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Tissue Culture Models

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Introduction

The use of tissue cultures as a research tool to investigate the pathophysiologic bases of diseases has become essential in the current age of molecular biomedical research. Although it will always be necessary to translate and validate the observations seen in vitro to the patient or animal, the ability to investigate the role(s) of individual variables free from confounders is paramount toward increasing our understanding of the physiology of the lung and the role of its cellular components in disease. Additionally, it is not feasible to conduct certain research in humans because of ethical constraints, yet investigators may still be interested in the physiologic response in human tissues; in vitro characterization of human tissue is an acceptable choice.

Tissue culture techniques have been utilized extensively to investigate questions pertaining to lung physiology and disease. The isolation and propagation of human bronchial epithelial cells has allowed investigators to begin to characterize the interactions and reactions that occur in response to various stimuli. Moreover, the culture of human airway smooth muscle has allowed researchers to investigate a pathologic cascade that occurs in asthma as well as other physiologic responses in the smooth muscle of the lung. Numerous lung cancer cell lines have been established to investigate their responses to chemotherapy and determine their biologic properties. Overall, the use of cultured human lung tissue has provided a windfall of information on the pathogenesis of diseases that affect the lung and on the basic physiology and development of the lung in general. Despite this wealth of information in the literature, this chapter is the first to discuss the use of tissue culture models to examine the physiology and pathologic basis of lung diseases. In light of this, we briefly discuss the history and principles behind the utilization of tissue culture. We then discuss the current use of tissue culture to examine many of the unanswered questions involved in pulmonary physiology and pathology.

History of Tissue Culture

The technique of tissue culture is generally accepted to have arisen following the experiment of Ross Harrison, around the turn of the twentieth century. In 1907, Harrison began by adapting a previously established bacteriology technique, the "hanging-drop" method, to culture a frog neuron.[1] In 1912, Alexis Carrel built upon this work by successfully culturing small tissue samples from an 18-day-old chick embryo heart, thereby becoming the first scientist to propagate mammalian cells in vitro.[2] Carrel's demonstration that cells could be passaged 18 times, remain viable over 3 months, and continue to maintain cardiac rhythm was the first to show that cardiac tissues in vitro could retain normal characteristics for a prolonged period of time. These elegant studies, conceived by Carrel, initiated the modern day art of *histoculture* as it is now known.[3,4]

Although only a small "sect" of researchers embraced early tissue culture as a methodology to investigate the pathogenesis of disease, it is appropriate to describe Carrel as the father of mammalian tissue culture. In fact, Sven Gard, in his presentation speech for the Nobel Prize in Physiology or Medicine in 1954 referred to tissue culture as a "tissue cult . . . with Carrel as their high priest." In that year, Drs. Weller, Enders, and Robbins shared the prize for their work in propagating poliovirus in tissue culture. This work was the first Nobel Prize awarded in medicine and physiology for work accomplished primarily utilizing tissue culture as a methodology.

An additional important milestone in the use of tissue culture in biomedical research was the establishment of the first human cell line. In 1951, cervical cancer cells

from Henrietta Lacks were cultivated into the first immortal cell line—"HeLa."[5] HeLa cells are still one of the most widely used cell lines today. Since the 1950s, tissue culture has become firmly established as a mechanism to answer many questions in biomedical research. Today, tissue culture is widely used to investigate diseases that affect the lung, and through this work we have been able to increase our understanding of the pathologic cascades that occur in lung diseases, as well as the normal physiologies of the lung.

Types of Tissue Culture

Tissue culture is a commonly used generic term for the in vitro cultivation of cells, attributed to the early cultures that generally consisted of heterogeneous cultures of crudely disaggregated tissues. Currently, many terms are used that can be encompassed by the term: organ culture, cell culture, primary explants, and ex vivo propagation all deal with the in vitro cultivation of cells or tissues. *Cell culture* in general can be applied either to primary cells (e.g., those with a finite life span) or to cell lines (e.g., HeLa cells). Additionally, these cultures can be either a homogenous or a heterogenous group of cells.

Primary cell culture involves the isolation of cells from a tissue by disaggregation. Single cell suspensions from tissues can be completed through either enzymatic digestion of extracellular matrix surrounding the cells—such as with ethylenediaminetetraacetic acid, trypsin, or collagenase—or mechanical disaggregation. These disaggregation procedures have the disadvantage of possibly injuring cells. If the cells of interest are adherent viable cells, they will be separated from nonviable cells when the medium is changed. Alternatively, viable cells can be separated from nonviable cells prior to culture by subjecting the single cell suspension to density gradient centrifugation (e.g., Hypaque). Primary cells have an advantage of possessing many of the biologic properties that they possessed in vivo because they are not transformed. Primary cells, unlike cell lines, are not immortal and have only a finite survival time in culture before becoming senescent. Variant cells, however, as well as those obtained from neoplastic tissue, may proliferate infinitely, thus becoming immortal in vitro. This will eventually allow the immortal cell to take over the culture and can be thought of as a cell line. In general, primary human cultures will survive for 30–80 passages in vitro, although this number is dependent on cell type, conditions, and possibly other unknown factors. Primary cells are widely used to examine the effects of toxins, infectious agents, or other cellular interactions that would not be feasible in vivo. Primary cells have a disadvantage of being a heterogeneous mixture of cells upon primary isolation, with the type of cell obtained generally a component of the disaggregation method used. The most common contaminant seen following isolation of primary cells is cells of mesenchymal origin (e.g., fibroblasts). However, advances have been made that allow the culture of homogenous populations of cells. For instance, cell surface molecules specific for the cells of interest may be tagged with monoclonal antibodies. Techniques such as fluorescence-activated cell sorting or the use of magnetic beads can be utilized to enrich the single cell suspension for the cell type of interest. Additionally, some investigators have recently exploited unique characteristics of certain cells, such as the presence of P-glycoprotein or multidrug resistance-associated proteins expressed on endothelial cells, to poison other contaminating cells in culture.[6]

Another type of primary cell culture is "primary explants." This type of culture is not subjected to a disaggregation procedure like the primary cell technique described earlier. Therefore, single cell suspensions do not occur. Briefly, tissue samples are dissected and finely minced. These tissue pieces are then placed onto the surface of a tissue culture plate. Following plating of tissue pieces, cells have been shown to migrate out of the tissue and onto the tissue culture surface.[7] This technique is useful when cells of interest may become damaged or lost in the disaggregation technique described earlier and is often used to culture human bronchial epithelial cells.[8]

Cell lines are another useful source of cells to investigate questions in biomedical research. These cells have the advantage of being immortal as opposed to the finite life spans that primary cells possess. Additionally, they are generally well studied and characterized, leaving few experimental variables to worry about. These cells however, are prone to dedifferentiation—a process by which they lose the phenotypic characteristics of the cell from which they began. Many of the early cell lines were established from tumor tissue and as such possess abnormal growth characteristics. Newer cell lines have been established by molecular techniques such as inserting a telomerase gene into a cell to allow it to replicate infinitely.[9] Because of the phenotypic changes that allow cell lines to replicate infinitely in culture, they are often a first choice for experiments; however, they are also highly criticized in light of their nonnatural phenotype.

Organ culture, as the name implies, involves ex vivo culture of the whole or significant portion of the organ. The main advantage to this type of culture is the retention and preservation of the original cell–cell interaction and extracellular architecture. This type of culture may be particularly important when experimental design necessitates the use of an ex vivo system, but researchers still need to retain the original organ architecture to answer questions posed. These types of cultures do not grow rapidly, however, and are therefore not suitable for experiments needing large numbers of a particular cell type.[10]

Advantages and Limitations of Tissue Culture

Tissue culture has become the penultimate tool of the reductionist biologist. The utilization of tissue culture as a research methodology has allowed investigators to study isolated interactions in its near-normal environment. These experiments by their very nature introduce artifacts; however, they do minimize the number of confounding variables that may affect a particular experiment. For instance, tissue culture allows investigators to determine the effects of one particular treatment on a particular cell type, which would not be feasible in vivo. Additionally, tissue culture models of disease allow investigators to obtain samples and make observations more readily than those done in vivo. However, it is the relative simplicity of experiments done in vitro that allows models of disease or physiology to come under frequent and warranted criticism. These models do not take into consideration the complexity of biologic systems. Diminishing possible confounding variables by culturing cells in vitro brings up the constant criticism of how applicable results are because of alterations of the normal cellular environment in vivo. For example, cell–cell interactions in vitro are reduced and unnatural. Moreover, the culture does not contain the normal heterogeneity and three-dimensional architecture that is seen in vivo. This said, however, tissue culture biology has proved to be successful in many ways.

Cell Culture and the Study of Disease Processes

We have briefly discussed the advantages that experimental systems using tissue culture affords researchers studying physiology and pathogenesis. Because of its ability to isolate individual variables and determine their role(s) in physiology, cell culture has become an integral tool in deciphering the pathologic cascades that occur in human disease. Diseases that affect lung are no exception.

Many diseases that affect the lung, and humans in general, are multifactorial. This begs the question how can cell culture, because of its reductionist nature only dealing with a minimal number of variables, help to solve the unknown questions and decipher the components involved in disease? Often, clinical observations, and the questions arising therein, have been the launching pad for investigation.

For instance, observations of massive inflammation in the bronchoalveolar lavage samples of patients with acute respiratory disease syndrome (ARDS), consistent with damage seen in histologic samples, prompted investigators to determine the role(s) of inflammation in the etiology of ARDS. Through the use of cell culture, investigators were able to determine individual interactions that occurred in the disease process. Investigators have utilized culture models employing microcapillary endothelial cells under flow conditions to understand the role of proinflammatory cytokines in the cytokinesis and emigration of neutrophils in disease. Using a model of pulmonary endothelium under flow conditions allowed investigators to demonstrate the importance of certain proinflammatory cytokines in ARDS.[11]

The role of inhaled toxicants in lung injury, and the mechanism(s) by which they cause disease, is another area of investigation that has utilized cell culture. Scientists have developed diverse and unique tissue culture systems that contain air–liquid barriers of lung epithelium and subjected these cells to various gaseous toxicants to determine what occurs following inhalation of various chemicals. Utilizing these types of systems, investigators are able to control the exposure time and other variables that may be difficult when determining inhaled toxicant effects in vivo. Moreover, the use of tissue culture, as opposed to an animal model, allows investigators to observe effects kinetically, without undue changes (e.g., sacrifice) and expense in the experimental model.[11]

A tissue culture model also permits an investigator to observe multiple changes in real time, such as cellular integrity, cell signaling and intracellular trafficking, protein expression changes, oxidant-induced cellular damage, and more. Deciphering each of these changes in an animal model would be extremely difficult; through employing a tissue culture model, researchers are able to tightly control the experimental system while isolating the events of interest. Further examples of how tissue culture models are currently being used to elucidate questions in lung physiology and disease are discussed later in the section on lung tissue cell lines.

Biology of the Cultured Cell

Culture Environment

Maintaining cells in vitro was initially a very difficult task. Many characteristics need to be fulfilled before a successful cell culture occurs. Some of these characteristics are dependent on the type of tissue being studied; others may depend on specific requirements of the individual cells. Various chemically defined media are now available commercially to support the growth and differentiation of numerous cell types. The creation of defined media has allowed investigators to culture a multitude of cell types while controlling the local environment to answer pertinent questions. For example, glucose can be removed

from a culture medium in order to study its effects on cellular metabolism, relative position in the cell cycle, and many other effects. Each chemical component is known in these media. Additionally, investigators can add growth factors to nourish their cell cultures.

The medium chosen when culturing cells in tissue culture must fit two main requirements: (1) it must allow cells to continue to proliferate in vitro, and (2) it must allow the preservation of the certain specialized functions of interest.[7] The most common medium formulations used currently in lung research are Dulbecco's modified Eagle's medium, minimum essential medium, RPMI 1640, and Ham's F-12. Occasionally, investigators develop new medium types to attain a formulation that optimizes their own experimental conditions. Fetal bovine serum is a common additive to most tissue culture media, although some investigators choose to forgo this additive for more defined supplementation. Additionally, others may choose sera from other sources such as human serum when culturing cells of human origin. Inactivation of complement by heat treating serum for 1 hr at 56°C was initially very popular in tissue culture. However, it has become clear that this treatment may in fact damage some of the proteinaceous growth factors present in the medium, rendering it less effective. Currently, many experts recommend heat inactivation only if the cell type of interest is particularly sensitive to complement.[12] More specific examples of medium utilized in lung tissue culture models are given later in the section on lung tissue cell lines.

When deciphering if the current culture conditions are sufficient for the experimental design, the investigator must determine which cellular characteristics are important. Not only are the general characteristics, such as adhesion, multiplication, and immortalization of cell types important, but so are tissue-specific characteristics. Of importance to pulmonary research, the lung is a unique environment to simulate in vitro because of the air–liquid interface. Recently, investigators have made use of culture insert wells (e.g., Transwells, Corning) in order to study this interaction.[6]

Cell Adhesion

Nearly all normal or neoplastic human epithelial cells will attach with relative ease to tissue culture surfaces. Most tissue culture models utilizing tissue of lung origin fit this description, with the notable exception of small cell lung carcinoma cell lines. However, for culture cells that may loosely adhere, or may not adhere at all, scientists coat tissue culture surfaces with extracellular matrix proteins. Incubating tissue culture surfaces with serum, as well as laminin, fibronectin, or collagen, prior to culture has been shown to improve attachment of finicky cells.[8] These treatments also help in replicating the normal attachment of cells to extracellular matrix proteins in vivo.

Development of Continuous Cell Lines

The development of continuous cell lines may be serendipitous, as was the development of early cell lines. In brief, many investigators would continue splitting primary cell cultures until one or more cell clones became immortal. Unfortunately, the changes that generally occurred in culture led to cells with abnormal phenotypes that had undergone dedifferentiation. Today, many investigators choose to use molecular biology techniques, exploiting our current knowledge of oncogenic viruses and enzymatic processes of cellular aging to transform primary cells in vitro to an immortal phenotype. It is known that the large T antigen present in the SV (Simian virus) 40 virus is capable of transforming cells to an abnormal phenotype.[11,13,14] Moreover, transfection of primary cells with a transposase enzyme has also been shown to induce an immortal phenotypic change while preserving most normal cellular functions and phenotypes.[11]

Dedifferentiation

A commonly encountered problem in tissue culture is dedifferentiation. This loss of phenotype may be insignificant to the research at hand or it may be critical, and it must be dealt with on a case by case basis. When a cell culture undergoes dedifferentiation it is often unclear whether undifferentiated cells took over the culture of terminally differentiated cells or whether a primary cell of interest became immortal under the culture conditions.

Functional Environment

The functional environment in which cells are cultured is critical when correlating experimental results to those seen in vivo. We previously alluded to the importance of the environment in which cells are cultured when discussing the advantages and limitations of tissue culture. Investigators have frequently strived to replicate integral in vivo environments in vitro in order to increase the significance of their experimental results.

The development of cell culture insert wells (e.g., Transwells, Corning) has allowed investigators to culture bronchial or alveolar epithelial cells at an air–liquid interface. This ability allows investigators to begin to replicate a significant aspect of these cells' functional environment in vitro, thereby increasing their understanding of the effects of gaseous particles on pulmonary epithelial cells. Alternatively, scientists have also cultured epithelial cells on a roller bottle apparatus. This method allows investigators to determine the amount of time the apical epithelial cell surface is in contact with the air.

Capillary cell cultures have also come under frequent criticism when cultured in a monolayer in a tissue culture

plate. Investigators have been able to utilize gel matrices in which capillary cells form tubule-like structures, more closely replicating the architecture these cells maintain in vivo. Additionally, endothelial cells are constantly under flow conditions in vivo. Addressing this condition in vitro has allowed investigators to look at the role of endothelial cells during inflammation—helping to increase the understanding of the role endothelium plays in acute lung injury.

At times, researchers may also choose to determine the effects of soluble factors (e.g., cytokines, hormones, neurotransmitters) from acute patients or animal models in a cell culture model. The milieu of soluble factors present in the serum that may play a role in a disease state is considerable. Moreover, these factors may have actions alone that are different when combined with other soluble factors. Reconstituting every factor presents a difficulty in vitro and leaves the possibility that an unknown factor may be missing. To address this, investigators have harvested sera from patients or animal models and used these samples as additives in their media formulations. For instance, through the use of serum samples from an animal model of smoke/burn injury–induced acute lung injury, investigators have demonstrated that use of arteriovenous CO_2 removal in acute lung injury significantly reduces apoptotic cell death in epithelial cells.[15]

Lung Tissue Cell Lines: Establishment and Significance

The diversity of research fields utilizing tissue culture models of lung diseases is extensive. In this section, we will give a brief overview of the main lung cell types that are being utilized in research today to answer pressing questions about lung physiology and the pathophysiology of pulmonary disease. Included in this discussion is also an overview of cell isolation and culture.

Normal Human Bronchial Epithelial Cells

The use of normal human bronchial epithelial (HBE) cells is extensively reported in the literature. Based on a method pioneered by Lechner et al.,[16] bronchial fragments obtained from surgery, autopsy, or biopsy specimens may be used as explants. The outgrowth of bronchial epithelial cells occurs readily from these explants when grown in medium supplemented with bovine pituitary extract and epidermal growth factor. Alternatively, these cells have also been demonstrated to grow in basal keratinocyte serum-free medium without supplementation; however, they demonstrate a slower growth rate and earlier senescence.[8]

Cultures of HBE cells are valuable for determining the responses to toxic inhaled pollutants. In vitro exposure systems based on these methods have several advantages. First, in vitro exposure systems can be stringently controlled and reproduced much better than in animal systems; second, individual determination of the cell types' responses to pollutants allows for a better characterization of the individual involvement of the cell type to a biologic response. Finally, in vitro determination of the responses to toxic agents allows investigators to observe the reactions of human cells when testing in humans is not feasible because of ethical restraints.

In vitro study of the responses of bronchial cells to gaseous pollutants is not without its difficulties. Wallaert et al.[17] have described these constraints well. Briefly, because of the gaseous nature of the pollutants, culture systems should be designed that allow significant exposure times to pollutants while also taking care to inhibit cells from drying out when exposed to air. To facilitate these experiments, roller bottle cultures have been developed that allow cells direct contact with the ambient air. Alternatively, cells have been grown on a membrane filter and cultured at an air–liquid interface, which allows constant exposure to the experimental treatment. The same type of experiments that are used to determine the responses of cells to inhaled toxicants have also been used to characterize responses to inhaled pharmaceuticals.

In addition to the characterization of responses to inhaled agents, epithelial cell cultures, notably alveolar epithelium obtained from fetal lung tissue, have allowed investigators to characterize the liquid transport phenotype that occurs in the developing lung. Characterization of the Cl^- ion secretion system, which occurs in the distal lung epithelium throughout gestation, has been shown to be integral in the stimulation of growth of the developing lung by regulating liquid secretion. Likewise, a phenotypic switch of Na^+ absorptive capacity has been described toward the end of gestation, which is important for preparation of the lung to function postpartum and beyond. These culture systems have elucidated important physiologic changes that occur in the developing lung. Similar experiments have demonstrated that while ion transport plays a crucial role in this process other hormones and neurotransmitters are also important.

Pulmonary Endothelial Cells

Pulmonary endothelial cells represent a unique type of endothelium because of their paradoxical responses to hypoxia. This uniqueness highlights the need to utilize cell culture models of pulmonary endothelium as opposed to other endothelia when interested in investigating their role(s) in pulmonary physiology. Several investigators have described the isolation and culture of pulmonary endothelial cells. Persistent pulmonary hypertension of the newborn, also known as neonatal pulmonary hyper-

tension, is caused by a disorder of the pulmonary vasculature from fetal to neonatal circulation, culminating in hypoxemic respiratory failure and death. The inciting events that culminate in neonatal pulmonary hypertension are multifactorial. Despite this, decreased production of vasodilator molecules such as nitric oxide and prostaglandin I_2 in the pulmonary endothelium has been shown to be a critical component of disease progression.[18]

Airway Smooth Muscle and Asthma

Primary cell cultures of human airway smooth muscle tissue can be obtained utilizing a method described by Halayko et al.[19] in which they isolated and characterized airway smooth muscle cells obtained from canine tracheal tissue. Briefly, airway smooth muscle cells were obtained by finely mincing tissue and subjecting it to an enzymatic disaggregation solution containing collagenase, type IV elastase, and type XXVII Nagarse protease. Following generation of a single cell suspension, cells may be grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Halayko et al.[20] obtained approximately 1.3×10^6 smooth muscle cells per gram of tissue using this method. Although Halayko et al.[21] pioneered this technique using trachealis tissue, many other investigators have obtained airway smooth muscle cells from a variety of biopsy specimens.

Airway smooth muscle hyperreactivity and hypertrophy has been known for nearly 100 years[2] to be an important end response of asthma. The use of airway smooth muscle in vitro has been vital toward delineating the pathologic steps that occur in asthma, as well as testing of potential therapeutics that may help to decrease the morbidity and mortality of asthma. Additionally, the relative paucity of in vivo models of asthma further illustrates the value of isolation and characterization of smooth muscle cells from asthmatic patients in vitro.

Using airway smooth muscle cell culture, investigators have characterized both the hypertrophic and hyperplastic growth of smooth muscle in individuals. Investigation of the potential stimuli that lead to airway smooth muscle proliferation and hypertrophy have led researchers to implicate the mitogen-activated protein kinase family members, extracellular signal-regulated kinase-1 and -2, and the phosphoinositol-3 kinase pathways in pathogenesis.[22] Additionally, mediators directing smooth muscle migration have also been observed in vitro and may play a role in the progression of asthma. Platelet-derived growth factor, fibroblast growth factor-2, and transforming growth factor-β (TGF-β) have all been shown to play a role in the migratory response of smooth muscle cells seen in asthma.[22] Additionally, contractile agonists such as leukotriene E_4 have been shown to potentiate the migratory responses seen with platelet-derived growth factor treatment.[22]

Human airway smooth muscle cell culture has also been utilized to investigate possible pharmacologic interventions for the treatment of asthma. β_2-Agonists have been shown to decrease the rate of DNA synthesis and likewise decrease the hyperplasia seen in airway smooth muscle cells in response to mitogenic stimuli through an increase in cyclic adenosine monophosphate. Like β_2-agonists, glucocorticoids have similar antiproliferative activities.

Lung Cancer Tissue and the Development of Novel Therapeutics

Culture of neoplastic cells from human tumors has allowed investigators to harvest a wealth of knowledge into the biology of lung cancers; moreover, these cultures have provided potential models to test potential therapeutics. The propagation of lung cancer cells in vitro has been covered in great depth previously.[8] In contrast to primary cell cultures, cultures of neoplastic cells are immortal, allowing their easy growth in culture with less chance of being overgrown by mesenchymal cells such as fibroblasts. The relative ease of growth in culture has led to many cell lines of lung cancer tissue. The National Cancer Institute, recognizing the need for a variety of lung cancer cell lines (both small cell and non–small cell), helped establish over 300 cell lines.[23] These lines are a wonderful resource for investigators given that they are extensively characterized, and many have full clinical data available. Moreover, many of these cell lines are now easily available through the American Type Culture Collection for a modest handling fee. Additionally, if investigators do not wish to use currently established lung cancer cell lines, obtaining clinical samples for use in tissue culture models is relatively easy. The same methods used to obtain biopsy specimens for clinical staging can also be used to begin cell cultures. Following culture and initial characterization of lung cancer cell lines, many investigators have demonstrated that lung cancer cell lines maintain a similar phenotype after establishment. Specifically, it has been verified that injection of lung cancer cell lines into nude mice exhibit similar histopathology to the original tumor, indicating minimal change occurred following establishment of the cell lines.

Small cell lung carcinoma (SCLC) cell lines have been established from a multitude of biopsy specimens, including bone marrow, lymph nodes, and pleural effusions.[8,24] Once viable cells have been obtained from clinical samples, cells are easily maintained in a basal cell culture medium such as RPMI 1640 in a humidified incubator at 37°C and 5% CO_2, although the initial isolations of SCLC lines utilized HITES and ACL-4 media.[25] Most established SCLC cell lines maintain a neuroendocrine phenotype in culture; however, Baillie-Johnson et al.[24] noticed considerable heterogeneity in the cell lines they

established, highlighting the significance that establishing a cell line from the clinical sample of interest may provide investigators with a line that possesses the exact phenotypic properties of interest.

Small cell carcinoma poses many difficulties to surgical treatment, owing to its early and widespread metastasis. Therefore, combination chemotherapy is generally utilized in treatment. Unfortunately, despite initial sensitivities, SCLC tumors become resistant to further treatment. Utilizing in vitro cultures of SCLC cell lines, Sethi et al.[26] began to describe how extracellular matrix proteins can protect SCLC against apoptosis-inducing chemotherapeutics through β_1-integrin–mediated survival signals. These data indicate that extracellular matrix proteins surrounding SCLC may play a role in the local recurrence seen in patients following chemotherapy in vivo and suggest novel therapeutics aimed at blocking these survival signals.

Non–small cell lung carcinoma (NSCLC) cell lines including squamous cell carcinoma, adenocarcinoma, and large cell carcinoma have all been established. Despite the fact that NSCLC cells comprise three distinct histologic cell types, all cell types can be established relatively easily. The primary treatment protocol for patients afflicted by NSCLC is generally surgical resection of the tumor; therefore, tumor cells for culture are readily available. These cell types can be grown under conditions similar to those described for SCLC.

Infectious Diseases

Infectious diseases play a unique role in lung pathology in light of their roles as either important contributors or consequences of many lung diseases. For instance, certain lung diseases may predispose patients to infection: patients afflicted with obstructive lung diseases, as well as cystic fibrosis patients, commonly suffer from severe and recurrent bacterial infections. Additionally, patients may become superinfected following a viral respiratory infection. Systemic infections, such as gram-negative bacterial sepsis, may lead to lung diseases such as ARDS.

Human Type II Alveolar Pneumocytes and Acute Lung Injury/Acute Respiratory Distress Syndrome

Pulmonary alveolar type II cells are a unique cell subset that carries out highly specialized functions that include synthesis and secretion of surfactant, a unique composition of lipoproteins that act to reduce surface tension at the alveolar air–liquid interface.[27] Defining the molecular mechanisms leading to production of surfactant by type II pneumocytes is important in many disease processes.

The pathogenic sequence that results in ARDS, the most severe manifestation of alveolar lung injury, is generally thought to be initiated by a systemic inflammatory response.[28] Despite this knowledge, there still exist many questions about the initial triggers and pathologic steps that occur in ARDS. Greater understanding of these steps may help to develop new treatment regimes. Currently, treatment of ARDS consists of mechanical ventilation, which helps to stabilize blood gases. However, mechanical ventilation itself may provoke further inflammation in the alveoli, thereby decreasing compliance and gas exchange in the alveoli.[29]

The cell type of particular interest in ARDS and diffuse alveolar damage is the type II pneumocytes.[30–34] Until recently, studies trying to decipher the pathologic sequence in acute lung injury have had to rely on standard lung epithelial cell lines. Recently, however, human type II alveolar epithelial cells (pneumocytes) have been successfully isolated from fetal human lung tissue by collagenase digestion.[35] Briefly, fetal lung tissues were minced and incubated in a serum-free medium containing dibutyryl cyclic adenosine monophosphate for 5 days. The tissue explants were then treated with collagenase and incubated with DEAE-dextran to eliminate contaminating fibroblasts. Cells were then plated onto tissue culture dishes treated with extracellular matrix derived from MDCK cells and cultured overnight in Waymouth's medium containing 10% serum. These steps resulted in relatively pure populations of human type II pneumocytes that were then cultured at an air–liquid interface. Using these methods, Alcorn et al.[35] were able to maintain a primary culture that retained the morphologic and biochemical characteristics of type II pneumocytes for up to 2 weeks.

Three-Dimensional Biology

Conventional Bioreactors and Three-Dimensionality: The Origins of Three-Dimensional Culture

Carrel postulated that tissue development was linked to access to nutrient supply, noting that peripheral cells grew readily, and internal cells became necrotic presumably based on their distance from the nutrient source. To circumvent this issue, Carrel implemented cultures on silk veils, preventing the plasma clots of the growth media from deforming or becoming spherical, thus facilitating the internal cell's ability to obtain nutrient replenishment. Many attempts were made in standard culture systems (bioreactors) and other culture apparatuses to escape the constraints of two-dimensional cell culture, with the intent of yielding high-fidelity human and mammalian tissues, and thus emphasizing the need for development of three-dimensional biology.

Another famous researcher, Leighton, improved on Carrel's techniques in the 1950s and 1960s. Leighton's major contribution to three-dimensional culture technology was the introduction of the idea of a sponge matrix as a substrate on which to culture tissues.[36,37] Leighton first experimented on cellulose sponges surrounded by plasma clots resident within glass tubes. He devised a system to grow 1- to 5-mm^3 tissue explants on sponges, using small amounts of chick plasma and embryo extract. After the mixture solidified on the sponge Leighton added the nutrient media and inserted the "histoculture" in a roller apparatus to facilitate nutrient mass transfer. He experimented with many sponge combinations, discovering that collagen-impregnated cellulose sponges were optimal for sustaining the growth of native tissue architecture.[3,38]

Leighton was successful in growing many different tissue types on the sponge-matrix cultures.[3,38] Leighton also found that C3HBA mouse mammary adenocarcinoma cells, when grown on sponge-matrix histoculture, aggregated "much like the original tumor, forming distinct structures within the tumors such as lumina and stromal elements, and glandular structures." An extremely important difference of this three-dimensional histoculture from the standard two-dimensional culture is the apparent quiescence of the stromal component and the balanced growth of these cells with regard to the overall culture. Leighton further advanced the concept of three-dimensional histoculture to histophysiologic gradient cultures.[39] These cultures are conducted in chambers that allow metabolic exchange between "the pool of medium and the culture chamber by diffusion across a membrane." Histophysiologic gradient cultures mimic, to some degree, diffusion in tissues.[38]

From the pioneering work of Carrel and Leighton, other methods of emulating three-dimensional cultures have been developed, such as embedding cells and tissues in collagenous gels of rat tail as per the techniques of Nandi and colleagues. Many of the advantages of three-dimensional cultures seen by Leighton, Nandi, and others may be attributed to permitting the cells to retain their normal shape and special associations.[3] This global concept will be important as we begin to understand and recall the physical and environmental characteristics of the rotating-wall vessel systems.

Other methods of three-dimensional culture encompass a technique known as *organ culture* or *culture on a filter*, a strategy developed by Strangeways[40] and Fell and Robinson.[41] Tissue explants were grown on lens paper in a watch glass containing liquid culture medium. Browning and Trier[42] found "that for some tissues, it is critical to keep the cultures at the air–liquid interface," thus allowing the tissues to experience conditions similar to the in vivo environment.

Another strategy is the use of three-dimensional cultures known as *proto-tissues*, or aggregates of cells, used to form spheroids. This technique was popularized by Sutherland and colleagues more than 20 years ago when they manipulated aggregates of cells into a spherical configuration by spinning agitation of the cells in spinner flasks.[43] This technique produced pseudo-tissue-like organoids useful for research evaluations. Each of these methodologies will be of benefit as we continue to examine strategies for achieving three-dimensional lung tissue constructs.[3,38]

Finally, membrane bioreactors are capable of retaining enzymes, organelles, and microbial, animal, and plant cells behind a membrane barrier, trapped in a matrix or adherent to the membrane surface. In 1963, Gallup and Gerhardt [44] first used the membrane bioreactor for dialysis culture of *Serratia marcescens*. Immobilized enzyme microencapsulation was pioneered by Chang,[45] but Butterworth et al.[46] first developed the enzyme membrane reactor to successfully accomplish starch hydrolysis with α-amylase. Likewise, for animal cell culturing, Knazek et al.[47] first cultured human choriocarcinoma cells on compacted bundles of Amicon fibers. Many reviews on the particular applications of hollow fiber and immobilized bioreactant bioreactors for enzyme catalysts, microbial cells, and animal cell culture are available.[48–53]

As presented previously, tissue-engineering applications of three-dimensional function and structure are well known in medical science research.[54] In microgravity three-dimensional aggregates form, facilitating the expression of differentiated organotypic assemblies. Investigations to determine the effect of composite matrices, spiked with esterified hyaluronic acid and gelatin, to augment osteochondral differentiation of cultured, bone marrow–derived mesenchymal progenitor cells and the effects of the matrix on cellular differentiation have been examined in vitro and in vivo.[54]

Briefly, empty and populated matrices cultured for 28 days, with and without TGF-β_1 demonstrated the following results. Cells implanted in the matrix produced a robust type II collagen extracellular matrix in vitro. Matrices placed in immunodeficient mice yielded no differentiation in empty constructs, osteochondral differentiation in loaded implants, and an enhanced level of differentiation in preimplantation in vitro–cultured matrices containing TGF-β_1. These results demonstrate the utility of three-dimensional matrix for presentation of bone mesenchymal progenitor cells in vivo for repair of cartilage and bone defects as well as indicate the efficacy for in vitro tissue engineering regimes.[54] These techniques lend themselves to microgravity and ground-based research tissue cultures alike.

Many earth-based laboratories are researching and developing hemopoietic bone marrow cultures of stem cell origin, and three-dimensional configurations are

providing promising results as illustrated by Schoeters and coworkers.[55] They report that murine bone marrow cells, cultured under long-term hemopoietic conditions, produce mineralized tissue and bone matrix proteins in vitro but only when precipitated by the presence of adherent bone stroma cells in three-dimensional collagen matrices. At a concentration of 8×10^6 stromal cells, mineralization occurs in 6 days. In contrast, two-dimensionally oriented marrow fragments at 1×10^7 cells require requires more than 10 days before mineralization can similarly be detected.[55]

Two-dimensional long-term marrow culture facilitates and enhances expansion of the stromal component and rudimentary differentiation of osteogenic-like cells in the adherent stromal layer as verified by type I collagen or cells positive for alkaline phosphatase. Production of osteonectin and osteocalcin, a bone-specific protein, combined with calcification is observed only in three-dimensional cultures. These studies demonstrate the need for and benefit of three-dimensionality and the application to the microgravity environment.[55] As we can see, this further reinforces the quest for three-dimensionality and the potential of modeling the microgravity environment.

Three-Dimensional Models for Physiological Study

Investigations clearly show the need for the application of three-dimensional study techniques in lung pathophysiologic studies. Interestingly, three-dimensional biology has facilitated full-scale investigations into most areas of tissue engineering, cell biology and physiology, immunology, and cancer research.

Anchorage-dependent cells are widely cultured on microcarriers.[56] Studies show that for the purposes of improved surface-to-volume ratio and scale up, the microcarrier suspension culture provides excellent potential for high-density cell growth.[57] In addition, microcarriers serve well as structural supports for three-dimensional assembly, the composite of which is the basis for three-dimensional tissue growth.[58]

Conventional culture systems for microcarrier cultures (i.e., bioreactors) use mechanical agitation to suspend microcarriers and thus induce impeller strikes as well as fluid shear and turbulence at the boundary layer between the wall and the fluid. Investigators have attempted to make a complete study of the most efficient bioreactor designs and agitation regimens.[59] They concluded that virtually all stirred-tank bioreactors operate in the turbulent regimen. It has been demonstrated that bead-to-bead bridging of cells is enhanced significantly at lower agitation rates in a stirred reactor.[60] Excessive agitation from either stirring or gas bubble sparging has been documented as a cause of cell damage in microcarrier cell cultures.[61,62] To overcome the problems induced by these mechanisms, investigators developed alternative culture techniques such as porous microcarriers to entrap cells,[63] increased viscosity of culture medium,[64] bubble-free oxygenation,[65] and improved methods for quiescent inoculation.[66,67] These steps decreased the damage attributed to turbulence and shear forces but failed to significantly rectify the problems. Reactor systems of substantially increased volume exhibit less agitation-related cell damage. This is presumably because of the decreased frequency of cell–microcarrier contact with the agitation devices in the systems. Research-scale investigations do not afford the luxury of experimenting with large-scale production systems. Therefore, if a large-volume system is indeed more quiescent, an improved bioreactor system should emulate the fluid dynamics present in the upper regions of large-scale reactors in which cells and microcarriers reside with minimal agitation. The problem, then, is to suspend microcarriers and cells without inducing turbulence or shear while providing adequate oxygenation and nutritional replenishment.

The term *rotating-wall vessel* comprises a family of vessels, batch fed and perfused, that embody the same fluid dynamic operating principles. These principles are (1) solid body rotation about a horizontal axis that is characterized by (a) colocation of particles of different sedimentation rates, (b) extremely low fluid shear stress and turbulence, and (c) three dimensional spatial freedom; and (2) oxygenation by active or passive diffusion to the exclusion of all but dissolved gasses from the reactor chamber, yielding a vessel devoid of gas bubbles and gas–fluid interface (zero head space).[68,69]

Three-Dimensional Models of Lung Disease

Lung Cancer

Current cell culture models have shortcomings resulting in unreliable tumor growth, uncharacteristic tumor development, nonhuman tumors, and inadequate methods of detection. Cells propagated under traditional culture conditions differ widely in their expression of differentiated markers, adhesion receptors, and growth factor receptors compared with cells in situ or those grown as tissue-like structures.[70,71] This is of concern because the phenotypic changes leading to malignant transformation often stem from alterations in the balanced and multifaceted roles of growth factors, receptors, and cytokines (reviewed by Herlyn et al.[71]). With increasing evidence of the importance of adhesive contacts, paracrine cross-talk between different cell types, and signaling cascades that link the cell with a complex substratum, there is now recognition that models must be developed that better simulate these complexities. There is still much to learn about the dynamic relationships among the different

phenotypes found in the normal lung and in lung cancers. Until a cell culture system is developed that allows differentiation to occur,[72] it is difficult to make any firm statement about relating effects in cell culture to clinical practice. Tissue engineering is very embryonic in development and currently nearly universally focused on building replacement tissues. A new technology developed at the NASA Johnson Space Center used to study colon cancer has been adapted to three-dimensional in vitro lung tissue culture models but has not been reported on to date.

Rotating-wall vessels are horizontally rotating cylindrical tissue culture vessels that provide controlled supplies of oxygen and nutrients with minimal turbulence and extremely low shear.[69] These vessels suspend cells and microcarriers homogeneously in a nutrient-rich environment, which allows the three-dimensional assembly of cells to tissue. Prior to seeding rotating-wall vessels (Synthecon, Inc, Houston, TX), cells were cultured in standard T flasks (Corning, Corning, NY) in GTSF-2 medium (1993 PSEBM) in a humidified 37°C, 5% CO_2 incubator. The rotating-wall vessels were seeded with 1–2 mg/mL Cultispher-GL microcarriers (Hyclone Laboratories, Inc., Logan, UT) followed by BEAS2-B or BZR-T33 cells (ATCC, Baltimore, MD) at a density of 2×10^5 cells/mL. Cultures were grown in the rotating-wall vessels for 14–21 days for formation of 3- to 5-mm diameter tumor masses. Rotating-wall vessel rotation was initiated at 25 rpm and increased as aggregate size became larger. Stationary control cultures were initiated under the same conditions using FEP Teflon bags (American Fluoroseal, Columbia, MD). At 24-hour intervals pH, dissolved CO_2, and dissolved O_2 were determined using a Corning 238 model clinical blood gas analyzer. Glucose concentration was determined using a Beckman 2 model clinical glucose analyzer (Beckman, Fullerton, CA). Cell samples were harvested every 48 hr and fixed with Omnifix (Xenetics, Tustin, CA) for immunohistochemistry or fixed with 3% glutaraldehyde/2% paraformaldehyde in 0.1 M cacodylic buffer (Electron Microscopy Sciences, Fort Washington, PA) for scanning electron microscopy.

Cancer models already developed by NASA investigators include growth and differentiation of an ovarian tumor cell line,[72–74] growth of colon carcinoma lines,[72] and three-dimensional aggregate and microvillus formation in a human bladder carcinoma cell line.[74] In support as an appropriate model for cancer, even the most rudimentary three-dimensional cellular structures exhibit different phenotypes than cell lines cultured under two-dimensional conditions. Properties such as responses to TGF-β, drug resistance to cisplatin or cyclophosphamide, and resistance to apoptosis are all altered in various types of cell aggregates.[75]

Many investigations sustain consistent evidence that cells growing in three-dimensional arrays appear more resistant to cytotoxic chemoagents than cells in monolayer culture.[38] Li et al. found that spheroids were more resistant to cytosine arabinoside by 11-fold and methotrexate by 125-fold when compared with single cell suspensions.[76] Further monolayer cultures of colon carcinoma cells were sensitive to piericidin C in contrast to responses within in vivo colon tumors or three-dimensional slices of tumors grown in vitro.[77] Numerous other investigations have revealed increased levels of drug resistance of spheroids compared with single cell monolayers.[3,38]

Questions of poor diffusion and insufficient drug absorption within spheroids and a relatively frequent high proportion of resting cells have clouded differences in drug resistance, which could be the result of nutrient deprivation and hypoxia. Heppner and colleagues executed precise experiments that confirmed three-dimensional structure and function as the causative agent and was responsible for drug resistance rather than simple inaccessibility to nutrients or the drug concentration. Heppner embedded tumor specimens or cell aggregates in collagen gels, exposed the culture to various cytotoxic drugs, and compared the drug responses of the same cells in monolayers. These experiments revealed an increased resistance in the three-dimensional tumor arrays of a remarkable 1,000-fold greater than in monolayer cultures, and a similar result was seen in three-dimensional histocultures in collagen. The tumor cells grew in the presence of drug concentrations that rendered monolayers to a viability less than 0.1% of control cultures. Amazingly, Heppner observed that the cells became sensitive again when replated as monolayers and finally showed that even when exposed to melphalan and 5-fluorouracil in monolayer cells transferred to collagen gels were again resistant based on three-dimensional architecture. Thus, the cells were exposed to the drugs as monolayers, facilitating access to the drugs, and, once the cells were transferred after drug exposure to a three-dimensional structure, high resistance to the drugs was sustained.[38,78–81]

Based on the caliber of data referenced above, Teicher et al.[82] serially passaged through multiple (10) transfers EMT-6 tumors in mice that were treated with thiotepa, cisplatin, and cyclophosphamide over a prolonged 6-month period, thus producing extremely drug-resistant tumors in vivo. When these tumors were grown as monolayer cultures, they were as drug sensitive as the parental cells. Kobayashi and colleagues[83] grew the same in vivo drug-resistant tumor cell lines as spheroids in three-dimensional arrays, and resistance was almost 5,000 times that of the parent line with selected drugs, an example being the active form of cyclophosphamide used in vitro. Similarly extreme resistance was also observed to cisplatin and thiotepa. This resistance was not seen in monolayer cultures, even when the monolayers were cultured on traditional extracellular matrix substrates. These

experiments reconfirmed that cells in a three-dimensional array are more drug resistant than monolayer cells in vitro and demonstrated that three-dimensional cellular configurations can and do become resistant to super pharmacologic doses of drugs by forming compact structures.[38]

Rotating-Wall Vessel Tumor Models

Several important human tumor models have been created in rotating-wall vessel cultures, specifically, lung, prostate, colon, and ovarian.[14,58,73,84] Many of these models involve cancers that are leading killers in our society. We present two such examples in this section, colon and prostate carcinoma. As previously reviewed, the literature indicates the remarkable difference between chemotherapeutic cytotoxicity in two-dimensional and three-dimensional cellular constructs, which may be predicated on a number of criteria. Therefore, a three-dimensional tumor model that emulates differentiated in vivo–like characteristics would provide unique insights into tumor biology.

Goodwin et al.[58] detail the first construction of a complex three-dimensional ex vivo tumor in rotating-wall vessel culture composed of a normal mesenchymal base layer (as would be seen in vivo) and either of two established human colon adenocarcinoma cell lines, HT-29, an undifferentiated line, and HT-29KM a stable, moderately differentiated subline of HT-29. Each of these engineered tumor tissues produced tissue-like aggregates (TLAs) with glandular structures, apical and internal glandular microvilli, tight intercellular junctions, desmosomes, cellular polarity, sinusoid development, internalized mucin, and structural organization akin to normal colon crypt development. Necrosis was minimal throughout the tissue masses up to 60 days of culture while achieving >1.0 cm in diameter. Other notable results included enhanced growth of neoplastic colonic epithelium in the presence of mixed normal human colonic mesenchyme. These results mimic the cellular differentiation seen in vivo and are similar to results obtained with other tumor types.

Prostate carcinoma has also been modeled in the rotating-wall vessel system by several investigators.[85–87] One of the most comprehensive descriptions of these engineered tissues is detailed by Wang et al.[88] In that review, the authors describe the ability of the rotating-wall vessel system to recapitulate human prostate carcinoma (LNCaP) and bone stroma (MG63) to illuminate the evolution of prostate tumorigenesis to the metastatic condition. In particular, the LNCaP and ARCaP models represented in the review are known to be lethal in the human, being androgen independent and metastatic. Rotating-wall vessel TLA engineering also allowed in-depth study of epithelial and stromal interactions, which are the facilitating elements of the continuance of LNCaP prostate-specific antigen production in vitro. When LNCaP was cultured in three dimensions without stroma, production of prostate-specific antigen ceased and metastatic markers were not observed. The authors outline the process of malignant transformation, demonstrating that these metastatic models are only possible in three-dimensional TLAs and are achieved by specific geometric relationships in three-dimensional configuration. Furthermore, they show through direct comparison with other culture systems the advantages of the rotating-wall vessel system to allow synergistic relationships to study this disease state.[88]

Unlike two-dimensional models, these rotating-wall vessel tumor tissues were devoid of metabolic and nutrient deficiencies and demonstrated in vivo–like architecture. These data suggest that the rotating-wall vessel affords a new model for investigation and isolation of growth, regulatory, and structural processes within neoplastic and normal tissues.

Rotating-Wall Vessel Normal Human Tissue Models as Disease Targets

In this section, we explore the utility of rotating-wall vessel TLAs as targets for microbial infection and disease. Several studies have been conducted recently that indicate that three-dimensional tissues respond to infective agents with greater fidelity and with a more in vivo–like response than traditional two-dimensional cultures. Nickerson et al.[89] describe the development of a three-dimensional TLA engineered from INT-407 cells of the human small intestine, which were used as targets for the study of *Salmonella typhimurium*. In this study, three-dimensional TLAs were used to study the attachment, invasion, and infectivity of *Salmonella* into human intestinal epithelium. Immunocytochemical characterization and scanning and transmission electron microscopic analyses of the three-dimensional TLAs revealed that the TLAs more accurately modeled human in vivo differentiated tissues than did two-dimensional cultures. The level of differentiation in the INT-407 TLAs was analogous to that found in previously discussed small intestine TLAs[72] and from other organ tissues reconstructed in rotating-wall vessels. Analysis of the infectivity studies revealed *Salmonella* attached and infected in a manner significantly different from that in control two-dimensional cultures. During an identical exposure period of infection with *Salmonella*, the three-dimensional TLAs displayed a minor loss of structural integrity when compared with the two-dimensional INT-407 cultures. Furthermore, *Salmonella* demonstrated a greatly reduced ability to adhere, invade, and induce the apoptotic event in these INT-407 three-dimensional TLAs than in two-dimensional cultures. This result is not unlike the in vivo

human response. Two-dimensional cultures were significantly damaged within several hours of contact with the bacteria; conversely, although "pot marks" could be seen on the surfaces of the three-dimensional TLAs, they remained structurally sound.

Cytokine analysis and expression postinfection of three-dimensional TLAs and two-dimensional cultures with *Salmonella* exhibited remarkable differences in expressed levels of interleukin (IL)-1α, IL-1β, IL-6, IL-1Ra, and tumor necrosis factor-α mRNAs. Additionally, noninfected three-dimensional TLAs constitutively demonstrated elevated levels of TGF-β_1 mRNA and prostaglandin E_2 compared with noninfected two-dimensional cultures of INT-407.[89]

As previously stated, traditional two-dimensional cell monolayers lack adequate fidelity to emulate the infection dynamics of in vivo microbial adhesion and invasion. The respiratory epithelium is of critical importance in protecting humans from disease. Exposed to the environment, the respiratory epithelium acts as a barrier to invading microbes present in the air, defending the host through a multilayered complex system.[90] The three major layers of the human respiratory epithelium are pseudostratified epithelial cells, a basement membrane, and underlying mesenchymal cells. Ciliated, secretory, and basal epithelial cells are connected by intercellular junctions and anchored to the basement membrane through desmosomal interactions. Together with tight junctions and the mucociliary layer, the basement membrane maintains the polarity of the epithelium and provides a physical barrier between the mesenchymal layer and the airway.[91,92] Infiltrating inflammatory and immune cells move freely between the epithelial and subepithelial compartments.

Airway epithelial cells play a vital role in host defense[90] by blocking paracellular permeability and modulating airway function through cellular interactions. Ciliated epithelial cells block invasion of countless inhaled microorganisms by transporting them away from the airways.[93] As regulators of the innate immune response, epithelial cells induce potent immunomodulatory and inflammatory mediators such as cytokines and chemokines that recruit phagocytic and inflammatory cells that remove microbes and enhance protection.[90,91,94,95]

Ideally, cell-based models should reproduce the structural organization, multicellular complexity, differentiation state, and function of the human respiratory epithelium. Immortalized human epithelial cell lines, such as BEAS-2B,[96] primary normal human bronchial epithelial cells,[97] and air–liquid interface cultures,[98] are used to study respiratory virus infections in vitro. Traditional monolayer cultures (two-dimensional) of immortalized human bronchoepithelial cells represent homogenous lineages. Although growing cells in monolayers is convenient and proliferation rates are high, such models lack the morphology and cell–cell and cell–matrix interactions characteristic of human respiratory epithelia. Thus, their state of differentiation and intracellular signaling pathways most likely differ from those of epithelial cells in vivo. Primary cell lines of human bronchoepithelial cells provide a differentiated model similar to the structure and function of epithelial cells in vivo; however, this state is short lived in vitro.[97,99] Air–liquid interface cultures of primary human bronchoepithelial cells (or submerged cultures of human adenoid epithelial cells) are grown on collagen-coated filters in wells on top of a permeable filter. These cells receive nutrients basolaterally, and their apical side is exposed to humidified air. The result is a culture of well-differentiated heterogeneous (ciliated, secretory, basal) epithelial cells essentially identical to airway epithelium in situ.[98,100] Although this model shows fidelity to the human respiratory epithelium in structure and function, maintenance of consistent cultures is not only difficult and time consuming but also limited to small-scale production and thus limits industrial research capability.

True cellular differentiation involves sustained complex cellular interactions[101–103] in which cell membrane junctions, extracellular matrices (e.g., basement membrane and ground substances), and soluble signals (endocrine, autocrine, and paracrine) play important roles.[104–107] This process is also influenced by the spatial relationships of cells to each other. Each epithelial cell has three membrane surfaces: a free apical surface, a lateral surface that connects neighboring cells, and a basal surface that interacts with mesenchymal cells.[108]

Recently viral studies by Goodwin et al.[109] and Suderman et al.[110] were conducted with rotating-well vessel–engineered TLA models of normal human lung. This model is composed of a coculture of in vitro three-dimensional human bronchoepithelial TLAs engineered using a rotating-wall vessel to mimic the characteristics of in vivo tissue and to provide a tool to study human respiratory viruses and host–pathogen cell interactions. The TLAs were bioengineered onto collagen-coated cyclodextran beads using primary human mesenchymal bronchial-tracheal cells as the foundation matrix and an adult human bronchial epithelial immortalized cell line (BEAS-2B) as the overlying component. The resulting TLAs share significant characteristics with in vivo human respiratory epithelium, including polarization, tight junctions, desmosomes, and microvilli. The presence of tissue-like differentiation markers, including villin, keratins, and specific lung epithelium markers, as well as the production of tissue mucin, further confirm these TLAs differentiated into tissues functionally similar to in vivo tissues. Increasing virus titers for human respiratory syncytial virus (*wt*RSVA2) and parainfluenza virus type 3 (*wt*PIV3 JS) and the detection of membrane-bound glycoproteins (F and G) over time confirm productive infections with

both viruses. Viral growth kinetics up to day 21 pi with *wt*RSVA2 and *wt*PIV3 JS were as follows: *wt*PIV3 JS replicated more efficiently than *wt*RSVA2 in TLAs. Peak replication was on day 7 for *wt*PIV3 JS (approximately 7 $\log_{10}$ particle forming units [pfu] per milliliter) and on day 10 for *wt*RSVA2 (approximately 6 $\log_{10}$ pfu/mL). Viral proliferation remained high through day 21 when the experiments were terminated. Viral titers for severe acute respiratory syndrome–coronavirus were approximately 2 $\log_{10}$ pfu/mL at 2 day pi.

Conclusion

Human lung TLAs mimic aspects of the human respiratory epithelium well and provide a unique opportunity to study the host–pathogen interaction of respiratory viruses and their primary human target tissue independent of the host's immune system, as there can be no secondary response without the necessary immune cells. These rotating-wall vessel–engineered tissues represent a valuable tool in the quest to develop models that allow analysis and investigation of cancers and infectious disease in models engineered with human cells alone.

We have explored the creation of three-dimensional TLAs for normal and neoplastic studies and finally as targets for microbial infections. Perhaps Carrel and Leighton would be fascinated to know that from their early experiments in three-dimensional modeling and the contributions they made has sprung the inventive spirit to discover a truly space age method for cellular recapitulation.

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