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Creation of a long-lifespan ciliated epithelial tissue structure using a 3D collagen scaffold

Yuchi Wang^{a,*}, Lid B. Wong^a, and Hua Mao^a

^aBioTechPlex Corporation, 1205 Linda Vista Dr., San Marcos, CA 92078 USA

Abstract

We describe a method of using a 3D collagen gel scaffold applied at the air-liquid interface to culture dissociated primary tracheal-bronchial ciliated cells into a ciliated epithelial tissue structure (CETS). This 3D collagen gel culture system enables the induction of ciliogenesis and continuously provides support, maintenance, development, differentiation and propagation for the growth of cilia into the CETS. The CETS developed by this system resembles the ciliary metachronal motility and morphological, histological and physiopharmacological characteristics of cells found in native and *in vivo* ciliated epithelia. The CETS can be sustained for months with a straightforward and simple maintenance protocol. The integrity of the functional ciliary activity of this CETS enables the evaluation of long-term effects of many pulmonary drug candidates without using animals.

1. Introduction

Ciliary activity, one of the most primitive forms of locomotion in nature, was first discovered in the mammalian respiratory tract by Purkinje and Valentin in 1834 [1]. Ciliated cells are adapted in the respiratory tract to form an epithelial lining of coordinated metachronal ciliary activity that provides the propelling force for the transport of mucus along the airways. This mechanism is central to the maintenance of patent airways and pulmonary homeostasis. Impaired mucociliary transport is an inherent pathological characteristic of patients with chronic obstructive pulmonary disease, asthma and cystic fibrosis [2].

A ciliated epithelial tissue or cell culture system that mimics *in vivo* metachronal ciliary activity is critical to the understanding of the physiology and biophysics of this phenomenon. However, the preservation of active functional cilia and the induction of ciliogenesis of airway epithelial cell cultures remains one of the most elusive culturing and tissue engineering problems. Cells dissociated from their natural milieu of 3D geometrical supporting structures usually do not grow into tissue-like cultures. This is particularly obvious in cells that exhibit contractility or motility functions such as cardiac myocytes and ciliated cells. Thus, to develop a tissue culture system for ciliated epithelial tissue structure (CETS), we explored a process in which we used different biomaterials as scaffolds, including collagen [3–6], chitosan [7], and gelatin [8], in combination with the air-liquid interface (ALI) culturing technique that has been uniquely developed for epithelial ciliated cell culture [9–13].

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*Corresponding author. BioTechPlex Corporation, 1205 Linda Vista Dr., San Marcos, CA 92078 USA. Tel.:7607441000; fax:760 744 0033, ywang@biotechplex.com.

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We herein describe a tissue engineering method in which we used dissociated primary mammalian airway ciliated epithelial cells and collagen, in association with the right constituents and the ALI culturing condition, to create a CETS. This 3D CETS with cilia exhibited vigorous, coordinated beating, viable cell interactions, polarity and morphological features closely resembling those found *in vivo* and has never before been reported. The CETS structure can be maintained in culture for a minimum of 4 months. Such a prolonged lifespan is especially useful for pulmonary cell-based drug discovery for the investigation of agents with long-acting physiopharmacological effects on mucociliary clearance. We have validated this method by using dissociated primary canine and porcine tracheal ciliated epithelial cells.

2. Materials and Methods

2.1. Preparation of scaffold matrix using collagen, chitosan, and gelatin

Type I collagen (0.3% at pH 3, ICN Biochemicals, Aurora, OH) was mixed with Dulbecco's Modified Eagle Medium (DMEM) and phosphate-buffered saline (PBS) to reach a final concentration of 2.0 mg/ml collagen in 10% PBS (v/v). A 1% solution of penicillin-streptomycin (10,000 units/ml penicillin, 10,000 g/ml streptomycin) was then added and the pH adjusted to 7–8 using 4.5% (w/v) NaHCO₃. The resulting collagen mixture was stored at 4 °C to prevent premature fibril assembly.

A day before culture, the collagen solution was gently vortexed to allow uniform distribution. For a polyethylene cell culture insert for a 24-well plate (Falcon, Becton Dickinson), a gel matrix <1 mm thick was formed with 100 µl of collagen solution incubated at 37 °C for 1 h. The gel was overlaid with DMEM and ready for cell seeding. Following the dispersion of the primary dissociated ciliated cells on the 3D gel surface, ciliated cells were allowed to attach to the surface and grow. No subculture of the cells was implemented.

To prepare the scaffold matrix using either chitosan or gelatin, we poured a 2% chitosan solution (1% acetic acid) or gelatin solution into the inserts. The respective 3D gels were polymerized with 5 N NaOH in a 37 °C incubator for 15 min and stored in the incubator at 37 °C and 5% CO₂. The gel was balanced with a PBS solution and washed several times with culture media with the pH adjusted to 7. Following the dispersion of the primary ciliated cells on the 3D gel surface, cells were allowed to attach to the surface and grow. Routine, conventional culture maintenance procedures such as exchanging the culture medium were implemented.

2.2. Harvesting primary tracheal-bronchial ciliated epithelial cells

Porcine tracheae were obtained from a local slaughterhouse. Canine tracheae were obtained from the University of Illinois Biological Resources Laboratory. These animals were killed at the end of formal protocols and the tracheae were harvested according to the approved animal protocols. These fresh tracheal tissues were stored in chilled M199. A total of 3 canine tracheae and 5 porcine tracheae were used.

The protocol for harvesting primary tracheal-bronchial ciliated epithelial cells has been described elsewhere. In brief, the tracheal and bronchial mucosae were dissected from the tracheae and bronchi. The resulting mucosae were washed in PBS and cut into small pieces of ~20 mm². They were digested at 4 °C for 14 h using 0.75% protease (Sigma) in M199. Clusters of ciliated cells were harvested by gently shaking the mucosae in M199 enriched with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin. The cells were allowed to settle for 1 h in a plastic petri dish to minimize fibroblast contamination. The resulting ciliated cells were clustered and floated in the media, whereas the fibroblasts adhered to the bottom of the petri dish. The resulting clusters of ciliated cells were collected and washed by centrifugation at

220g for 5 min in M199 and then dispersed in M199. In each of the tracheae, 5 to 10 samples of ciliated cell cultures were obtained.

2.3. Generation of CETS

After overnight incubation, the solidified collagen gel on the culture insert was rinsed with the culture medium. After the cells were seeded ($2-4 \times 10^4$ cells per insert) on the collagen gel, the culture inserts were placed in a 24-well culture plate. Cells were cultured in a media-submerged condition, that is, with medium (bronchial epithelial growth medium [BEBM; Cambrex, Walkersville, MD] + 10% FCS + 10^{-7} M retinoic acid) on both apical and basolateral compartments of the insert. After 3–4 days, an ALI culture was established by lowering the level of the medium in the apical compartment of the insert to the level of the collagen gel. The cells were kept in a 37 °C, 5% CO₂ incubator with 100% humidity. Culture medium was renewed every 2–3 days. For CBF (ciliary beat frequencies) measurements, the cells were temporarily transferred to a 2-well chambered coverglass (Lab-Tek Chambered Coverglass, Nalge Nunc).

2.4. Scanning electron microscopy (SEM)

Inserts were washed twice in PBS prior to fixation in 300 l/insert containing 2% glutaraldehyde in $1 \times$ PBS at room temperature for 2 h. The samples were then fixed using 1% osmium tetroxide in the dark for another hour before they were rinsed and held for 5 days at 4 °C. The resulting membranes were loaded into a dryer holder and dehydrated with ethanol at increasing concentrations of 50%, 75%, 95%, 100%, 100% and 100% for 7 min at room temperature. After the samples were processed by a Samdri 760 critical point dryer, they were removed from the holder and mounted with carbon tape and sputter coated (Hummer VI sputter coater) for 5 min with gold/palladium. SEM pictures were viewed using a Hitachi S2700 SEM coupled with IXRF EDS2004 software.

2.5. Histology of CETS

The samples were fixed in 4% paraformaldehyde that was dissolved in 0.1 M PBS and embedded in a hydrophilic resin. The sample was cut into 1- and 2-mm sections and stained with toluidine blue and Weigert's resorcin-fuchsin [14].

2.6. Video recording of ciliary activity of CETS

A CETS sample was selected randomly for video recording. The sample was placed in an inverted microscope (Diaphot, Nikon, Japan) with a CCD camera (Model NX18AS, NEC, Japan) attached to the video port of the microscope. The CETS image was recorded every 2–4 weeks up to 4 months after ciliated cell seeding using a 40 $\times$ objective. At the end of 4 months, vigorous ciliary beating was observed in all samples but no further recordings were made.

2.7. Measurements of ciliary beat frequencies using dynamic laser light scattering

CBF was measured by a laser light-scattering technique using an inverted microscope [15]. The CETS sample was transferred to a 2-well chambered coverglass and placed on an inverted microscope stage. Hank's solution (100 μ l) was placed on both sides of the insert. An attenuated He-Ne laser beam ($\lambda = 632.8$ nm) was focused on the beating cilia from the top using a 100 mm focal length plano-lens. The backscattered light was focused by a 40 $\times$ inverted microscope objective such that the focal spot was coincident with the beating cilia. The focal spot was estimated to encompass 1–5 cells of the cilia. The backscattered photons were collected following optical and spatial filtering and directed to a photon-counting photomultiplier tube. The heterodyne photon signals containing the embedded ciliary beat period were converted to standard logic pulses using a pulse-amplifier-discriminator and routed to a photon counter housed in a personal computer. Photon counts were analyzed for the predominant frequency

of each 512-channel sequence sampled at 4 ms. This procedure resulted in a CBF measurement each second continuously.

2.8. Basal CBF measurements

CETS samples that exhibited vigorous ciliary beating were selected for the study. Studies were performed using CETS derived from 7 canine CETS samples. Baseline CBF measurements were made for an average of 5 min. Three minutes of CBF data were presented as mean $\pm$ SE.

3. Results

3.1. The collagen scaffold material

We evaluated 4 tissue culture protocols (Table 1). In each of the protocols, culture inserts for the ALI were used to establish ciliated cell cultures. With a ciliated cell culture using only ALI (Protocol 1), a limited ciliogenesis with a sparse ciliated cell structure was observed, consistent with all previous reports [9,11,13]. In Protocols 2 and 3, ciliated cells were cultured on the respective chitosan gel and gelatin gel on the apical chamber of the ALI inserts. There was no observable difference in the quantity of ciliogenesis compared with ALI culture alone, as in Protocol 1. In Protocol 4, ciliated cells were cultured using the collagen matrix. Remarkable ciliogenesis was observed 2 weeks after seeding the cells.

3.2. SEM of canine CETS

Ciliated epithelial cells were initially cultured at submerged conditions. Cells covered almost all the visible surfaces of the collagen gel matrix within 3 days of cell seeding. At this time, most of the ciliated cells lost their original cilia. Two weeks into the ALI cultures, ciliogenesis occurred in some of the cells. Vigorous ciliary activity was easily observed under the microscope and confirmed with SEM, as shown in Fig. 1a.

After 6 weeks, ciliary activity was easily observed everywhere on the surface and edges of the gel. All the visually observable gel surfaces were covered with the regenerated cilia, as shown in Fig. 1b. The relative dimensions of the cilia, the cell bodies and the gel were easier to compare from the cross-sections of the ciliated cell gel system, as shown in Fig. 1c. The cilia were abundant and exhibited vigorous activity (Fig. 1d). Notable was the porous nature of the collagen matrix that not only provided appropriate support for the CETS to grow, but also allowed free diffusion of the media to nourish the CETS. One view of the ciliated surface showed well-coordinated movement of ciliary activity, with dense cilia covering the entire surface (Fig. 1e). An expanded view demonstrated that the cells were columnar in shape (Fig. 1f).

3.3. Histology of porcine CETS

Four samples of porcine CETS were used to process the pictures. An example of a histological picture generated from a 5-week-old porcine CETS is shown in Fig. 2a. The asymmetrical locations of the cilia at the apical surface of the epithelial cells demonstrate that the gel matrix system provides appropriate support to establish polarization of the epithelial cells. The tip of the cilia appears to form a coordinated metachronal movement across the ciliated surface. An expanded view of the picture is shown in Fig. 2b. It illustrates the location of the nucleus and the packed structure at the root of the cilia, which appears to be the kinetosomes [16] of the ciliated cell bodies that are located exclusively at the apical region of each ciliated cell. The juxtaposition of the cells suggests that they are tightly coupled such that intercellular communication is possible, as shown extracellularly via the metachronal activity among cells. The firm attachment of the cells to the collagen substrate demonstrates that the baseline membranes are also likely intact.

3.4. Video recording of canine CETS

The video recording captures the vigorous ciliary beating of the canine CETS. A snapshot of this video is shown in Fig. 3. The surface of the collagen gel was covered with vigorously beating cilia moving in a coordinated fashion, as indicated by the fluid movement. Vigorous ciliary activity was obvious in every microscopic view when the samples were moved under the microscope objective. In many instances, metachronal ciliary movement seen in local fields was the same as that reported *in vivo*. As noted from the video and the snapshot, some ciliated cells migrated from the cells grown on the collagen gel matrix to the membrane surface of the insert. This observation is similar to that seen in ciliated cell culture using an organ culturing technique by planting a piece of ciliated epithelial tissue at the surface of thin collagen; the primary ciliated cells migrated and spread throughout the collagen surface.

3.5. Ciliary activity of canine CETS

The ciliary activity determined by dynamic laser light scattering showed a remarkable clear oscillating signal, similar to those observed in isolated single cell culture, organ culture or native ciliated epithelia. As shown in Fig. 4, the ciliary activity was well coordinated and synchronous. There are two notable observations in this example. First, in an individual ciliary cycle, clear, distinct forward and recovery strokes of the cilia can be discerned, as previously reported for a single cilium movement [17]. Second, an envelope of the metachronal wave is obvious in all measurements, as illustrated in the hand-drawn graphics and characterized by the movement of the tips of the cilia in transporting secretions [18].

3.6. Basal CBF of canine CETS

We summarized 3 of the 5 min of basal CBF measurements for 7 canine CETS samples. CBF measurements ranged from 1.8 to 12 Hz, with a mean of 4.2 ± 0.1 Hz ($n = 1234$). These data were consistent with all previous measurements made for other species of ciliated cells, including human [19]. These data imply that the cilia of these ciliated cells were beating at their intrinsic, basal autorhythmic rate and that their spontaneous oscillatory mechanisms were also intact [17].

4. Discussion

Biodegradable synthetic polymers and natural polymers are the two types of common scaffold materials used for structural support in tissue engineering [20]. For example, synthetic poly (N-isopropylacrylamide) has been applied successfully to generate cultures of electrically communicating 3D cardiac tissues [21,22]. However, synthetic polymers may not interact well with ciliated epithelial cells and they are laborious to produce. Our initial focus was on natural scaffold materials, such as collagen derived from known animal origin. Collagen is a major constituent of the extracellular matrix (ECM) and is a biodegradable protein that can be extracted and purified from the connective tissues of animals. Its main function is to maintain tissue integrity and to provide appropriate tension. As a structural support, collagen has been shown to provide a milieu for cell growth and has enabled cells to organize naturally with an *in vivo*-like morphological characteristics. The electron micrographs in the present study demonstrated unequivocally that the heterotypic cell-to-cell interactions, intercellular communications and polarity of the ciliated cells were well developed and preserved using collagen as the scaffold material to support the CETS system. This method obviates the complexities in most of the other reported cell, tissue and organ culturing techniques. As noted, we also evaluated gelatin, chitosan and collagen-coated culture inserts alone or in combination to create the CETS because they are natural polymers used as tissue engineering scaffolds. These media were unremarkable and thus, the data are not reported. Chitosan is a polysaccharide with characteristics similar to agarose, alginate and hyaluronic acid. Gelatin, collagen derivatives, is derived by denaturing the structure of collagen into single-stranded

molecules to form polymeric polypeptide scaffolds. The reason for the failure of gelatin and chitosan to produce the CETS is uncertain.

Collagen appeared to be ideal to create the CETS cultures. Collagen, a core constituent of the ECM that provides the means by which adjacent cells communicate with each other, as shown in cardiac tissue engineering [23–26], could have unique properties for ciliated cells. However, the concentrations of the collagen, the culture media, the buffer solution and the neutralizing agent, as well as the process of gel formation at the right temperature, appeared to be critical for developing the CETS system. Although the cell media can be selected from Ham F10, Ham's-F12, DMEM, M199, basal medium Eagle, BEBM or LHC-9, we chose DMEM to implement the protocol. The addition of retinoic acid to the culture medium to cause the cells to develop into an outgrowth of cilia is essential. Our results (data not shown) showed that retinoic acid is one of the key elements in promoting differentiation of epithelial cells into ciliated epithelial cells and is consistent with previous reported findings [27,28]. Similarly, buffer solution can be chosen from Hank's balanced salt solution, PBS, saline solutions, Dulbecco's modified PBS, or HEPES-buffered salt solution. We chose PBS and maintained its final concentration at 5–20%. The neutralizing agent can be selected from NaHCO_3 , NaHPO_4 or NaOH . We chose 4.5% (w/v) NaHCO_3 to adjust the final pH to 7–8. The collagen solution should be maintained at 25–40 °C for a sufficient period for the collagen gel to form, and the gel should also be hydrated with the culture medium for a sufficient period. Most important, the CETS should be created using a collagen concentration of 2.0–5.0 mg/ml. A proper pore size for the collagen gel at this concentration appears to provide the best supporting matrix and the free diffusion of nutrients required to sustain the CETS.

We also tested the tissue culturing system using human bronchial epithelial cell lines, including BEAS-2B and CRL-2777, both available from ATCC. Ciliogenesis was not induced in these cells. It appears that the combination of primary ciliated cells with a demonstrated motility and the interplay between the composition and concentration of collagen is requisite for developing the CETS. The mechanism awaits further delineation.

One of the major applications of the CETS system is the feasibility of performing long-term physiopharmacological studies that are central to asthma drug discovery. Autonomic and anti-inflammatory agents are the two principal classes of drugs in asthma treatment and drug discovery. Accordingly, we evaluated the CBF responses of fenoterol and interleukin-4 (IL-4) using the 3D CETS system. CBF data were presented as the average of all CBF measurements made during the 5-min intervals. Fenoterol is a long-acting β_2 adrenergic agonist that induces broncho-relaxation for longer than 12 h in people with asthma [29,30]. This drug was tested using a 6-week porcine CETS sample. Each of the 5-min CBF measurements made at 2 h, 4 h, 6 h and 24 h following the application of 1 μM fenoterol showed an increase in CBF. Fenoterol stimulated CBF to a peak of $1.4 \times$ the baseline ~4 h following its application (Fig. 5a). This stimulation of CBF was sustained at the end of the experiment 24 h later. The temporal response of CBF stimulated by fenoterol is consistent with the pharmacokinetics of this long-acting β_2 adrenergic agonist. IL-4 is an inflammatory interleukin that has been implicated in late-phase broncho-constriction found in atopic asthma [31]. Similarly, IL-4 applied topically at 20 ng/ml to a 6-week porcine CETS system caused CBF to decrease from the baseline at an average rate of 8% per day in a 6-day experiment. The β_2 adrenergic and IL-4-mediated signal transduction mechanisms appear to be intact for the regulation of CBF in these preparations. These data demonstrate that evaluation of long-term effects of drug candidates on pulmonary diseases can now be performed using this culture system.

At present, ciliated cells are largely cultured in a thin collagen layer using cells derived from either protease-digested primary ciliated epithelial cells [19] or freshly excised ciliated tissue explants [18] using specialized bronchial epithelial media such as BEBM. Ciliated epithelial

cells in this 2D collagen structure lose their histological characteristics, differentiation capabilities and most of their cilia afterwards. The CETS system is robust with the use of common cell-culturing media. These data support the concept that a 3D structural support that mimics the *in vivo* condition is critical for ciliated cell culture.

5. Conclusions

The airway epithelial tissue system reported herein closely mimics the *in vivo* morphological characteristics and function of ciliated epithelia. In our studies, a simple 3D tissue engineering approach to generate a CETS has been described. This collagen gel scaffold, ALI tissue culture system enables dissociated primary ciliated cells to develop and differentiate into a 3D CETS. The CETS system is robust and straightforward to maintain. It also appears to preserve the integrity of the cellular receptor-mediated signal transduction mechanisms present *in vivo*. The maintenance of active, functional cilia in airway epithelial cells for a prolonged period of more than 4 months in culture in this CETS system has potentially wide applications in pulmonary toxicology and drug discovery research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

(A) SEM of canine CETS formed from culturing of dissociated canine primary tracheal epithelial cells after 2 weeks. (B),(C), (D) (E) (F) were derived from SEM of 6 weeks old canine CETS (1.5 months).

(B) A sample of ciliated epithelial cells grown on the gel matrix.

(C) A cross-section of collagen matrix with ciliated epithelial cell layer.

(D) An enlarged cross-section view showing cilia, columnar cell layer and collagen.

(E) A topical view of the collagen gel matrix to illustrate the densely packed ciliated surface.

(F) An expanded columnar view of Figure 1D.

Fig. 2. Histology of a 5 week old porcine CETS. (A) The arrow indicates the metachronal coordinated ciliary activities. (B) An expanded view of (A) to show the apical localization of the cilia in each cell and the arrow shows the anchor of the ciliary basal bodies in the cytoplasm where kinetosomes are usually located.

Fig. 3. Snap-shot of a video of ciliary activities of a 2 month old canine CETS. The upper arrow indicates the migration of the ciliated cells to the culture insert membranes that also exhibit vigorous beating. A movie about figure 3 is published as supporting information.

Fig. 4.

An example of scattered photon counts CBF measurements of canine CETS. The elliptic circle (broken line) illustrates in each beat cycle, it consists of a forward stroke and a recovery stroke as reported. The wavefront envelop covers the tip of each ciliary cycle is consistent with the previously reported metachronal wave period

Fig. 5.

(A) An example of CBF temporal responses of 6 week porcine CETS to femoterol for 24 hrs.

(B) An example of CBF temporal responses of 6 week porcine CETS to IL-4 for 6 day.

Table 1

We evaluated difference culture protocols. Remarkable ciliogenesis was observed two weeks after seeding the cells.

	Tissue Engineering Culture Method For CETS	Ciliogenesis (+ refers to the level of ciliogenesis)
1	Air-liquid culture (ALI)	++
2	3D chitosan gel + ALI	++
3	3D gelatin gel + ALI	++
4	3D collagen gel + ALI	++++