Induction of Ciliated Cells from Avian Embryonic Stem Cells Using Three-Dimensional Matrix

Yuchi Wang, Ph.D., Lid B. Wong, Ph.D., and Hua Mao, M.D., Ph.D.

We have devised a simple three-dimensional (3D) tissue-culturing method to induce ciliogenesis from avian embryonic stem (ES) cells by using avian fertilized eggs. Unlike the previous reported techniques, this method does not require trypsinization, which would reduce the viability of the cells; it also does not require an air-liquid interface to induce ciliogenesis and to maintain the growth of the induced ciliated cells. ES cells seeded and attached on this collagen-coated chitosan 3D gel grew spontaneously and robustly. Following 2 weeks in culture with inhibition of embryoid body formation, cells with noticeable and vigorous beating cilia were observed. We measured the ciliary beat frequencies of these ES-differentiated ciliated cells for 40 days. These results were consistent with all reported measurements made for other species of ciliated cells, including human, from our previous study. These data imply that the cilia of these ES-derived ciliated cells, beating at their intrinsic basal autorhythmic rate, preserve the integrity of the regulatory mechanisms of ciliary beat frequency. In conclusion, we have shown that ES cells cultured in a 3D tissue-engineered scaffold is a promising approach for developing an *in vitro* cell model that closely mimics the *in vivo* ciliated cell natural milieu. This cell model can potentially be the source of ciliated cells for cell-based high-throughput screening and discovery of pulmonary drugs.

Introduction

IN PERSONS with chronic obstructive pulmonary disease, asthma, bronchiectasis, cystic fibrosis, and ciliary dyskinesia, impaired airway epithelial cell functions, such as reduced mucociliary clearance, have a central pathological role in their recurrent respiratory tract infections. Airway epithelial cell function with active cilia is of central interest to airway pharmacology and drug discovery. Not only can ciliated epithelial cells exhibit dysfunction in their primary protective role, but these cells have also been implicated in the transduction of signals from the airway lumen to smooth muscle and endothelial cells. Airway ciliated epithelial cells whose physiological cell function can be used to screen a wide range of receptor-mediated signal transduction mechanisms for a variety of agonists and antagonists will enhance pulmonary drug discovery processes.^{1,2}

Airway ciliated epithelial cells are specialized to transport secretions in the airways. Morphologically, cilia are located at the apical surface of the membrane. In their natural habitat, cilia are immersed in an air-liquid interface (ALI) milieu with the basolateral membranes of the ciliated cells nourished by the capillary bed. Physiologically, the asymmetrical location of ion pumps and transporters between the apical and basolateral membranes of these polarized ciliated cells is responsible for the transport of ions and water across the epithelia.¹

In most situations, ciliated cells cultured in submerged media transform within 3 weeks from pseudo-stratified columnar cells to cuboidal monolayers, with loss of their cilia and microvilli.³ Under these conditions, the apical surface of the ciliated cells becomes smooth and indistinguishable from the basal surface. The induction of reciliation of these epithelial cells has been more successful with recent ALI-based culturing techniques. However, at present, generally only ~25% of the cells reciliate within 30 days.^{3,4} This process has been difficult to reproduce and the yield of ciliated cells varies from laboratory to laboratory.^{5,6} The practical aspects of these culturing techniques prohibit wide availability and applications of these *in vitro* models for pulmonary drug screening, drug discovery, and toxicological studies.

Embryonic stem (ES) cells are pluripotent cells derived from the cell mass of the blastocyst stage embryos. They can be maintained in an undifferentiated state using leukemia inhibitory factor (LIF). These undifferentiated cells have the potential to differentiate into a broad spectrum of cells with appropriate induction conditions.^{7,8} Thus, ES cells can potentially be the source of ciliated cells for pulmonary drug screening. Many studies demonstrate that three-dimensional (3D) culture of ES cells increases the production of extracellular matrix (ECM) as well as cell adhesion, resulting in increased signaling and enhanced expression of genes that function in promoting cell differentiation.⁹ It is this ECM that

provides the structural integrity of tissue.¹⁰ The scaffold provides physical cues for cell orientation and spreading, and pores provide space for remodeling of tissue structures.¹¹ In addition, a 3D scaffold-based culture provides the physiological microenvironment and biomolecular signals for the scaffold to mimic the structure and properties of human tissue to direct tissue formation by upregulating key growth factors, transcription factors, and genes related to cell differentiation.¹²

For these reasons, we developed a new protocol, described herein, using a 3D cell culture matrix (scaffold) that supported the differentiation of ES cells into ciliated cells and ciliated cell growth. In this study, we used collagen-coated chitosan as a 3D matrix. Many different biomaterials have been previously investigated for tissue engineering or drug delivery applications.^{13,14} These biomaterials include synthetic and natural materials, including metals, ceramics, and polymers. An ideal scaffold should be biocompatible, with a high affinity for cells to attach and proliferate, and have an appropriate biodegradation profile and mechanical strength. Chitosan and collagen are two naturally occurring biopolymers that have recently received most research attention.¹⁵ Both polymers are biodegradable and have excellent biocompatibility properties that make them highly desirable biomaterials to be used as a matrix to support cell and tissue growth.¹⁶ In addition, they appear to be capable of being transformed into many different forms, including thin films and porous and gel structures.^{17–20} In this study, we used these biopolymers to form a gel structure as a model system to support cell growth and development. The gel model is transparent; thus, it is readily applicable for most optical-based cellular function assays. We have tested this gel model by inducing ciliogenesis in ES cells derived from avian fertilized eggs. Ciliary beat frequency (CBF) was used as a physiological functional assay to determine the viability of these ES-differentiated ciliated cells over 40 days using laser light scattering.²¹ The stimulatory responses of CBF, as a measure of functional integrity of the cells, were tested by activating the β_2 adrenergic receptors to increase intracellular cyclic adenosine monophosphate (cAMP) using terbutaline. These data demonstrate that culturing ES cells with 3D tissue engineering scaffolds is a promising approach to develop an *in vitro* cell model that closely mimics *in vivo* ciliated cell development.

Materials and Methods

Agents used

The ES-Dulbecco's modified Eagle's medium (DMEM) consisted of DMEM (ATCC, Manassas, VA) supplemented with 2.0 mM L-alanyl-L-glutamine (ATCC), 1% nonessential amino acids (ATCC), 0.1 mM β -mercaptoethanol (Invitrogen Life Technologies, Carlsbad, CA), 1000 U/mL mouse LIF (Chemicon, Temecula, CA), and 15% fetal bovine serum (FBS; ATCC). Bronchial epithelial cell growth medium was obtained from Biowhittaker (Watersville, MD). Gelatin (0.1%), chitosan, and collagen were all obtained from Sigma-Aldrich (St. Louis, MO). Trypsin (0.025%) was obtained from ATCC.

Creation of the 3D substrate system

A 4% chitosan solution was made by dissolving chitosan powder with 1% acetic acid. A 3D gel material was prepared by polymerizing the chitosan solution using 5 N NaOH at 37°C for 15 min. With the razor stamp as a pattern creator, a

series of grooves made of 0.3% collagen solution was generated on top of a pH-balanced 3D collagen coating chitosan gel, as shown in steps 1 and 2 of Figure 1.

Blastodermal and stem cell culture

Blastodermal cells were harvested from stage IX to XI avian embryos (*Gallus gallus domestica*).²² In brief, the surface of the fertilized, unincubated egg was cleaned with 70% ethanol and opened ventrally. The yolk was separated from the albumen. The blastoderm was harvested, transferred to chilled phosphate-buffered saline (PBS), and incubated for 10 min at 4°C with 0.025% trypsin. Embryos, pooled at one embryo per milliliter of ES-DMEM, were centrifuged at 1000 rpm for 10 min. The resulting cell pellet was allowed to slowly and mechanically dissociate in the ES-DMEM. The blastoderm cells were seeded in ES-DMEM on a collagen-precoated Petri dish and maintained at 37°C in 5% CO₂ and 90% humidity. Half of the medium was replaced with fresh ES-DMEM after 24 h in culture. To further culture the stem cells seeded in 0.1% collagen-coated tissue culture plates in an undifferentiated state, we maintained them in ES-DMEM consisting of DMEM supplemented with 10% FBS, 0.1 mM nonessential amino acids, 0.1 mM β -mercaptoethanol, 2 mM L-glutamine, and 1000 U/mL LIF.

Generation of airway ciliated epithelia from the ES cells

Confluent cultures of undifferentiated ES cells were detached from the substrate. To induce these ES cells to differentiate spontaneously without embryoid body formation, we seeded undifferentiated ES cells ($2 \times 10^3/\text{cm}^2$) on a collagen-chitosan 3D gel matrix and cultured them with the ES-DMEM without the LIF (day 0). Under these culture conditions, most of the ES cells were attached to the matrix surface in 8–10 days, with the cells spreading over the matrix surface. After ES cells were cultured in the grooved 3D gel system for 2 weeks, they differentiated into cilia cells (see Supplementary Movie S1, available online at www.liebertonline.com). The sample was placed in an inverted microscope (Diaphot-TMD; Nikon, Tokyo, Japan) equipped with a CCD camera (Model 4192; Cohu, Poway, CA) and the image was recorded after ciliated cell seeding using either a 10 $\times$ or 25 $\times$ objective. At this time, the ES-DMEM was replaced with the bronchial epithelial cell growth medium without serum (at day 15), and the medium was exchanged with fresh medium every 3 days.

Immunohistochemistry and scanning electron microscopy

Samples were plated on coverslips and were washed several times in PBS. Then the samples were fixed with freshly prepared 3% paraformaldehyde for 30 min at room temperature. For the identification of the outgrowth of cilia on the insert, anti- β -tubulin-antibody (ICN Biomedicals, Aurora, OH) was used. After washing off the primary antibodies, the sample was incubated with biotinylated, linked antibodies (Abs) for 20 min at room temperature and followed with the reactions of streptavidin-horseradish peroxidase conjugates. Color development was achieved by adding 3-amino-9-ethylcarbazole (Sigma-Aldrich) as a substrate. The cells were observed under the microscope.²³

Samples were washed twice in PBS and fixed in 3.5% glutaraldehyde in 0.15 M phosphate buffer (pH 7.4) at room

FIG. 1. Procedure for the creation of the 3D substrate system. 3D, three-dimensional; PBS, phosphate-buffered saline.

temperature for 1 h. They were then dehydrated in graded ethanol series and treated with a series of different ratios of ethanol with hexamethyldisilazane. After air-drying at room temperature, the samples were mounted on an aluminum tube, sputter-coated with platinum/palladium, and analyzed using a scanning electron microscope.

Measurements of CBFs using laser light scattering

CBF was measured by a laser light scattering technique using an inverted microscope.^{21,24} Part of the 3D ciliated cell culture was cut from gel, placed in a two-well coverglass

chamber, and then placed on an inverted microscope stage (Diaphot-TMD; Nikon). One hundred microliters of Hank's solution 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 dynamically Doppler-shifted light scattered by the beating cilia was observed using a 40 $\times$ inverted microscope objective such that the focal spot was coincident with the beating cilia. Depending on the confluent growth of the cultured monolayer, the focal spot encompassed the cilia of one to five cells. 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.

Experimental protocols

Ciliated cells exhibiting vigorous beating were selected for the study. Three samples were used. CBF measurements were made in each sample for the first 7 days and then at days 14, 21, 28, and 40. Eleven baseline measurement experiments were performed per sample for 40 days. At days 21, 28, and 40, after the measurement of baseline CBF for 5 min in each sample, cumulative applications of 1 and 10 μ M terbutaline were added to each sample 5 min apart.^{2,21}

Data analysis

CBF was derived from all values in the 5 min measurement intervals. The mean data derived from each of the 5 min measurement data are expressed as arithmetic means and standard deviations. Analysis of variance was applied to the raw data to determine the statistical significance of each experimental condition.

Results

Differentiation of ES cells into ciliated cells on 3D gel in vitro

Figure 2A shows a photograph of the chitosan–collagen matrix prior to seeding of cells. Figure 2B shows a phase-contrast micrograph of a gel pattern with 250- μ m-wide grooves, with the ES cells seeded on the surface of the grooves. ES cells seeded in the grooves were attached only on the circumferential surface of the grooves. Following 6 weeks in culture, cells began to spread all over the grooves. After 2 weeks, cells with noticeable beating cilia were observed (Fig. 2C). They were all video recorded (see Supplementary Movie S1, available online at www.liebertonline.com). Most of these ciliated-like cells were localized in the area of expanded outgrowth. This observation is consistent in all of the ES cultures. Cilia outgrowth occurred in the epithelial cells as shown by positive anti- β -tubulin staining²³ of cilia on the gel (Fig. 2D). The length of the cilia was also gradually increased. After 1 month, most of the cells were covered with regenerated cilia and cells subsequently became more columnar. Cell differentiation continued with more cells becoming ciliated epithelial cells; the length of the cilia continued to increase. After 6 weeks of culture, the cells were ciliated, with the cilia length being about 6–7 μ m. Vigorous ciliary beating was observed in all samples. These grown cilia showed a rhythmic ciliary beating pattern similar to that observed under the light microscope in primary epithelial cell culture.

For 6 weeks in our conditions, the pictures of scanning electron micrographs of ciliated cells grown are shown in Figure 2E–G. Stages of ciliogenesis were captured as illustrated in Figure 2E, H. Figure 2F, G show enhanced magnification. Most of the cilia reached a mature length of approximately 6–7 μ m (Fig. 2F, G). After 10 weeks of culture, over 80% of the cultured cell surface was covered with mature cilia in most areas of the culture (Fig. 2H).

Basal CBF of avian ES-derived ciliated cells

As noted, after 2 weeks of culture, beating cilia were observed in some cells. This is defined as day 1 for the CBF measurement experiment. Although CBF was measured for only 40 days, vigorous beating cilia were observed for longer than 3 months (measurements were not made). As shown in Figure 3, the mean of CBF was 6.02 ± 0.07 Hz at day 1. There were no significant differences in the baseline measurement in the first 7 days. After 3 weeks in culture, CBF gradually declined to 5.79 ± 0.13 Hz. At day 30, CBF decreased to 5.49 ± 0.03 Hz. At day 40, CBF was 5.23 ± 0.05 Hz. These data are consistent with all previous measurements for other species of ciliated cells, including human.²⁴ These data imply that the cilia of these ES-derived ciliated cells were beating at their intrinsic, basal autorhythmic rate.^{1,21,24}

Physiological responses of CBF to terbutaline

Terbutaline measurements were made for all three samples at days 21, 30, and 40 for a total of nine measurements (Fig. 4). The mean of the basal CBF of nine experiments was 5.65 ± 0.5 Hz. Application of 1 μ M terbutaline increased CBF to 6.01 ± 0.2 Hz ($p < 0.1$, compared with baseline). The second application of 10 μ M terbutaline caused an increase of CBF to only 5.75 ± 0.47 Hz ($p < 0.5$, compared with baseline). This is consistent with the previous findings that high concentration of β_2 adrenergic agonist is toxic to the ciliated cells.^{2,25}

Discussion

Ciliated cells derived from stem cells have been reported recently using mouse ES cells.^{26,27} Coraux *et al.* induced ES cell differentiation into ciliated cells by using an ALI that mimicked the adult tracheal–bronchial milieu.²⁶ This is different from the ciliated cell differentiation that occurs when cells are submerged in embryonic fluids during normal fetal development. Nishimura *et al.* improved upon the mouse ES cell differentiation technique by obviating the need for a complex ALI ciliogenesis induction protocol.²⁷ Using knockout serum replacement media to substitute for FBS, they were able to mimic the embryonic stage of development, which enabled ciliated cell differentiation from ES cells in submerged culture conditions. The compelling advantage of the technique described herein is the straightforward protocol of using a 3D tissue engineering approach. The protocol did not require ES cells to be first induced to differentiate into Clara cells using type I collagen for 8 days followed by ALI cultures,²⁶ and the cells did not need to be in well-defined knockout serum replacement media²⁷ to be in submerged culture. ES cells needed only to be induced in the 3D gel system from the first day and maintained in submerged cultures. Ciliogenesis occurred spontaneously.

It is notable that similar morphological epithelialization was observed in cells in these cultures as was observed in those cultured in serum-free medium with retinoic acid.⁵ The avian ES cell-induced ciliated epithelia differentiated into a multilayered, pseudostratified-like cell organization in the 3D gel in submerged culture conditions. This is consistent with the observations of Nishimura *et al.*, in which ALI culturing conditions did not seem necessary to promote optimal epithelial cell differentiation from ES cells into a

FIG. 2. (A) A 10 $\times$ photograph of the chitosan–collagen matrix. (B) A 10 $\times$ optical micrograph of epithelial cell growth from ES cells on 250 μ m grooves after 6 weeks of seeding of the cells. (C) Ciliogenesis started at 2 weeks after seeding of the cells. Cilia started beating at the edge of the gel (top view) (see Supplementary Movie S1; available online at www.liebertonline.com). Black arrow shows the position of the beating cilia. (D) Anti- β -tubulin staining of cilia after 2 weeks of culture. White arrow shows the anti- β -tubulin stain of the cilia. (E) Scanning electron micrographs of epithelial cell growth from ES cells on matrix after 6 weeks. (F) An enlarged top view showing cilia. (G) Mature cilia reached a length of approximately 6–7 μ m. (H) Over 80% of the cultured cell surface was covered with mature cilia after 10 weeks of culture. ES, embryonic stem. Color images available online at www.liebertonline.com/ten.

pseudostratified organization in the 3D gel system.²⁷ In general, tissues regenerate when structural guidance supports are provided.¹³ A suitable 3D substrate to support organizational cultures has been applied to liver cells, cardiac cells, and solid tumors to attain morphological structure and maintain the integrity of intercellular signaling mechanisms.^{15,28–30} Collagen, a major constituent of the ECM,³¹ has consistently been shown to support the development of the embryoid bodies. The microfilaments supporting structural collagen likely enable the cells to grow and organize naturally into an *in vivo*-like morphology. In this study, avian ES cells seeded in a collagen-coated chitosan scaffold developed into an *in vivo*-like ciliated cell phenotype. It sustained vig-

orous ciliary beating and exhibited predictable physiopharmacological responses.

Ciliogenesis was based on the observations of cilia outgrowth with vigorous beating on the cell surface. The vigorous beating of the cilia was similar to those found in the native epithelia and was documented with video recording in all samples. We have further validated the cultures by measuring the basal CBFs for 40 days using laser light scattering. The intrinsic variations of the basal CBFs in this cell culture model are consistent with the inherent characteristics of ciliary activity reported in the previous observations.^{1,21,24} To test the drug effect on CBF, the baseline of each sample usually served as its own control. As the

FIG. 3. Basal CBF of three avian ES-derived ciliated cell samples measured from day 1 to 40. An average basal CBF value was derived from each of the 5 min measurement intervals for each sample at each day. At each day, a mean CBF value was obtained from the average basal CBF values of the three samples. They were presented as the basal CBF (mean $\pm$ standard deviation) plotted against the day. CBF, ciliary beat frequency.

full potential and utility of this new cell culture model is dependent on the validation of the functional integrity of the receptor-mediated intra- and interregulatory mechanisms of ciliary activity, we have tested this ciliated cell model using terbutaline to stimulate CBF. The results are consistent with all the previously reported findings, that is, high doses of β_2 adrenergic agonists caused toxic^{2,25} or attenuated CBF³² responses. Future studies can now be conducted using a con-

FIG. 4. Cumulative stimulatory responses of CBF by 1 and 10 μ M terbutaline of the three avian ES-derived ciliated cell samples measured at days 21, 30, and 40. At each measurement day, an average CBF value was derived from each of the 5 min measurement intervals per experimental condition for each sample. A mean CBF value was then obtained from the CBF averages of each of the experimental conditions of the three samples. They are the basal CBFs and the CBF stimulatory responses induced by 1 and 10 μ M terbutaline. They were presented as the CBFs (mean $\pm$ standard deviation) of each experimental condition. * p < 0.5, compared with basal CBF; ** p < 0.1, compared with basal CBF.

ventional "agonists-antagonists" experimental design² to delineate the other signal transduction mechanisms^{23,32-38} in regulating CBF, including the cyclic guanosine monophosphate (cGMP)/protein kinase G (PKG)-dependent cholinergic stimulatory pathway,³⁷ the nitric oxide-cyclic adenosine monophosphate (cAMP) dependent stimulatory pathway,³³ the purinergic-intracellular calcium stimulatory pathway,^{35,36} and the protein kinase C (PKC)-dependent inhibitory pathway.²⁴ One of the other major applications of this ciliated cell model is the feasibility of performing long-duration physiopharmacological studies that are central to asthma drug discovery, such as the desensitization of protein kinase A (PKA)³⁸ and long-acting β_2 adrenergic agonist³⁴ effect on ciliary activity.

Many studies reported that the interfacial surface properties, including the topography and chemistry, are of prime importance in establishing the responses of tissues to biomaterials.^{39,40} We created groove structures to mimic the circumferential geometry of the trachea. Our results indicated that there was no difference in terms of ciliated cell outgrowth when the widths of the grooves were decreased from 250 to 100 μ m (data not shown).

The utility of ciliary function to screen drugs for the pharmaceutical industry has been proposed.⁴¹ However, it has never been extensively adopted. The lack of suitable available cultured ciliated cells is one of the major technical issues that explain why ciliated cells have not been widely used for drug discovery, drug screening, and toxicology studies. Ciliated epithelial cells are one of the very few cell types that express their intracellular regulatory mechanisms extracellularly via mechanical vibratory motion, in this case CBF. In the upper airways, over 50% of the epithelial cells are ciliated. For an investigation of the fundamental mechanisms in ciliated epithelial cell physiology, which can subsequently lead to the discovery of new drugs, a cell line culture that maintains the mechanisms of ciliogenesis and can be activated on demand is highly desirable. The intracellular regulatory mechanisms of epithelial cells with intact cilia better represent the mechanisms in native epithelia than do the epithelial lines presently available that have lost most, if not all, of their cilia. Our ciliated cell cultures will provide faster and cheaper alternatives to the use of standard rodent³⁸ and rabbit⁴² toxicity tests. Ciliated cells induced from avian ES cells resolve many of these issues.

The use of avian ES cells to derive functional ciliated cells offers many practical advantages over mammalian cells. The avian embryo develops outside the mother, with minimal incubation time. This minimizes variations of external neural-humoral conditions during development and provides more consistent and controlled biological conditions. Harvesting avian embryonic cells avoids the ethical issues involved with studying mammalian embryonic cells. The use of avian ES cells for the induction of ciliated cells allows large numbers of cells to be harvested at minimal cost, a major consideration in drug discovery when thousands of compounds need to be screened each day by high-throughput screening (HTS) technologies. Cell-based HTS assays have been used for target validation. However, it is hard to evaluate the physiological status of the cultured cells in many of these cell-based HTS platforms. Using beating ciliated cells as a functional assay in HTS, we will be able to demonstrate viability of the basic physiological functions of

these cells. This will be the first such approach in cell-based HTS. The gel system described herein can also potentially be a forerunner for the development of artificial human trachea^{43–45} using human ES cells.

Conclusion

A method for producing a ciliated cell culture system from avian stem cells has been developed for the first time. The cells were cultured on 3D gel scaffolds. Beating cilia were observed after 2 weeks and they exhibited vigorous beating for 3 months. Measurements of basal CBF have been made for 40 days since the first appearance of the moving cilia in the culture. The notable stimulation of CBF by terbutaline indicates that β_2 adrenergic receptors are functionally expressed in these ES-induced cells. The tissue engineering approach with stem cells enables the culture and maintenance of these cells for physiopharmacological screening. Unlike other reported methods, our protocol is straightforward and does not require ALI to sustain the cultures. This method provides an innovative new platform technology that also has broad applications for the chemical industry and for safe pharmacological drug discovery.

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Disclosure Statement

No competing financial interests exist.

References

- Wong, L.B., and Yeates, D.B. Dynamics of the regulation of ciliated epithelial function. *Comments Theor Biol* **4**, 183, 1997.
- Wong, L.B., Miller, I.F., and Yeates, D.B. Regulation of ciliary beat frequency by autonomic mechanisms: *in vitro*. *J Appl Physiol* **65**, 1895, 1988.
- Neugebauer, P., Endepols, H., Mickenhagen, A., and Walger, M. Ciliogenesis in submersion and suspension cultures of human nasal epithelial cells. *Eur Arch Otorhinolaryngol* **260**, 325, 2003.
- Kaartinen, L., Nettesheim, P., Adler, K.B., and Randell, S.H. Rat tracheal epithelial cell differentiation *in vitro*. *In Vitro Cell Dev Biol Anim* **29**, 481, 1993.
- De Jong, P.M., van Sterkenburg, M.A., Hesseling, S.C., Kempenaar, J.A., Mulder, A.A., Mommaas, A.M., Dijkman, J.H., and Ponc, M. Ciliogenesis in human bronchial epithelial cells cultured at the air-liquid interface. *Am J Respir Cell Mol Biol* **10**, 271, 1994.
- Ostrowski, L.E., and Nettesheim, P. Inhibition of ciliated cell differentiation by fluid submersion. *Exp Lung Res* **21**, 957, 1995.
- Placzek, M.R., Chung, I.M., Macedo, H.M., Ismail, S., Mortera Blanco, T., Lim, M., Cha, J.M., Fauzi, I., Kang, Y., Yeo, D.C., Ma, C.Y., Polak, J.M., Panoskaltis, N., and Mantalaris, A. Stem cell bioprocessing: fundamentals and principles. *J R Soc Interface* **6**, 209, 2009.
- Kirouac, D.C., and Zandstra, P.W. The systematic production of cells for cell therapies. *Cell Stem Cell* **3**, 369, 2008.
- Agrawal, C.M., and Ray, R.B. Biodegradable polymeric scaffolds for musculoskeletal tissue engineering. *J Biomed Mater Res* **55**, 141, 2001.
- Liu, H., and Roy, K. Biomimetic three-dimensional cultures significantly increase hematopoietic differentiation efficacy of embryonic stem cells. *Tissue Eng* **11**, 319, 2005.
- Vacanti, J.P., and Langer, R. Tissue engineering: the design and fabrication of living replacement devices for surgical reconstruction and transplantation. *Lancet* **354**, SI32, 1999.
- Liu, H., Lin, J., and Roy, K. Effect of 3D scaffold and dynamic culture condition on the global gene expression profile of mouse embryonic stem cells. *Biomaterials* **27**, 5978, 2006.
- Lee, J., Cuddihy, M.J., and Kotov, N.A. Three-dimensional cell culture matrices: state of the art. *Tissue Eng Part B Rev* **14**, 61, 2008.
- Ratner, B.D., and Bryant, S.J. Biomaterials: where we have been and where we are going. *Annu Rev Biomed Eng* **6**, 41, 2004.
- Torisawa, Y.S., Shiku, H., Yasukawa, T., Nishizawa, M., and Matsue, T. Multi-channel 3-D cell culture device integrated on a silicon chip for anticancer drug sensitivity test. *Biomaterials* **26**, 2165, 2005.
- Wang, Y.C., Lin, M.C., Wang, D.M., and Hsieh, H.J. Fabrication of a novel porous PGA-chitosan hybrid matrix for tissue engineering. *Biomaterials* **24**, 1047, 2003.
- Badyrak, S.F. The extracellular matrix as a biologic scaffold material. *Biomaterials* **28**, 3587, 2007.
- Wang, Y.C., Kao, S.H., and Hsieh, H.J. A chemical surface modification of chitosan by glycoconjugates to enhance the cell-biomaterial interaction. *Biomacromolecules* **4**, 224, 2003.
- Yunoki, S., Ikoma, T., Monkawa, A., Marukawa, E., Sotome, S., Shinomiya, K., and Tanaka, J. Three-dimensional porous hydroxyapatite/collagen composite with rubber-like elasticity. *J Biomater Sci Polym Ed* **18**, 393, 2007.
- Lee, J.H., Kim, H.J., Kim, H., Lee, S.J., and Gye, M.C. *In vitro* spermatogenesis by three-dimensional culture of rat testicular cells in collagen gel matrix. *Biomaterials* **27**, 2845, 2006.
- Mao, H., and Wong, L.B. Fluorescence and laser photon counting: measurements of epithelial $[Ca^{2+}]_i$ or $[Na^+]_i$ with ciliary beat frequency. *Ann Biomed Eng* **26**, 666, 1998.
- Eyal-Giladi, H. The gradual establishment of cell commitments during the early stages of chick development. *Cell Differ* **14**, 245, 1984.
- Wong, L.B., Miller, I.F., and Yeates, D.B. Regulation of ciliary beat frequency by 590 autonomic mechanisms: *in vitro*. *J Appl Physiol* **65**, 1895, 1988.
- Wong, L.B., Park, C.L., and Yeates, D.B. Neuropeptide Y inhibits ciliary beat frequency in human ciliated cells via nPKC, independently of PKA. *Am J Physiol* **275**, C440, 1998.
- Mao, H., Wang, Y., Yuan, W., and Wong, L.B. Ciliogenesis in cryopreserved mammalian tracheal epithelial cells cultured at the air-liquid interface. *Cryobiology* **59**, 250, 2009.
- Coraux, C., Nawrocki-Raby, B., Hinnrasky, J., Kileztky, C., Gaillard, D., Dani, C., and Puchelle, E. Embryonic stem cells generate airway epithelial tissue. *Am J Respir Cell Mol Biol* **32**, 87, 2005.
- Nishimura, Y., Hamazaki, T.S., Komazaki, S., Kamimura, S., Okochi, H., and Asashima, M. Ciliated cells differentiated from mouse embryonic stem cells. *Stem Cells* **24**, 1381, 2006.
- Landry, J., Bernier, D., Ouellet, C., Goyette, R., and Marceau, N. Spheroidal aggregate culture of rat liver cells: histotypic reorganization, biomatrix deposition, and maintenance of functional activities. *J Cell Biol* **101**, 914, 1985.
- Riccalton-Banks, L., Liew, C., Bhandari, R., Fry, J., and Shakesheff, K. Long-term culture of functional liver tissue:

- three-dimensional coculture of primary hepatocytes and stellate cells. *Tissue Eng* **9**, 401, 2003.
30. Bartholoma, P., Gorjup, E., Monz, D., Reininger-Mack, A., Thielecke, H., and Robitzki, A. Three-dimensional *in vitro* reaggregates of embryonic cardiomyocytes: a potential model system for monitoring effects of bioactive agents. *J Biomol Screen* **10**, 814, 2005.
 31. Eschenhagen, T., Fink, C., Remmers, U., Scholz, H., Wattchow, J., Weil, J., Zimmermann, W., Dohmen, H.H., Schafer, H., Bishopric, N., Wakatsuki, T., and Elson, E.L. Three-dimensional reconstitution of embryonic cardiomyocytes in a collagen matrix: a new heart muscle model system. *FASEB J* **11**, 683, 1997.
 32. Verdugo, P., Johnson, N.T., and Tam, P.Y. β -Adrenergic stimulation of respiratory ciliary activity. *J Appl Physiol* **48**, 868, 1980.
 33. Jain, B., Rubinstein, I., Robbins, R.A., Leise, K.L., and Sisson, J.H. Modulation of airway epithelial cell ciliary beat frequency by nitric oxide. *Biochem Biophys Res Commun* **191**, 83, 1993.
 34. Lindber, S., Khan, R., and Runer, T. The effects of formoterol, a long-acting β_2 -adrenoceptor agonist, on mucociliary activity. *Eur J Pharmacol* **285**, 275, 1995.
 35. Lieb, T., Frei, C.W., Frohock, J.I., Bookman, R.J., and Salathe, M. Prolonged increase in ciliary beat frequency after short-term purinergic stimulation in human airway epithelial cells. *J Physiol* **538**, 636, 2002.
 36. Salathe, M., and Bookman, R.J. Mode of Ca^{2+} action on ciliary beat frequency in single ovine airway epithelial cells. *J Physiol* **520**, 851, 1999.
 37. Wyatt, T.A., Spurzem, J.R., May, K., and Sisson, J.H. Regulation of ciliary beat frequency by both PKA and PKG in bovine airway epithelial cells. *Am J Physiol* **275**, L827, 1998.
 38. Wyatt, T.A., Gentry-Nielsen, M.J., Pavlik, J.A., and Sisson, J.H. Desensitization of PKA-stimulated ciliary beat frequency in an ethanol-fed rat model of cigarette smoke exposure. *Alcohol Clin Exp Res* **28**, 998, 2004.
 39. Brunette, D.M., and Chehroudi, B. The effects of the surface topography of micromachined titanium substrata on cell behavior *in vitro* and *in vivo*. *J Biomech Eng* **21**, 49, 1999.
 40. Chou, L., Firth, J.D., Uitto, V.J., and Brunette, D.M. Substratum surface topography alters cell shape and regulates fibronectin mRNA level, mRNA stability, secretion and assembly in human fibroblasts. *J Cell Sci* **108**, 1563, 1995.
 41. Romet-Haddad, S., Marano, F., Blanquart, C., and Baeza-Squiban, A. Tracheal epithelium in culture: a model for toxicity testing of inhaled molecules. *Cell Biol Toxicol* **8**, 141, 1992.
 42. Lindberg, S., and Mercke, U. Capsaicin stimulates mucociliary activity by releasing substance P and acetylcholine. *Eur J Respir Dis* **68**, 96, 1986.
 43. Vacanti, C.A., Paige, K.T., Kim, W.S., Sakata, J., Upton, J., and Vaccanti, J.P. Experimental tracheal replacement using tissue-engineered cartilage. *J Pediatr Surg* **29**, 201, 1994.
 44. Yanagi, M., Kishida, A., Shimotakahara, T., Matsumoto, H., Nishijima, H., Akashi, M., and Aikou, T. Experimental study of bioactive polyurethane sponge as an artificial trachea. *ASAIO J* **40**, M412, 1994.
 45. Kuriloff, D.B., and Fayad, J.N. Tracheal autograft prefabrication using microfibrillar collagen and bone morphogenetic protein. *Arch Otolaryngol Head Neck Surg* **11**, 1385, 1996.

Address correspondence to:

Yuchi Wang, Ph.D.

BioTechPlex Corporation

1205 Linda Vista Dr.

San Marcos, CA 92078

E-mail: ywang@biotechplex.com

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