-3T3 fibroblasts in $F_{med} + Y$ medium. Emphasis was placed on ensuring that a great amount of time did not elapse between thawing and plating.[36]

2.5 | ALI culture

To explore differentiation potential, cryopreserved passage 2 cells were thawed and cultured on irradiated fibroblasts for one passage. Passage 3 cells were seeded on collagen-coated (the collagen was made 1X by adding 20X stock) transwell membranes (0.33 cm^2 culture area; Corning, Glendale, AZ) at a density of 4×10^4 cells/well in 0.15 ml hALI-p medium with Y-27632.[37] Therefore, 120,000 cells were seeded per square centimeter. Y-27632 was removed the following day to allow cell proliferation. At confluence, media was changed to supplemented Pneumacult ALI medium (StemCell Technologies, Vancouver, Canada) to the basal compartment only to establish the ALI. ALI cultures were fed M, W, F and fixed on day 21 (Figure 1). Membranes were fixed with 4% paraformaldehyde, 3% sucrose, and 1X PBS fixation solution and stored at 4°C prior to immunofluorescent analysis.

2.6 | Clone-forming cell frequency assay

Cryopreserved cells were assessed for the presence of clone-forming cells at the time they were seeded for ALI culture. Clone-forming cell frequency (CFCF) is an assay that is a limiting dilution method.[38] This assay reports progenitor cell frequency as the CFCF × 1000. Therefore, if CFCF × 1000 equals 1000, then all cells created a clone. Whereas, if CFCF × 1000 equals 750, then only three quarters of the cells formed a clone.[13] CFCF has been used to measure the quantity of progenitor cell frequency *in vitro* and *in vivo* (Figure 1).[5,33,37,39]

2.7 | Immunofluorescent staining of ALI membranes

Fixed membranes were blocked with PBS containing BSA and Triton X-100, then probed with primary antibodies to acetylated tubulin (mouse anti-ACT, 1:8000; Sigma) and Mucin 5b (rabbit anti-MUC5b, 1:500; Sigma) to visualize differentiation into ciliated and goblet epithelial cell types. Following an overnight incubation at 4 °C, membranes were washed in 1X PBS solution and probed with secondary antibodies to anti-mouse IgG (AF488, 1:500; Invitrogen/Thermo Fisher, Waltham, MA) and anti-rabbit IgG (AF594, 1:500; Invitrogen/Thermo Fisher, Waltham, MA). DAPI (1:1000 dilution of 1 mg/ml stock solution; Sigma) was added as a counterstain to visualize all cellular nuclei. Membranes were again washed with 1X PBS solution, then mounted on slides with Fluoromount G medium (Southern Biotech, Birmingham, AL). Images were captured on DAPI, Alexafluor-488, and Alexafluor-594 channels at 40× magnification (6 fields/membrane) and quantified using the ImageJ software (NIH, Bethesda, MD).

3 | RESULTS

No adverse events were associated with biopsy collection. Our complete specimen cohort included 31 patients. Of the total 29 brush biopsies that were obtained, 16 (55%) were successfully cultured to passage 1 and then cryopreserved at passage 2. Biobanked samples from 10 donors were recovered and differentiation potential and progenitor frequency were quantified using ALI and CFCF (Figure 2).

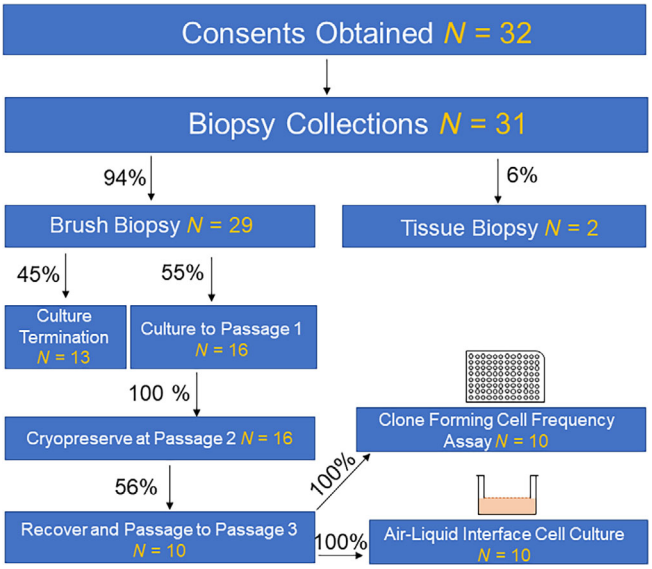

FIGURE 2 Complete specimen cohort

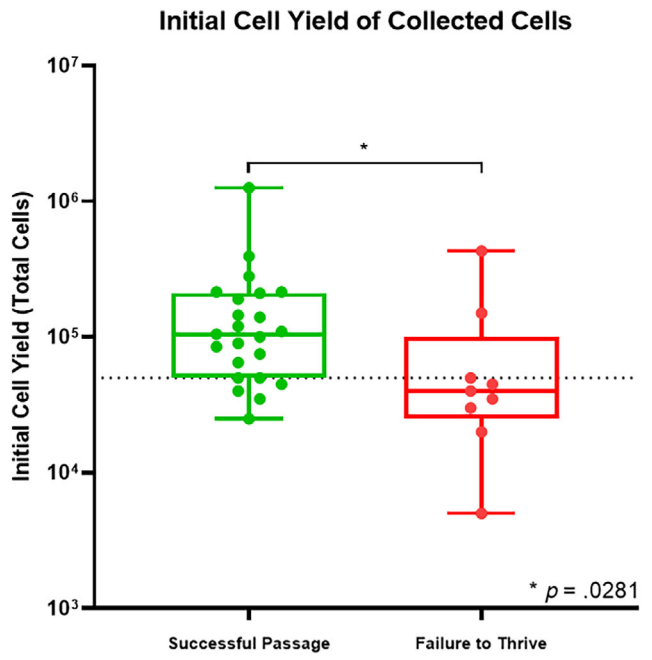

FIGURE 3 Initial cell yield as a predictor of successful culture to passage 1. Cultures with an initial cell yield of greater than 5×10^4 cells were more likely to survive to passage 1 and subsequent cryopreservation. For the purposes of the study, "Failure to Thrive" was defined as the development of 3 or fewer colonies after 14 days in culture.

In assessing samples that were successfully cultured, those with higher initial cell yields were more likely to achieve this benchmark. Cultures with an initial cell yield of greater than 5×10^4 cells were more likely to survive to passage 1 and subsequent cryopreservation ($p = .0281$). For this study, "failure to thrive" was defined as the development of 3 or fewer colonies after 14 days in culture (Figure 3).

TABLE 1 Demographic information of nine patient samples successfully recovered for assessment

	Frequency	Percentage
Gender		
Female	3	33%
Male	6	67%
Race		
White	6	67%
Black	3	33%
Prematurity		
<28 Weeks	2	22%
28–32 Weeks	3	33%
33–37 Weeks	1	11%
Full-term	3	33%
Tracheostomy medical history		
Current tracheostomy	1	11%
None	8	89%
Decannulated tracheostomy	0	–
Underlying medical conditions		
Neurologic disease	3	33%
Chromosomal syndrome	1	11%
Pulmonary disease	3	33%
GI disease	3	33%
Past airway surgery	2	22%
Congenital malformation	2	22%
Cardiac disease	0	–

Of the 10 brush biopsy samples that were thawed, 9 (90%) were capable of differentiation by ALI. Of the nine unique donors, 33% were female ($N = 3$), the average age was 2.4 ± 2.4 years, and 67% ($N = 6$) were white. Prematurity varied greatly with 22% ($N = 2$) patients having a gestational age less than 28 weeks, 33% ($N = 3$) of patients between 28 and 32 weeks, 11% ($N = 1$) of patients between 33 and 37 weeks, and the remaining 33% ($N = 3$) at full term. Only 11% ($N = 1$) have a current tracheostomy and the remaining 89% ($N = 8$) have no tracheostomy history. The nine donors also had a variety of underlying medical conditions including neurologic diseases, chromosomal syndromes, pulmonary disease, gastrointestinal diseases, congenital malformations, and past airway surgeries (Table 1).

In evaluating the procedure information of the nine donors, the primary indication for the bronchoscopy included dysphonia not yet diagnosed ($N = 1$, 11%), aspiration not yet diagnosed ($N = 2$, 22%), surveillance after airway surgery ($N = 1$, 11%), supraglottic obstruction/lesion ($N = 2$, 22%), tracheal obstruction/lesion ($N = 1$, 11%), and the bronchoscopy being part of another airway procedure ($N = 2$, 22%). Four donors (44%) had laryngotracheal pathology. Laryngotracheal interventions at the time of specimen collection included microdebrider laser surgery ($N = 1$, 11%), supraglottoplasty ($N = 1$, 11%), and no intervention ($N = 7$, 78%). Finally, the grade view during the bronchoscopy was grade 1 ($N = 8$, 89%) or grade 2 ($N = 1$, 11%)

TABLE 2 Bronchoscopy procedure information of nine patient samples successfully recovered for assessment

	Frequency	Percentage
Primary indication for DLB		
Dysphonia NYD	1	11%
Aspiration NYD	2	22%
Surveillance after airway surgery	1	11%
Supraglottic obstruction/lesion	2	22%
Tracheal obstruction/lesion	1	11%
Part of other airway procedure	2	22%
Stridor NYD	0	—
Recurrent croup	0	—
Failure to wean off ventilator	0	—
Failure to extubate	0	—
Rule out foreign body	0	—
Glottic obstruction/lesion	0	—
Subglottic obstruction/lesion	0	—
Laryngotracheal pathology findings during DLB		
Subglottic stenosis	1	11%
Laryngomalacia	2	22%
Subglottic cyst	1	11%
No findings	5	56%
Tracheal stenosis	0	—
Tracheomalacia	0	—
Laryngotracheal intervention at time of cell collection		
Microdebrider laser surgery	1	11%
Supraglottoplasty	1	11%
No Intervention	7	78%
Balloon dilation	0	—
Injection of steroids	0	—
Grade view during DLB		
Grade 1	8	89%
Grade 2	1	11%
Grade 3	0	—
Grade 4	0	—

(Table 2). No correlation between clinical information and successful culture in ALI has been made at this time.

While all 10 recovered cultures were able to proliferate to confluence on transwell membranes, 9 of 10 cultures (90%) demonstrated differentiation capacity at 21 days of culture, as indicated by detection of ciliated cells (ACT+) and mucous cells (MUC5B+; Figure 4). Bronchial epithelial cells were recovered to act as a control and comparison to our tracheal epithelial cell cultures. Bronchial cells are the gold standard and are often used for cell differentiation studies. The bronchial epithelial culture had uniform coverage on the membrane, as seen in the high mean expression and low standard deviation for ACT and MUC5b staining (Figure 4). The tracheal epithelial cell cultures from our pediatric population were much more variable in ACT and MUC5b expression across the nine successfully recovered cultures (Figure 4). Since our results show that the recovered cultures were able to retain the ability to differentiate, further study is needed to determine whether the extent of the differentiation is related to disease.

The CFCF assay showed that CFCF is highly variable in tracheal epithelial cells with a mean progenitor cell frequency value of 175 (SD: 130; Figure 5). We report that 90% of the cultures that successfully differentiated at passage 3 had a CFCF value between 39 and 281. The one unsuccessful culture had the highest CFCF value of 366. In addition, for cultures where CFCF was determined for the cells at passage 1, there was a decline in the number of cells as passage number increased. This finding of CFCF decreasing with passage has been described and aligns with previous literature.[33]

4 | DISCUSSION

A formalized approach to the banking of tracheal basal cells from pediatric patients has not been established. Being able to collect and cryopreserve tracheal basal cells would enable in vitro study of these cells to enhance cellular therapy options for tracheal restoration. Ensuring that these cryopreserved cells maintain their ability to differentiate is an important measure.

We report that tracheal epithelial basal cells obtained from pediatric patients can be collected by brush biopsy during bronchoscopy procedures and are able to be successfully cryopreserved for clinical research. Showing that our biobanked cells are capable of differentiation, this indicates that this methodology is feasible and additional efforts should be put toward utilizing this model system. It should be noted that these collections can be completed safely, as no adverse events were associated with biopsy collection. Successful culture of the collected cells can be impacted by an inability to develop colonies (Figure 3). In addition, since colony development may be influenced by the initial cell yield, brushing techniques that maximize the collection of cells, while not compromising the safety of the patient, should be utilized. In the cell culturing process, care should be taken not to overgrow p0 cultures to ensure greater probability of success in additional passages.

In developing the most successful method of recovering cryopreserved tracheal epithelial basal cells, upon recovery from cryopreservation, passaging the cells was required to successfully establish ALI cultures. The two methods that were tested were direct seeding versus utilizing an intermediate passage. In the direct seeding experiment, the cells did not grow to confluence after 14 days in the culture medium, however passaging the cells after thawing proved successful. The recovered cells were capable of differentiation into expected epithelial cell types, including ACT+ ciliated cells and MUC5+ mucous-producing cells, thereby affirming that these cells maintain the ability to support the restoration of a new epithelium. The one donor that was not capable of differentiation by ALI, was found to have laryngotracheal pathology during the bronchoscopy. In this way, additional donors are needed to determine if this pathology contributed to the unsuccessful ALI assay. Furthermore, in cases where cultures are

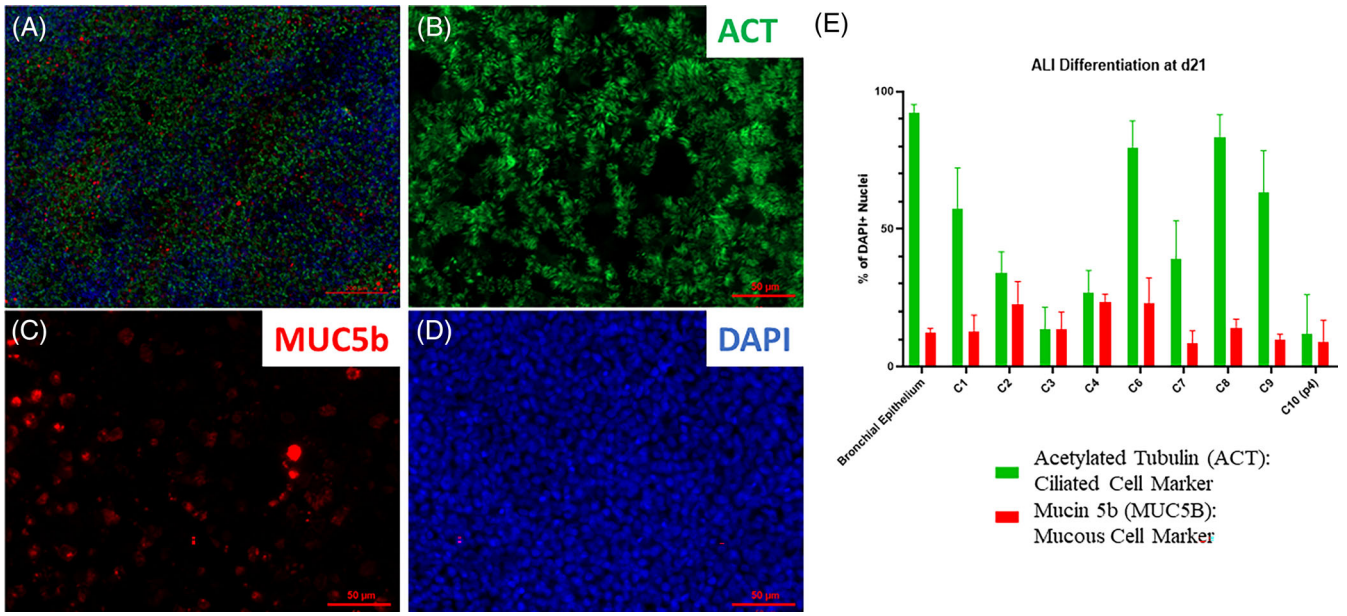

FIGURE 4 Recovered tracheal basal cells were capable of differentiation into expected epithelial cell types after 21 days of air–liquid interface (ALI) culture. A representative image (A) at 10× magnification of 1 culture capable of differentiation. Panels (B)–(D) were representative images of Culture A at 40× magnification. (B) Ciliated cells expressing acetylated tubulin (ACT, green). (C) Mucous-producing cells expressing Mucin 5b (MUC5b, red). (D) DAPI (blue) counterstaining to visualize cell nuclei. (E) ALI differentiation of a bronchial epithelial cell culture (ACT: 92.4% ± 2.9%, MUC5b: 12.5% ± 1.6%, $N = 6$) and tracheal epithelial cell cultures (ACT: 45.7% ± 27.6%, MUC5b: 15.0% ± 8.1%, $N = 53$) at 21 days. C5 is the culture that did not differentiate; therefore, it is not included in the data.

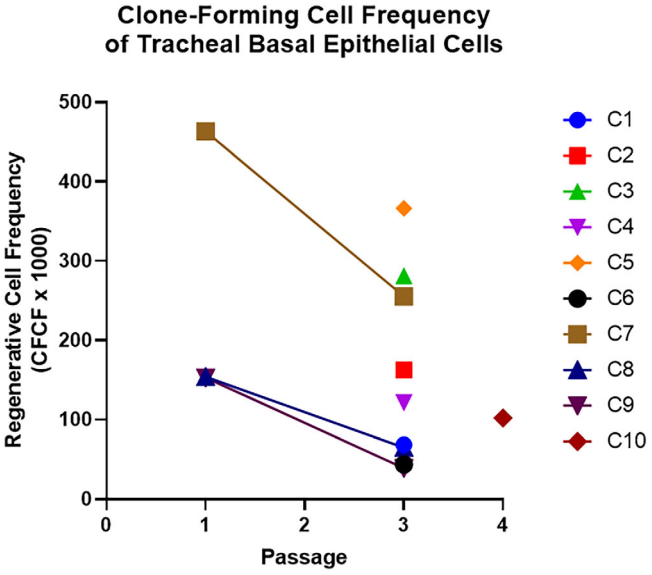

FIGURE 5 Clone forming cell frequency is highly variable in tracheal epithelial cells. Recovered cultures were assessed for the presence of clone-forming cells at the time they were seeded for air–liquid interface (ALI) culture. The number of clone-forming cells varied among the cultures.

failing, it would be beneficial to check CFCF values to ensure that cultures are not being overgrown and utilizing all their mitotic potential. Overall, because we were able to show that there is differentiation, it indicates that the cultures are polarized and that there is epithelium, marked by the presence of ciliated and goblet cells.

There is a need for future work, so as to make advancements toward creating a robust *in vitro* tool to evaluate the performance of candidate biomaterials that are being considered for use in tracheal replacement procedures. Overall, tracheal epithelial basal cell biobanking has potential as a means of studying epithelial cell behavior in the pediatric population.

5 | CONCLUSION

Pediatric tracheal basal cells can differentiate into ciliated cells and goblet cells *in vitro*, and these populations can be successfully collected and cryopreserved for future use. Initial cell yield is a predictor of successful culture for cryopreservation. Future studies can utilize this method to collect, culture, and cryopreserve specimens, which can then be used for studies involving cellular therapy options for tracheal restoration, graft design, and disease modeling.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose for this study.

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