18
Tissue Culture Models

Roger A. Vertrees, Jeffrey M. Jordan, Travis Solley, and Thomas J. Goodwin

Introduction

The use of tissue cultures as a research tool to investigate the pathophysiological bases of diseases has become essential in the current ag 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 and their role 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 physiological 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 physiology and disease. The isolation and propagation of human epithelial cells has allowed investigators to begin to characterize the interactions and reactions that occur in response to various stimuli. Moreover, the culture of other human tissue has allowed researchers to investigate pathological cascades as well as other physiological responses. Combining cultured endothelial cells and leukocytes together in vitro under laminar flow conditions has helped elucidate the critical interactions that occur in rolling and emigration of leukocytes during the inflammatory response. Cultured embryonic stem cells that had been subjected to various growth conditions have advanced our understanding of cellular differentiation and growth.

Numerous cancer cell lines have been established to investigate their responses to chemotherapy and determine their biological properties. Overall, the use of cultured human tissue has provided a windfall of information on the pathogenesis of diseases that affect the body. 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 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[2] 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.[3] 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.[4,5]

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."[6] 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 body, and through this work we have been able to increase our understanding of the pathological cascades that occur in diseases, as well as normal physiologies.

P.T. Cagle and T.C. Allen (eds.), *Basic Concepts of Molecular Pathology*, Molecular Pathology Library 2,
DOI: 10.1007/978-0-387-89626-7_18,

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 concern 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 lifespan) or to cell lines (e.g., HeLa cells). Additionally, these cultures can be either a homogeneous or a heterogeneous 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 before culture by subjecting the single-cell suspension to density-gradient centrifugation (e.g., Hypaque). Primary cells have an advantage of possessing many of the biological 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 development 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 are cells of mesenchymal origin (e.g., fibroblasts). However, advances have been made that allow the culture of homogeneous 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.[7]

Another type of primary cell culture are "primary explants." This type of culture is not subjected to a disaggregation procedure as is 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.[8] 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.[9]

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 lifespans 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.[10] 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.[11]

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 biological 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 are 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 discussed the advantages that experimental systems using tissue culture afford 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 pathological cascades that occur in human disease.

Many diseases that affect humans 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 histological 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.[12]

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.[12]

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, and oxidant-induced cellular damage. Deciphering each of these changes in an animal model would be extremely difficult; by 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 physiology and disease are discussed later.

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 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.[8] The most common medium formulations used currently 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 h 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.[13]

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.

Cell Adhesion

Nearly all normal or neoplastic human epithelial cells will attach with relative ease to tissue culture surfaces. 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, before culture has been shown to improve attachment of finicky cells.[9] 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.[12,14,15] 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.[12]

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 striven to replicate integral in vivo environments in vitro 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 various diseases.

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- or 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.[16]

Epithelial Cell Culture Models

Epithelial cells provide many important and equally diverse physiological roles in vivo. Toward the end of replicating these roles in vitro, investigators have collected numerous epithelial cells, both primary and immortalized, to help advance our understanding of the physiological significance.

Gastrointestinal Epithelial Cell Cultures

Results from a three-dimensional organotypical tissue culture experiment from adult murine colon performed by Bareiss et al[17] showed that this model could be useful in studying epithelial cell–cell interactions, cellular signaling, and microbiological infections. Primary cell cultures of bovine colon epithelium can further be used in toxicological studies in vitro as a model for the colon epithelium.[18]

Hepatocyte Tissue Cultures

Hepatocytes play an integral role in the metabolism of numerous drugs, nutrients, and other constituents in vivo. In vitro culture of hepatocytes has facilitated extending our understanding of the physiological steps important in mediating these physiological functions. Additionally, in vitro culture has allowed investigators to understand the importance of viral infection, drug interactions, and other physiological signals that alter metabolism and normal cellular function.

Hepatocytes in Drug Metabolism

Studies performed by Nussler et al[19] concluded that the use of a collagen sandwich or three-dimensional membrane bioreactor can be used to study drug metabolism of human hepatocytes. Additionally, to study drug metabolism in human hepatocyte cells, Zeilinger et al[20] developed a three-dimensional bioreactor culture model of liver cells under continuous medium perfusion with decentralized mass exchange of integral oxygenation.

Hepatocellular Carcinoma

Zen et al,[21] using tissue culture methods, discovered support for the maintenance of the cancer cell hierarchy in human hepatocellular carcinoma. Utilizing a radial flow bioreactor system, Kosuge et al[22] prompted differentiation of human hepatocellular carcinoma cells in three-dimensional culture. The resulting differentiated cells could be useful for improvements in genetic or pharmacological reinforcement and for the monitoring of bioartificial livers.[22]

Nervous System Tissue Cultures

To potentially use reconstructed functional cortical-like tissues for drug screening, in detection of environmental toxins, and in neural cell therapy, Ma et al[23] cultured neural stem and progenitor cells on three-dimensional type I collagen gels and then transferred the cell-collagen constructs to three-dimensional bioreactors. Additionally, using three-dimensional collagen gel cultures, Ma et al[24] succeeded in using neural stem and progenitor cells to recapitulate the central nervous system stem cell development demonstrating a functional synapse and neuronal network formation in a three-dimensional matrix. A tissue-like primary cortical cell culture using Matrigel and serum free Start V medium was developed by Braun et al[25] to be used in the investigation of basic cell-cell interactions in vitro.

Lymphatic Tissue Culture

The complexity of the lymphatic system has made the engineering of functional tissues difficult, but progress has been made in the engineering of lymphatic capillaries and the development of a bioreactor designed to culture lymph nodes.[26]

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 hypertension, 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.[27]

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 which include synthesis and secretion of surfactant, a unique composition of lipoproteins that act to reduce surface tension at the alveolar air–liquid interface.[28] 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.[29] Despite this knowledge, there still exist many questions about the initial triggers and pathological 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.[30]

The cell type of particular interest in ARDS and diffuse alveolar damage is the type II pneumocyte.[31–35] Until recently, studies trying to decipher the pathological 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.[36] 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 Weymouth'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[36] were able to maintain a primary culture that retained the morphological 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.[2,3]

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.[37,38] Leighton first experimented on cellulose sponges surrounded by plasma clots resident within glass tubes. He devised a system to grow tissue explants 1–5 mm^2 in area 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.[4,39]

Leighton was successful in growing many different tissue types on the sponge-matrix cultures.[4,39] 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 histophysiological gradient cultures.[40] These cultures are conducted in chambers that allow metabolic exchange between "the pool of medium and the culture chamber by diffusion across a membrane." Histophysiological gradient cultures mimic, to some degree, diffusion in tissues.[39]

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. Clusters of mammary cells were initiated into cultures, and Nandi and colleagues observed three-dimensional formation of ductile structures and sustained growth over a period of several weeks concordant with proliferation at the edge of the cell mass with minimal fibroblast contamination.[41] Thus, mammary tissue cultured in the collagen gels resembled, histologically, in vivo tissue, differing significantly from two-dimensional monolayer cultures. Nandi et al suggested an important criterion for successful growth of mammary tissue-like structures in collagen gels, versus the

monolayer culture, was maintenance of mammary cell shape in three dimensions as opposed to conventional monolayer culture. 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.[4] 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[42] and Fell and Robison.[43] Tissue explants were grown on lens paper in a watch glass containing liquid culture medium. Browning and Trier[44] 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.[45] 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.[4,39]

Bioreactors and three-dimensional systems have been and are used by medical researchers and by pharmaceutical production plants to create mimics of human, mammalian, microbial, and plant physiology. As reviewed, many approaches have been employed to create these synthetic physiologies; however, none of these has been entirely satisfactory for broad-based mammalian cell culture use.

The impact of molecular genetic engineering in the early 1980s was for the most part confined to pharmaceutical proteins such as interferon, growth hormones, and insulin molecules. During processing of these valuable, but low-yield, products, emphasis was placed on downstream recovery rather than on bioconversion or bioprocesses, let alone three-dimensional tissue development. Advances in modern biotechnological product recovery are expanding to medium- and high-volume enzyme products and limited forms of bulk products. In the world of high-volume, high-value products, cell culture bioreactor productivity contributes an important role in determining the economic influence of bioprocessing. Ideal bioprocessing bioreactors will in future have high reactor productivity with an in situ separation capability of products.[46]

Membrane bioreactors fit this description and already contribute remarkably to the bioprocessing industry. 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[47] initially used the membrane bioreactor for dialysis culture of *Serratia marcescens*. Immobilized enzyme microencapsulation was pioneered by Chang,[48] but Butterworth et al[49] first developed the enzyme membrane reactor to successfully accomplish starch hydrolysis with α-amylase. Similarly, for animal cell culturing, Knazek et al[50] 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.[46,51–55]

Prolonged human residence in microgravity results in the atrophy of healthy skeletal muscle. Continued space exploration requires a better understanding of this phenomenon.[56] Muscle wasting is likely caused by changes at the system level marked by increased circulating glucocorticoids and decreased circulating growth hormone and at local levels as seen by decreased myofiber resting tensions. Differentiated skeletal muscle cultures have served as model systems for gaining a better understanding of the roles of exogenous and endogenous cytokines and of muscle fiber tension in regulating muscle cell growth. This effort has led to the beginning of tissue engineering studies by the space science community. Engineering these tissues into three-dimensional bioartificial muscle (BAM) constructs has extended their utility for space flight investigations as a prelude to developing countermeasures for the microgravity environment. Serendipitously, these bioreactor models in part mimic diseases of the musculature such as dystrophy.[57]

BAMs have been sustained in a viable condition for as much as a month in perfused hollow-fiber bioreactor cartridges.[58] Further, investigators have documented[57] that growth hormone and/or insulin-like growth factors are potential protein therapeutics, which may in part attenuate skeletal muscle degeneration in space as well as show promise for the treatment of muscle-wasting diseases of the terrestrial community.[57]

An associated application of space bioscience to an Earth-based disease is the investigation of bone degeneration. The microgravity effect on bone decalcification has been documented beginning with early space flights and subsequently found to mimic conditions analogous to osteoporosis frequently seen in aging females. Over the past three decades, the relationship between cellular morphology and metabolism and the role of physical stress in bone loss has been studied. Physiomechanical stresses induce shape changes in osteoblasts, possibly mediated by reorganization of focal contacts. Thus, gravity variations (Gz) have been studied to understand their influence on osteoblast adhesion of ROS 17/2.8 rat osteosarcoma cells during 15–30 parabolic flights. Nontrivial flight-induced cell shape changes consisting of decreased area concordant with contact plaque reordering have been observed; however identical periods of continuous mechanical stress induced by centrifugation (2 Gz) or clinostatic rotation (Gz randomization) had no discernible effect on cell adhesion. Synchronization of ROS 17/2.8 G2/M accomplished via treatment in nocodazole inhibited

the flight-induced decreases in adhesion parameters. Investigators thus concluded that ROS 17/2.8 cells possess the ability to sense Gz triggers and that adaptation is related to cytoskeletal function.[59]

As presented previously, tissue engineering applications of three-dimensional function and structure are well known in medical science research.[60] 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.[60]

Briefly, empty and populated matrices cultured for 28 days with and without transforming growth factor (TGF)-β1 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-β1. These results demonstrate the utility of a 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.[60] These techniques lend themselves to microgravity and ground-based research tissue cultures alike.

Many earth-based laboratories are researching and developing hematopoietic bone marrow cultures of stem cell origin, and three-dimensional configurations are providing promising results, as illustrated by Schoeters and coworkers.[61] They report that murine bone marrow cells, cultured under long-term hematopoietic 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.[61]

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.[61] 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 pathophysiological studies. Interestingly, three-dimensional biology has facilitated full-scale investigations into most areas of tissue engineering, cell biology and physiology, immunology, and cancer research.

Several important factors are necessary when considering the successful recapitulation of a three-dimensional mammalian tissue. These factors include specific elements of differentiation which, when replicated accurately, result in the functionality of the original tissue being mimicked. Figure 18.1 graphically represents the stages of "assembly" and differentiation needed to result in a functionally accurate tissue. These stages are three-dimensional assembly, three-dimensional growth, cellular matrix formation [extracellular matrix (ECM) and basement membranes], differentiation (associated with cellular specialization), and vascular formation (or pseudo-vasculature).

To accomplish the successful recapitulation of an ex vivo tissue, a series of defined steps are necessary. The creation of a three-dimensional structure is accomplished by systematic tissue engineering of the desired tissue architectures. Figure 18.2 demonstrates the assembly process and illustrates some of the variables that may be employed in the engineering process.

The assembly of complex functional mammalian tissues in 1× gravity is problematic because of the effects of shear stress, turbulence, and inadequate oxygenation in conventional cell culture systems. This study describes the culture and three-dimensional assembly of baby hamster kidney (BHK) mammalian cells on microcarriers under controlled oxygenation, low shear stress, and turbulence in the NASA-designed integrated rotating-wall vessel (RWV).[62–64] Anchorage-dependent cells are widely cultured on microcarriers.[65] 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.[66] In addition; microcarriers serve well as structural supports for three-dimensional assembly, the composite of which is the basis for three-dimensional tissue growth.[67]

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.[68] 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.[69] Aggregates of as many as 12–15 microcarriers

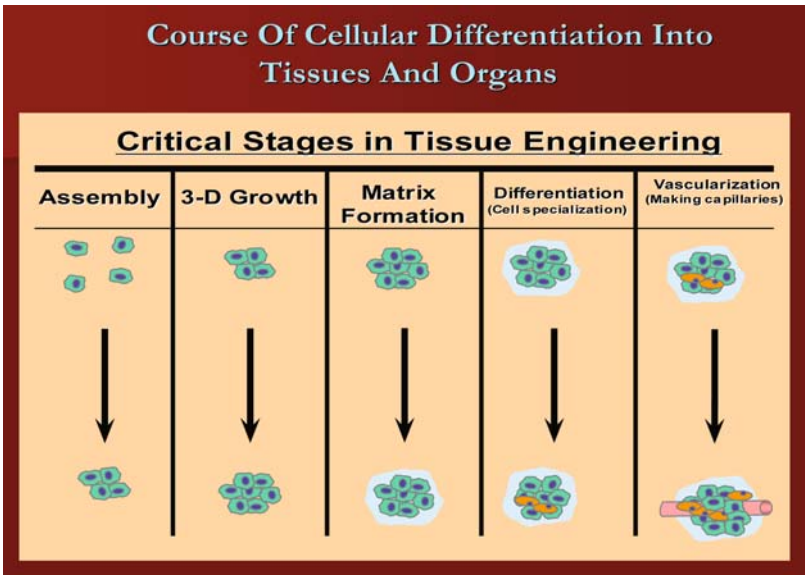

FIG.18.1. Stages of cellular differentiation requisite for tissue functionality.

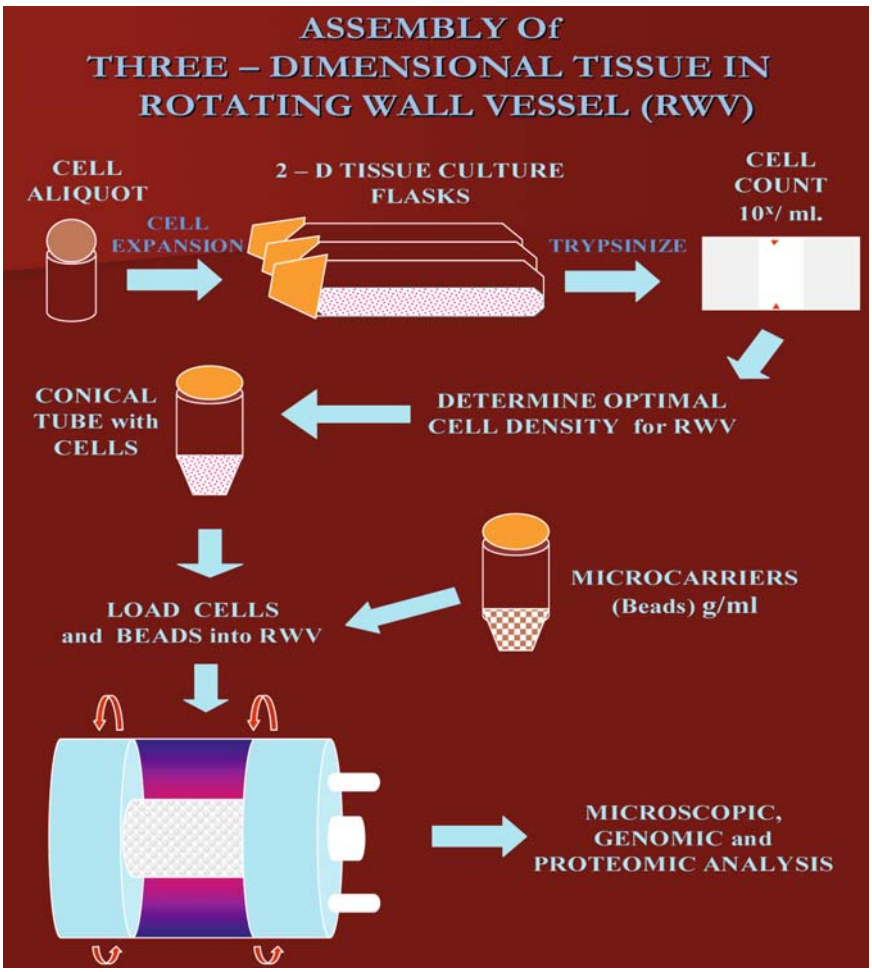

FIG. 18.2. Development and tissue assembly process for engineering of a three-dimensional tissue.

bridged with bovine embryonic kidney cells (BEK) have been reported. Excessive agitation from either stirring or gas bubble sparging has been documented as a cause of cell damage in microcarrier cell cultures.[70,71] To overcome the problems induced by these mechanisms, investigators developed alternative culture techniques such as porous microcarriers to entrap cells,[72] increased viscosity of culture medium,[73] bubble-free oxygenation,[74] and improved methods for quiescent inoculation.[75,76] 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, 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. Microcarriers and cells in large-volume reactors are semibuoyant, rendering them practically weightless, a condition that we hypothesize may be analogous to the environment of microgravity. It is stated that "Unless a cell culture is growing in an environment free of gravitational forces, moderate levels of agitation are required to suspend microcarriers that are not neutrally buoyant."[73] The ability to grow cells in a rudimentary horizontally rotated chamber has been previously demonstrated and is intended to simulate in the laboratory the effects of weightlessness or microgravity on cells. It was shown that human embryonic kidney (HEK) cells attach to microcarriers in microgravity (Space Shuttle experiment) and that attachment and spreading may be enhanced in a weightless environment.[77] Review articles of cell biology performed in space flight experiments delineate the growth of many cell types in an environment devoid of gravitational influence.[78] The problem, then, is to suspend microcarriers and cells without inducing turbulence or shear while providing adequate oxygenation and nutritional replenishment. One environment that possesses these attributes is the microgravity of space flight. Another is the system referenced here, which randomizes the forces of gravity by classical methods, thus simulating some aspects of microgravity.

The term *rotating-wall vessel* (RWV) 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) (Fig. 18.3).[79,80]

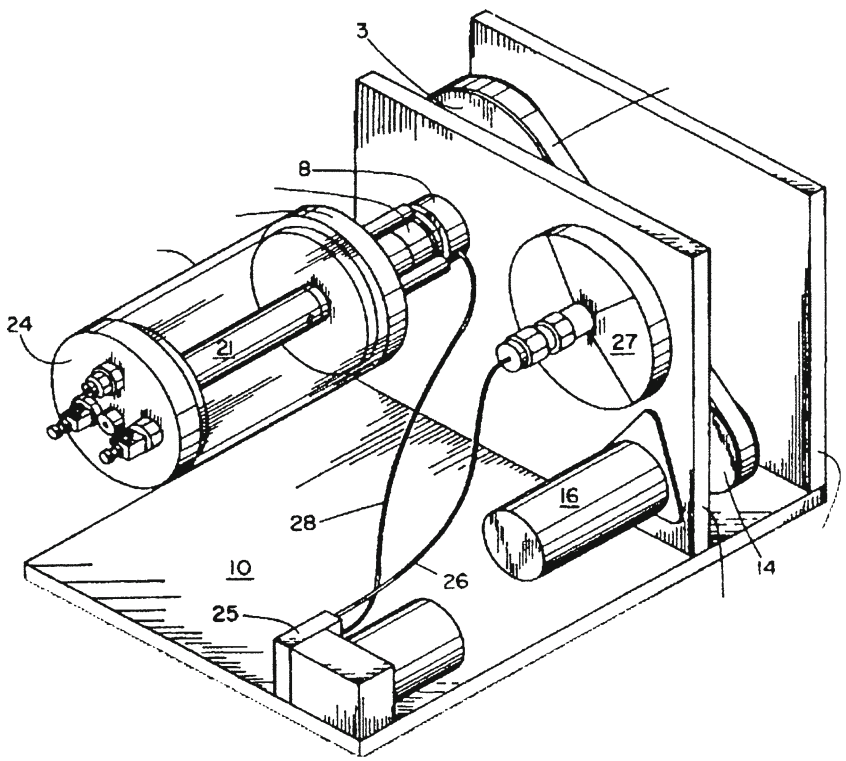

FIG. 18.3. NASA Rotating-Wall Bioreactor. United States Patent: Schwarz et al.[64] (Courtesy of NASA).

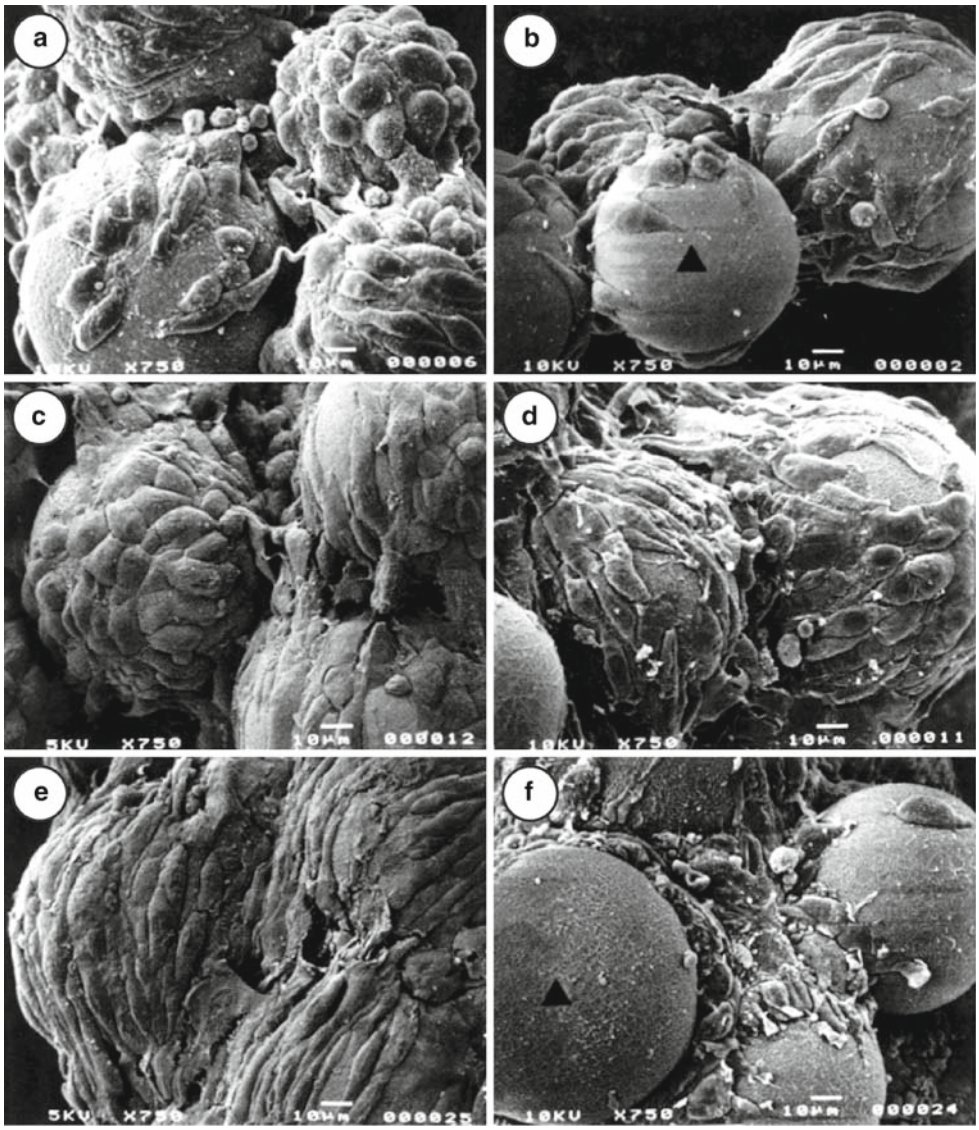

FIG. 18.4. Three-dimensional baby hamster kidney (BHK) tissues created in rotating-wall vessel (RWV) culture. (From Goodwin TJ, et al.[62] Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc).

The baby hamster kidney (BHK) model (Fig. 18.4) described and illustrated here has been used extensively as a means of transfecting viral and bacterial DNA into cells to accomplish gene splicing protocols. These three-dimensional models are proving extremely valuable in viral, microbial, and genetic research.

Normal Tissue Models

As reviewed in several of the previous sections, investigators have cultured bone, cartilage (chondrocytes), muscle tissue, cells of the immune system, and others. Baker and Goodwin in 1997 employed the RWV to culture chondrocytes (Fig. 18.5) for 36 days to observe the influence of low-shear and quiescent culture conditions on the development of three-dimensional differentiation and extracellular matrix formation in bovine chondrocytes.

Chondrocytes from bovine cartilage were inoculated into the RWV with 5 mg/ml Cytodex-3 microcarriers in nonadherent petri dishes with identical conditions and were initiated with microcarriers as standard tissue culture controls. Differentiated chondrocytes were observed in all sections of RWV material through 36 days, while few were observed in the sections of petri dish material."[81] These results indicate that the unique conditions provided by the RWV afford access to cellular processes that signify the initiation of differentiation as well as production of normal matrix material. These experiments demonstrated the ability to create a synthetic model of cartilage or bone without leaving Earth's orbit.

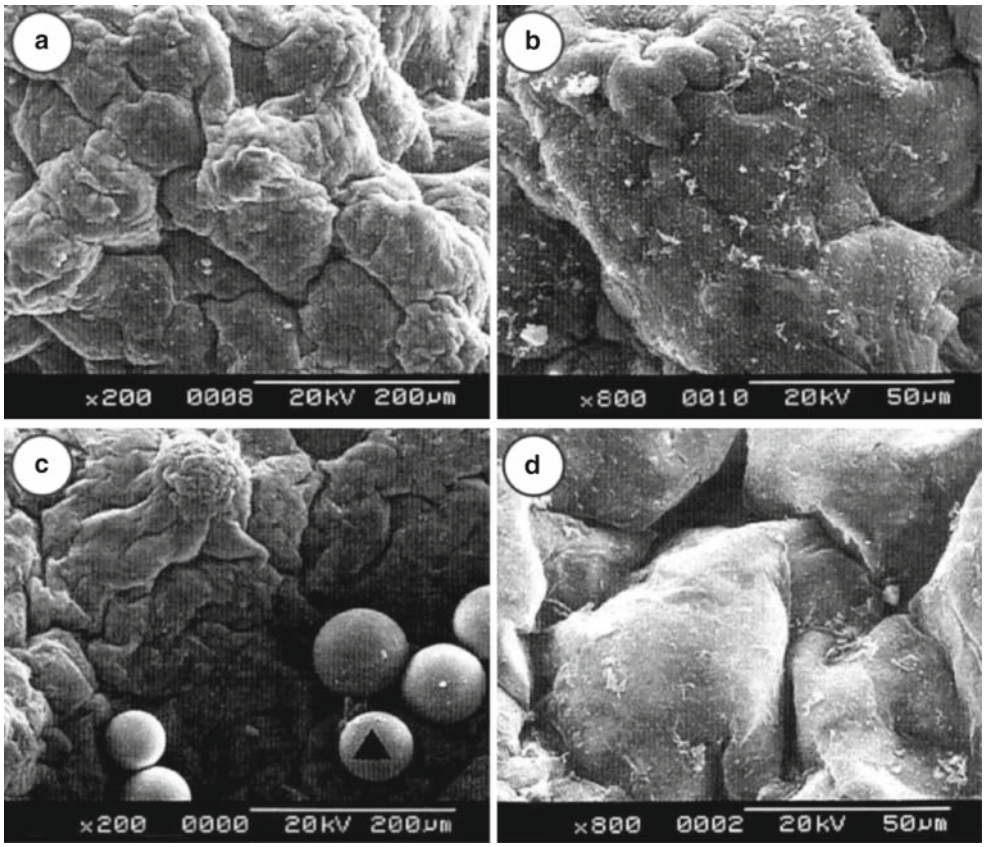

FIG. 18.5. Chondrocytes. (From Baker TL, Goodwin TJ.[81] Reprinted by permission of Springer Berlin/Heidelberg).

Figure 18.6 illustrates a 34-day time course of three-dimensional culture of normal human chondrocytes (HCH). Increased Actin (alexa 555 phalloidin; red) and nuclear staining (syto16; green) is observed over the time course, resulting in yellowish-red staining by day 34. Goodwin et al[63] describe the results of experiments where normal human small intestine epithelium and mesenchyma were cultured in rotating-wall vessel cultures. They found that the rotating-wall vessels are a major advance toward constructing a functionally accurate, large-scale, in vitro, three-dimensional (3-D) tissue model of the small intestine. Because of the presence of large tissue-like masses that express differentiated epithelial, mesenchymal, and developing endothelial cells, a multitude of possibilities are afforded for cell biological investigations, and further, functional epithelial cell brush borders with extracellular matrix and basal lamina components that represent ordering of tissue and cellular polarity were nurtured by the molecular conditions and physical orientations of the culture system. An important additional finding was that this 3-D model demonstrates a significantly diminished requirement of complex culture media, which suggests specific cell-cell interactions and the production of paracrine and autocrine factors essential to the growth and development of these fragile tissues.[63]

The role of basement membranes and extracellular matrix and their relationship to epithelial mesenchymal development and differentiation are the subjects of considerable research. Current studies indicate that the stromal component exerts a strong and driving influence over developing intestinal mucosa.[82–84] Stallmach et al[85] have shown that only organ-specific mesenchyme will produce differentiation in epithelium from a given organ site and that embryonic mesenchyme of the same age but from different organs was ineffective. Studies of rat small intestine development have detailed a shift in the membrane molecular components of crypt cells as measured by monoclonal antibody binding in fetal versus newborn rat epithelium. Quaroni[86] postulates that the presence of specific markers in the crypts indicates undifferentiated crypt cells, which may be able to perform specific intestinal functions similar to that of the villus cells. Additional information shows that a single fetal epithelial cell type in the last 2–3 days of gestation may express the function of more than one cell, thereby giving rise to DNA synthesis and proliferation.[87]

Cocultures of small intestine produced in the RWV were initiated with adult epithelium composed of dividing and terminally differentiated cells and predominantly adult mesenchymal cells with one exception, a 2-month-old female

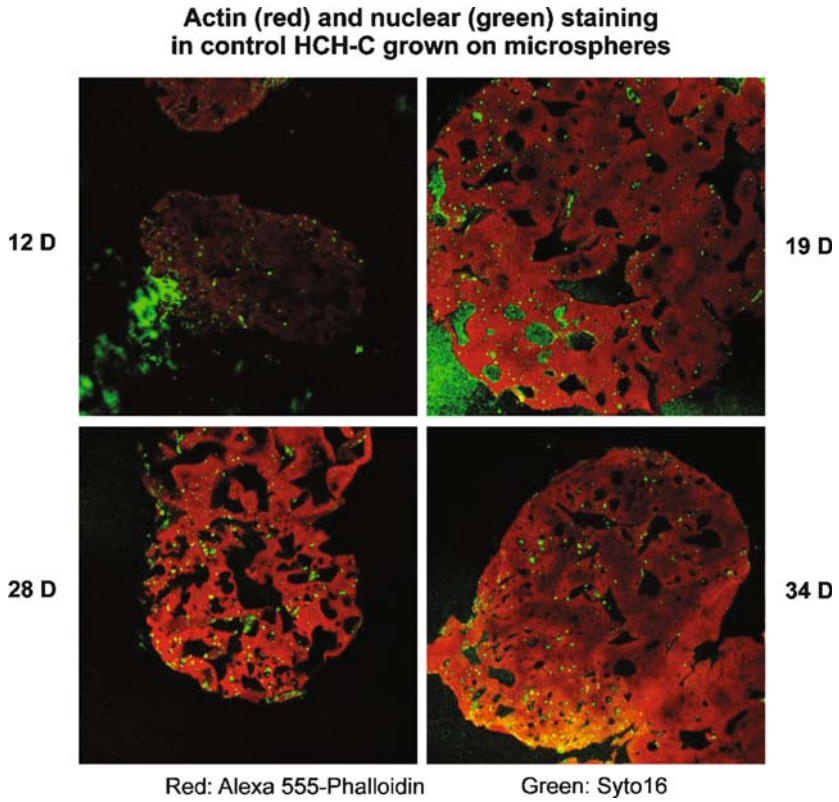

FIG. 18.6. Human articular chondrocytes stained for actin-binding protein (*red*) and nuclear protein (*green*).

donor. Levels of epithelial cell (keratin-positive) and mesenchymal cell-specific (vimentin-positive) immunostaining decreased slightly but consistently over the course of the RWV experiments, while total numbers of cells increased. Levels of endothelial cell (Factor VIII-positive) and epithelial cell (villin-positive) staining remained reasonably constant, while staining for angioblasts (endothelial precursor cells) developed toward the end of each experiment. Functional brush border markers such as sucrase were also present in this coculture system. Other brush border-specific monoclonal antibodies, kindly supplied by Dr. Andrea Quaroni (Cornell University, Ithaca, NY), have similarly proved positive in more recent studies (unpublished results). Collectively, these results indicate that the model, although not histotypically correct, exhibits many facets of functional normal small intestine. Growth conditions, however, may still be skewed slightly in favor of the mesenchymal versus the epithelial cell types. Work will continue on further definition of the correct nutritional and growth-matrix requirements directed toward attaining the best representation possible. Interestingly, all the RWV cocultures exhibited laminin, fibronectin, and type IV collagen production, as well as large amounts of proteoglycan. Hahn states that only undifferentiated, highly proliferative intestinal epithelial cells (usually fetal) synthesize the aforementioned proteins.[88] Of these, only laminin appeared to promote differentiation of intestinal epithelial cells. Type IV collagen and fibronectin had no effect.

The literature reviewed and the data obtained here would suggest that this model of human small intestine embodies many aspects of differentiation observed in other in vitro and in vivo cell and organ models. Primary distinctions would be (1) the overall scale of the model, (2) the ability to culture epithelium for long periods (in excess of 40 days) without loss of functional cell markers, and (3) the ability of the system to respond to extensive analyses and manipulations without the termination of a given experiment.

The significance of a complex three-dimensional in vitro culture system for the growth of normal small intestine should not be underestimated. The molecular basis and clinical treatment of diseases such as inflammatory bowel disease (Crohn's, ulcerative colitis), malabsorptive syndromes (short-gut syndrome), numerous infectious diseases, and tumors of the small bowel may be investigated with the advent of this new technology. For example, the recent demonstration that the human immunodeficiency virus can replicate in human small intestine and columnar epithelium may be impacted through studies of virus-cell interactions in the RWV culture system.[89] This hypothesis is currently being tested. Additionally, general application of this culture model may lead to advances in understanding growth and differentiation in developing organisms and the potential treatment of a myriad of clinical conditions as well as tissue renewal (Figs. 18.7 and 18.8).

The RWV systems have been demonstrated as useful for the development of sophisticated models, which emulate facets of

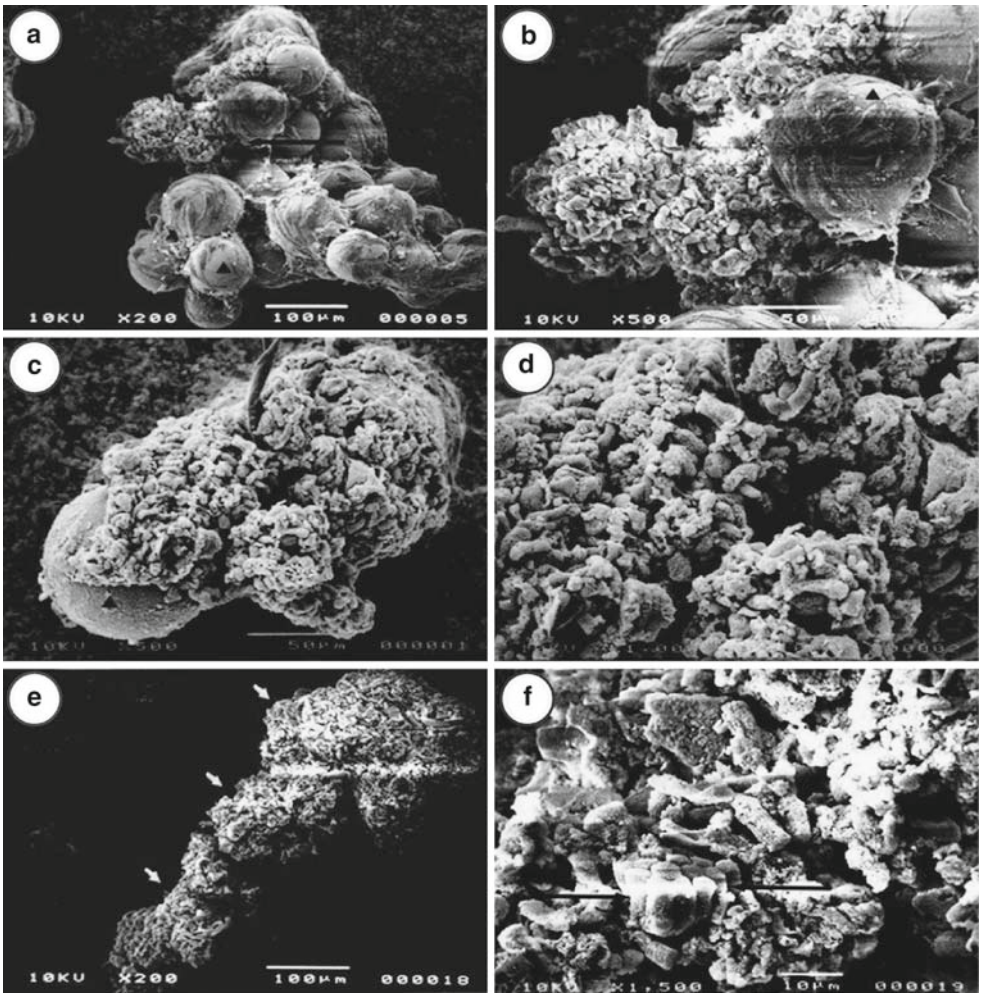

FIG.18.7. Early-stage scanning electron micrographs of human small intestine RWV cocultures. (From Goodwin et al.[63] Reprinted by permission of the Society for Experimental Biology and Medicine).

microgravity and at the same moment present characteristics of in vivo tissues. We have examined the production of normal tissue models for research and experimentation; as we will see in the next section, the RWV has the ability to serve as a tremendous tool to construct synthetic tumor tissues, which allows an in-depth understanding of tumor biology and oncology.

Three-Dimensional Models of Lung Disease

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.[90,91] This factor 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[90]. 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,[62] it is difficult to make any conclusive 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.

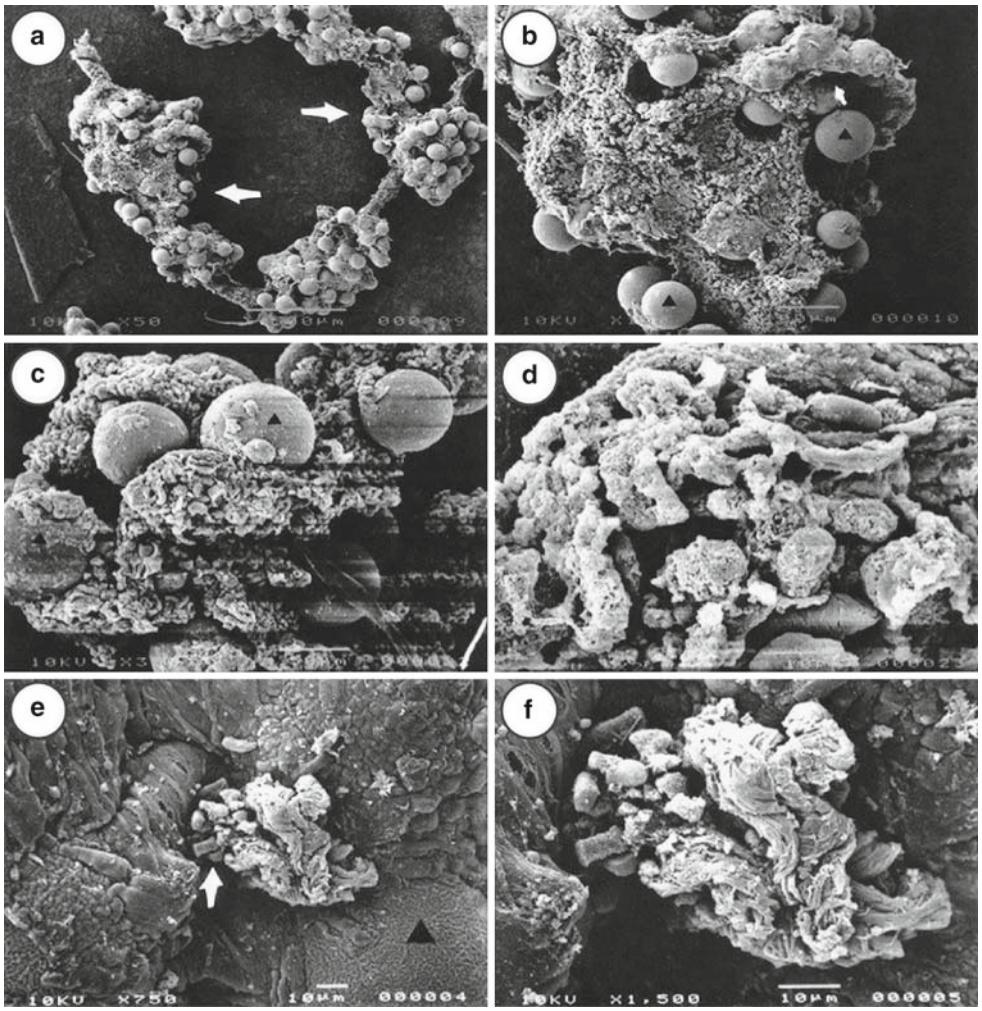

FIG.18.8. Scanning electron micrographs of mid- and late-stage human small intestine RWV cocultures. (From Goodwin et al.[63] Reprinted by permission of the Society for Experimental Biology and Medicine).

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.[79] These vessels suspend cells and microcarriers homogeneously in a nutrient-rich environment, which allows the three-dimensional assembly of cells to tissue. Before seeding rotating-wall vessels (Synthecon, Houston, TX, USA), cells were cultured in standard T flasks (Corning, Corning, NY, USA) 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, Logan, UT, USA) followed by BEAS2-B or BZR-T33 cells (ATCC, Baltimore, MD, USA) 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, USA). At 24-h 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 clinical glucose analyzer. Cell samples were harvested every 48 h and fixed for immunohistochemistry or for scanning electron microscopy.

Cancer models already developed by NASA investigators include growth and differentiation of an ovarian tumor cell line,[62,92,93] growth of colon carcinoma lines,[62] and three-dimensional aggregate and microvillus formation in a human bladder carcinoma cell line.[93] 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.[94]

Many investigations sustain consistent evidence that cells growing in three-dimensional arrays appear more resistant

to cytotoxic chemoagents than cells in monolayer culture.[39] Li et al[95] found that spheroids were more resistant to cytosine arabinoside by 11-fold and methotrexate by 125-fold when compared with single-cell suspensions. 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.[96] Numerous other investigations have revealed increased levels of drug resistance of spheroids compared with single-cell monolayers.[4,39]

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 which 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 culture, 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.[39,97–100]

Based on the caliber of data referenced above, Teicher et al[101] serially passaged through multiple (ten) 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[102] 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-pharmacological doses of drugs by forming compact structures.[39]

Rotating-Wall Vessel Tumor Models

Several important human tumor models have been created in rotating-wall vessel cultures, specifically, lung, prostate, colon, breast carcinoma, and ovarian.[15,67,92,103] 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.

Colon Cancer

Goodwin et al[67] detailed the first construction of a complex three-dimensional ex vivo tumor in rotating-wall vessel (RWV) 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, were grown in RWV culture. RWVs were used in conjunction with multicellular cocultivation to develop a unique in vitro tissue modeling system. Cells were cultivated on Cytodex-3 microcarrier beads, with and without mixed normal human colonic fibroblasts, which served as the mesenchymal layer. Culture of the tumor lines in the absence of fibroblasts produced spheroid-like growth and minimal differentiation. In contrast, when tumor lines were cocultivated with normal colonic fibroblasts, initial growth was confined to the fibroblast population until the microcarriers were covered. The tumor cells then commenced proliferation at an accelerated rate, organizing themselves into three-dimensional tissue masses that achieved 1.0- to 1.5-cm diameters. 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 RWV system by several investigators.[104–106] One of the most comprehensive descriptions of these engineered tissues is detailed by Wang et al[107] In that review, the authors describe the ability of the RWV 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 RWV system to allow synergistic relationships to study this disease state.[107]

The final two carcinoma models to be reviewed here are the subject of original ongoing research, breast carcinoma in Dr. Goodwin's lab and lung carcinoma in Dr. Vertrees' lab. As we have seen with the previous data, the modeling of three-dimensional tissues is not only achievable, but also desirable to create an improvement in the fidelity of the tumor model for research and diagnostic purposes. Construction of three-dimensional tumor models using RWVs has revealed striking similarities to tissues grown in vivo.[63,67]

As a demonstration of the capability of these advanced tumor models to produce unique results, we have developed three-dimensional breast carcinoma models and subjected them to assault by lymphokine-activated killer (LAK) cells. Tumor-infiltrating lymphocyte (TIL) and lymphokine-activated killer (LAK) cell research has primarily dealt with tumor cells in single suspension cultured in the presence of LAK cells in close proximity using 96-well plates. Although a substantial amount of literature exists in this field, no literature could be found investigating three-dimensional tumor models and the kill response elicited in the presence of LAK cells. We hypothesized that not only would the three-dimensional models more closely resemble in vivo tissue histologically, but they also could demonstrate a useful application of using three-dimensional models. Three breast carcinoma cell lines were chosen for this investigation: BT-20, an undifferentiated estrogen receptor (ER)– cell line; MCF-7, a moderately differentiated and metastatic ER+ cell line; and T-47D, an ER+ cell line. Each cell line was cultured in the RWV for a minimum of 21 days. Mononuclear cells were isolated from peripheral blood lymphocytes and placed in T-flasks at a cell density of 1.5×10^6 cells/ml in the presence of rhIL-2. A modified procedure based on the standard ^{51}Cr release assay was developed to quantitate the activity of LAK cell invasion of three-dimensional (3-D) RWV-grown tumor aggregates. Cells from T-flasks (2-D controls) or RWV-grown aggregates (3-D) were labeled with ^{51}Cr and seeded into 12-well plates with LAK cells at a 10:1 ratio to the tumor cells. We chose the lowest ratio of effector cells to target cells compared to standard suspension cell protocols of using 10:1, 100:1, or 1,000:1. Spontaneous release controls were initiated identically without LAK cells. Supernatant samples were taken at 24-h and 48-h intervals and counted using a gamma counter. Maximum release data were obtained by lysing the entire cell population using Triton X-100. Cytokine analysis of the supernatant and flow cytometry analysis of the cytokine receptors were performed from samples taken at the same time points from a duplicate nonradiolabeled assay.

Comparative data accumulated on the two estrogen receptor (ER)-positive and one ER-negative cell lines indicated that, in contrast to generally accepted data obtained from standard two-dimensional LAK assay, three-dimensional tumor aggregates present significantly different kill rates for all the breast carcinoma lines than do their two-dimensional analogues. Additionally, these altered kill ratios are associated with dramatic response changes in both the receptor and soluble molecular expression for the cytokines interleukin (IL)-8, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, and epidermal growth factor (EGF).

In Fig. 18.9, a well-developed histology section of MCF-7 is seen with organizational structure before LAK invasion. The scanning electron microscopy (SEM) panel in Fig. 18.10 demonstrates the adherence and invasion of LAK cells at 24 and 48 h post inoculation onto a mature, three-dimensional culture of breast carcinoma. Concomitantly, at 48 h LAK cells are seen digesting the surrounding tumor cells (Fig. 18.10).

Vertrees et al[108] reported on a study that compared various characteristics between an immortalized (not malignant) and its malignant transfected counterpart grown both as 2-D and 3-D cultures (Figs. 18.11 and 18.12). Electron microscopy identified significant mitochondrial and granular endoplasmic reticular pathology in the 2-D cells not seen in the 3-D cells.[108] The degree of differentiation determined by immunohistochemistry shows that ultrastructure and antibody expressions were more representative of control tissue when cells (both immortalized and transfected) were grown in 3-D culture than when grown as a 2-D culture. Electron microscopy identified the presence of lipid inclusion and lamellar (surfactant) bodies indicative of type II pneumocytes, a differentiation not seen in monolayer cultures. Fluorescence microscopy allowed for determination of individual migrating cells (unpublished data). The coculture experiment (simultaneous cultures of normal and transformed lung cells) in the three-dimensional environment demonstrates the ability of the transformed cell to migrate through the preexisting normal cells.

In contrast to 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 tissue-like aggregates (TLA)s as targets for microbial infection and disease. Several studies have been conducted

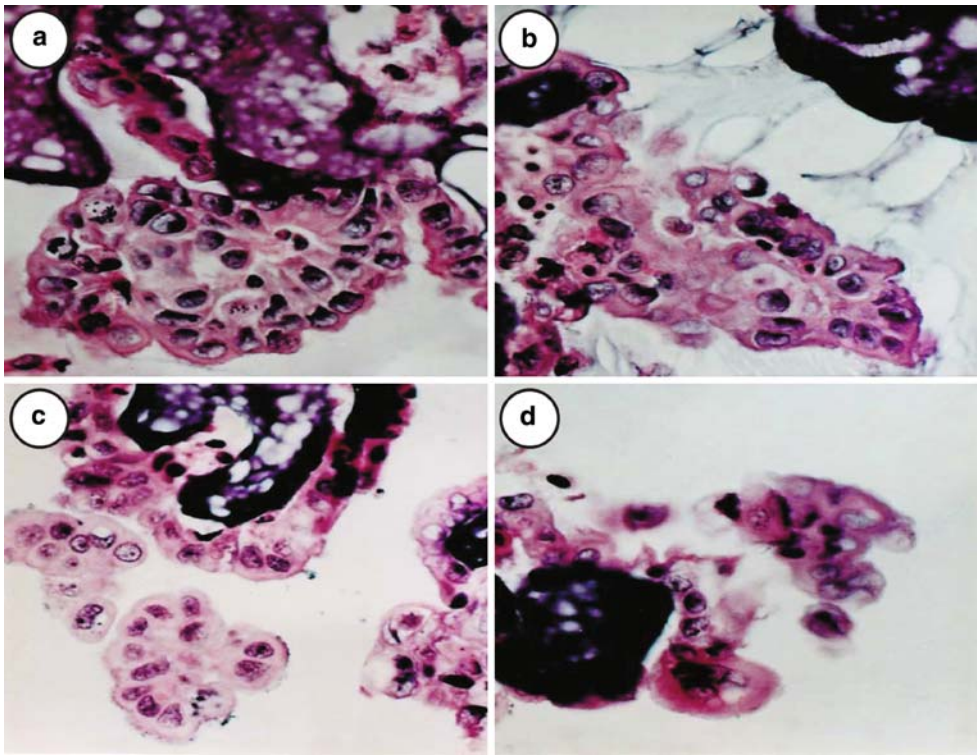

FIG. 18.9. Hematoxylin and eosin (H&E)-stained sections (×400) demonstrating the superior organization of the RWV-grown cells at days 14 and 21 (**a** and **c**) versus the static control cultures at days 14 and 21 (**b** and **d**). (From Goodwin, 2001: original work; supported by NASA grant #962-23-0132).

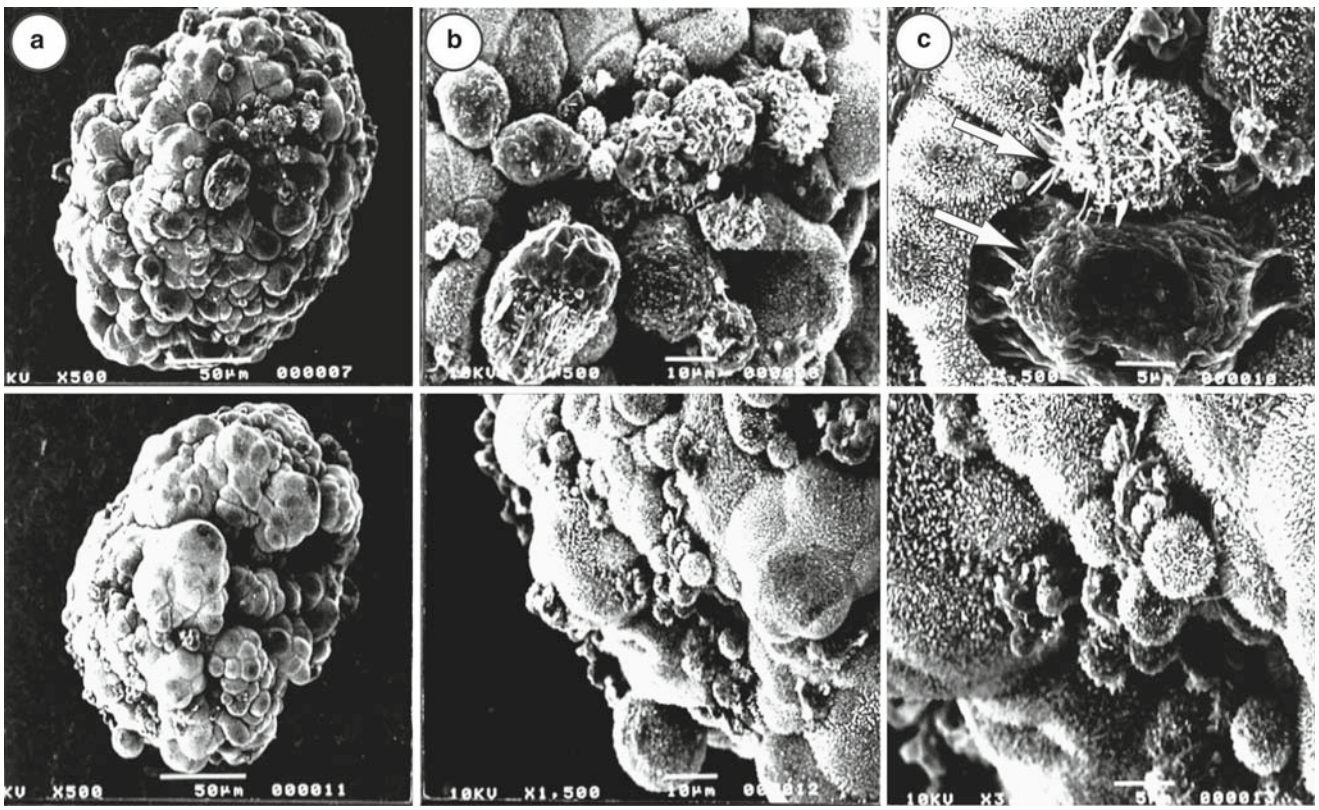

FIG.18.10. Scanning electron microscopy at 48 h post-LAK invasion. Notice the increased invasion potential of the LAK cells exposed to the RWV-grown cells (**a**) as compared to the static control cultures (**b**). (From Goodwin et al., 2001: original work; supported by NASA grant #962-23-0132).

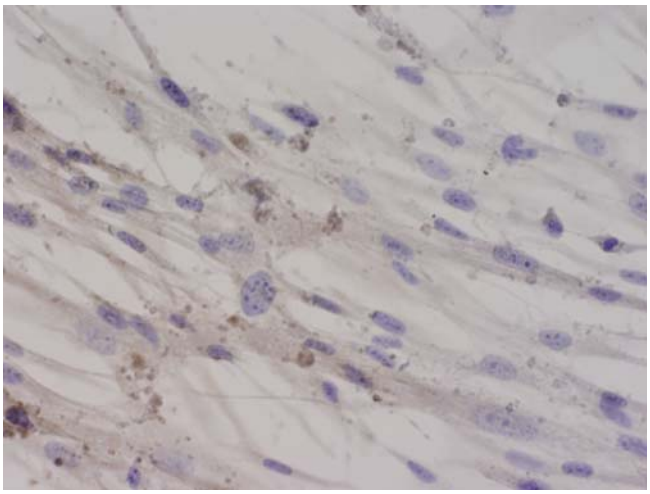

FIG. 18.11. Micrograph of a human lung cancer grown in two-dimensional (2-D) cultures. Note the lack of any architecture resembling that of lung.

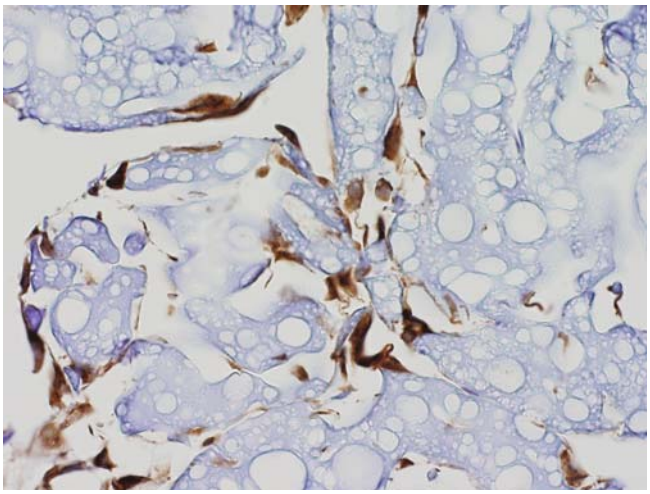

FIG. 18.12. Micrograph of a human lung cancer in three dimensions (3-D). Note the overall spherical geometry and lung architecture

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[109] 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 modeled human in vivo differentiated tissues more accurately 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[62] 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-Ra, and TNF-α mRNAs. Additionally, noninfected three-dimensional TLAs constitutively demonstrated elevated levels of TGF-β1 mRNA and prostaglandin E_2 compared with noninfected two-dimensional cultures of INT-407.[109]

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.[110] The three major layers of the human respiratory epithelium are pseudo-stratified 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.[111,112] Infiltrating inflammatory and immune cells move freely between the epithelial and subepithelial compartments.

Airway epithelial cells play a vital role in host defense[110] 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.[113] 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.[110,112,114,115]

Ideally, cell-based models should reproduce the structural organization, multicellular complexity, differentiation state, and function of the human respiratory epithelium.

Immor-talized human epithelial cell lines, such as BEAS-2B,[116] primary normal human bronchial epithelial cells,[117] and air-liquid interface cultures,[118] are used to study respiratory virus infections in vitro. Traditional monolayer cultures (two-dimensional) of immortalized human bronchoepithelial cells represent homogeneous 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.[117,119] 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.[118,120] 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[121–123] in which cell membrane junctions, extracellular matrices (e.g., basement membrane and ground substances), and soluble signals (endocrine, autocrine, and paracrine) play important roles.[124–127] 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 which interacts with mesenchymal cells.[128]

Viral studies by Goodwin et al[129] and Suderman et al[130] were conducted with RWV-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 wtRSVA2 (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 days post inoculation.

Conclusion

Although great strides have been made in recent years in the understanding of the biology of cancer, the role that cellular differentiation and three-dimensional structural organization play in metastasis and malignancy is still unclear. A model that more closely links the expression of specific antibodies in normal lung cells, transformed lung cells, and cancerous lung cells to cellular biology may provide new information about cancer. The development of three-dimensional cell cultures in bioreactors may ultimately provide a model that facilitates discovery and interpretation of more relevant information for the expression of an antibody and its role in cellular pathobiology of the lung.

Human lung tissue-like aggregations mimic aspects of the human respiratory epithelium wall and provide a unique opportunity to study the host-pathogen interaction of respiratory viruses and their primary human target tissue independent of the host 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 tissue-like aggregates 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.

References

1. Clements JA, King RJ. Composition of surface active material. In: Crystal RG, ed. *The Biochemical Basis of Pulmonary Function*. New York: Marcel Dekker; 1976:363–387.
2. Carrel A. On the permanent life of tissues outside the organism. *J Exp Med.* 1912;15:516–528.
3. Huber HL, Koessler KK. The pathology of bronchial asthma. *Arch Intern Med.* 1922;30:689–760.

4. Hoffman RM. Three-dimensional histoculture: origins and applications in cancer research. *Cancer Cells.* 1991;3:86–92.
5. Sherwin RP, Richters A, Yellin AE, Donovan AJ. Histoculture of human breast cancers. *J Surg Oncol.* 1980;13:9–20.
6. Scherer WF, Syverton JT, Gey GO. Studies on the propagation in vitro of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. *J Exp Med.* 1953;97:695–710.
7. Look DC, Walter MJ, Williamson MR, et al. Effects of paramyxoviral infection on airway epithelial cell Foxj1 expression, ciliogenesis, and mucociliary function. *Am J Pathol.* 2001;159:2055–2069.
8. Freshney RI. *Culture of Animal Cells: A Manual of Basic Technique*. 3rd ed. New York: Wiley-Liss; 1994.
9. Tsao M, Viallet J. Preclinical models of lung cancer: cultured cells and organ culture. In: Lenfant C, ed. *Biology of Lung Cancer*. New York: Marcel Dekker; 1998:215–246.
10. Walboomers JM, Meijer CJ, Steenbergen RD, van Duin M, Helmerhorst TJ, Snijders PJ. Human papillomavirus and the development of cervical cancer: concept of carcinogenesis. *Ned Tijdschr Geneeskd.* 2000;144:1671–1674.
11. Adamson IY, Young L, Bowden DH. Relationship of alveolar epithelial injury and repair to the induction of pulmonary fibrosis. *Am J Pathol.* 1988;130:377–383.
12. Krump-Konvalinkova V, Bittinger F, Unger RE, Peters K, Lehr HA, Kirkpatrick CJ. Generation of human pulmonary microvascular endothelial cell lines. *Lab Invest.* 2001;81:1717–727.
13. Rep M, van Dijl JM, Suda K, Schatz G, Grivell LA, Suzuki CK. Promotion of mitochondrial membrane complex assembly by a proteolytically inactive yeast Lon. *Science.* 1996;274:103–106.
14. Gruenert DC, Finkbeiner WE, Widdicombe JH. Culture and transformation of human airway epithelial cells. *Am J Physiol.* 1995;268:L347–L360.
15. Vertrees RA, Das GC, Coscio AM, Xie J, Zwischenberger JB, Boor PJ. A mechanism of hyperthermia-induced apoptosis in ras-transformed lung cells. *Mol Carcinog.* 2005;44:111–121.
16. Vertrees RA, Nason R, Hold MD, et al. Smoke/burn injury-induced respiratory failure elicits apoptosis in ovine lungs and cultured lung cells, ameliorated with arteriovenous CO2 removal. *Chest.* 2004;125:1472–1782.
17. Bareiss PM, Metzger M, Sohn K, et al. Organotypical tissue cultures from adult murine colon as an in vitro model of intestinal mucosa. *Histochem Cell Biol.* 2008;129:795–804.
18. Follmann W, Weber S, Birkner S. Primary cell cultures of bovine colon epithelium: isolation and cell culture of colonocytes. *Toxicol In Vitro.* 2000;14:435–445.
19. Nussler AK, Wang A, Neuhaus P, et al. The suitability of hepatocyte culture models to study various aspects of drug metabolism. *Altex.* 2001;18:91–101.
20. Zeilinger K, Sauer IM, Pless G, et al. Three-dimensional coculture of primary human liver cells in bioreactors for in vitro drug studies: effects of the initial cell quality on the long-term maintenance of hepatocyte-specific functions. *Altern Lab Anim.* 2002;30:525–538.
21. Zen Y, Fujii T, Yoshikawa S, et al. Histological and culture studies with respect to ABCG2 expression support the existence of a cancer cell hierarchy in human hepatocellular carcinoma. *Am J Pathol.* 2007;170:1750–1762.
22. Kosuge M, Takizawa H, Maehashi H, Matsuura T, Matsufuji S. A comprehensive gene expression analysis of human hepatocellular carcinoma cell lines as components of a bioartificial liver using a radial flow bioreactor. *Liver Int.* 2007;27: 101–108.
23. Ma W, Tavakoli T, Chen S, et al. Reconstruction of functional cortical-like tissues from neural stem and progenitor cells. *Tissue Eng Part A.* 2008;14:1673–1686.
24. Ma W, Fitzgerald W, Liu QY, et al. CNS stem and progenitor cell differentiation into functional neuronal circuits in three-dimensional collagen gels. *Exp Neurol.* 2004;190:276–288.
25. Braun H, Buhnemann C, Neumann J, Reymann KG. Preparation of a tissue-like cortical primary culture from embryonic rats using Matrigel and serum free Start V Medium. *J Neurosci Methods.* 2006;157:32–38.
26. Hitchcock T, Niklason L. Lymphatic tissue engineering: progress and prospects. *Ann N Y Acad Sci.* 2008;1131:44–49.
27. Shaul PW. Regulation of vasodilator synthesis during lung development. *Early Hum Dev.* 1999;54:271–294.
28. Gikas EG, King RJ, Mescher EJ, et al. Radioimmunoassay of pulmonary surface-active material in the tracheal fluid of the fetal lamb. *Am Rev Respir Dis.* 1977;115:587–593.
29. Goodwin MN Jr. Selected anatomic burn pathology review for clinicians and pathologists. *Aviat Space Environ Med.* 1989;60 :B39–B43.
30. Lionetti V, Recchia FA, Ranieri VM. Overview of ventilator-induced lung injury mechanisms. *Curr Opin Crit Care.* 2005;11:82–86.
31. Balamugesh T, Kaur S, Majumdar S, Behera D. Surfactant protein-A levels in patients with acute respiratory distress syndrome. *Indian J Med Res.* 2003;117:129–133.
32. Herrera MT, Toledo C, Valladares F, et al. Positive end-expiratory pressure modulates local and systemic inflammatory responses in a sepsis-induced lung injury model. *Intensive Care Med.* 2003;29: 1345–1353.
33. Imai Y, Parodo J, Kajikawa O, et al. Injurious mechanical ventilation and end-organ epithelial cell apoptosis and organ dysfunction in an experimental model of acute respiratory distress syndrome. *JAMA.* 2003;289:2104–2112.
34. Schiller HJ, Steinberg J, Halter J, et al. Alveolar inflation during generation of a quasi-static pressure/volume curve in the acutely injured lung. *Crit Care Med.* 2003;31:1126–1133.
35. Song ZF, Yu KL, Shan HW, Ma J. Evaluation of mechanical ventilation for patients with acute respiratory distress syndrome as a result of interstitial pneumonia after renal transplantation. *Zhongguo Wei Zhong Bing Ji Jiu Yi Xue.* 2003;15:358–361.
36. Alcorn JL, Smith ME, Smith JF, Margraf LR, Mendelson CR. Primary cell culture of human type II pneumonocytes: maintenance of a differentiated phenotype and transfection with recombinant adenoviruses. *Am J Respir Cell Mol Biol.* 1997;17:672–682.
37. Leighton J, Justh G, Esper M, Kronenthal RL. Collagen-coated cellulose sponge: three dimensional matrix for tissue culture of Walker tumor 256. *Science.* 1967;155:1259–1261.
38. Leighton J, Tchao R, Stein R, Abaza N. Histophysiologic gradient culture of stratified epithelium. *Methods Cell Biol.* 1980;21B: 287–307.
39. Hoffman RM. The three-dimensional question: can clinically relevant tumor drug resistance be measured in vitro? *Cancer Metastasis Rev.* 1994;13:169–173.
40. Leighton J. Structural biology of epithelial tissue in histophysiologic gradient culture. *In Vitro Cell Dev Biol.* 1992;28A: 482–492.

41. Yang J, Richards J, Bowman P, et al. Sustained growth and three-dimensional organization of primary mammary tumor epithelial cells embedded in collagen gels. *Proc Natl Acad Sci U S A.* 1979;76:3401–3405.
42. Strangeways T. *Tissue Culture in Relation to Growth and Differentiation*. Cambridge, England: Heffer and Sons; 1924.
43. Fell HB, Robison R. The growth, development and phosphatase activity of embryonic avian femora and limb-buds cultivated in vitro. *Biochem J.* 1929;23:767–845.
44. Browning TH, Trier JS. Organ culture of mucosal biopsies of human small intestine. *J Clin Invest.* 1969;48:1423–1432.
45. Inch WR, McCredie JA, Sutherland RM. Growth of nodular carcinomas in rodents compared with multi-cell spheroids in tissue culture. *Growth.* 1970;34:271–282.
46. Chang HN, Furusaki S. Membrane bioreactors: present and prospects. *Adv Biochem Eng Biotechnol.* 1991;44:27–64.
47. Gallup DM, Gerhardt P. Dialysis fermentor systems for concentrated culture of microorganisms. *Appl Microbiol.* 1963; 11:506–512.
48. Chang TM. Semipermeable microcapsules. *Science.* 1964;146: 524–525.
49. Butterworth TA, Wang DI, Sinskey AJ. Application of ultrafiltration for enzyme retention during continuous enzymatic reaction. *Biotechnol Bioeng.* 1970;12:615–631.
50. Knazek RA, Gullino PM, Kohler PO, Dedrick RL. Cell culture on artificial capillaries: an approach to tissue growth in vitro. *Science.* 1972;178:65–66.
51. Belfort G. Membranes and bioreactors: a technical challenge in biotechnology. *Biotechnol Bioeng.* 1989;33:1047–1066.
52. Chang HN. Membrane bioreactors: engineering aspects. *Biotechnol Adv.* 1987;5:129–145.
53. Gekas VC. Artificial membrane as carrier for the immobilization of biocatalysts. *Enzyme Microb Technol.* 1986;8:450–460.
54. Hopkinson J. Hollow fiber cell culture in industry. In: Mattiasson B, ed. *Immobilized Cells and Organelles*. Boca Raton, FL: CRC Press; 1983.
55. Kitano H, Ise N. Hollow fiber enzyme reactors. *Trends Biotechnol.* 1984;2:5–7.
56. Vandenburgh HH, Shansky J, Del Tatto M, Lee P, Meir J. *Tissue Engineering Organs for Space Biology Research*. First Biennial Space Biomedical Investigators Workshop; 1997.
57. Vandenburgh H, Del Tatto M, Shansky J, et al. Attenuation of skeletal muscle wasting with recombinant human growth hormone secreted from a tissue-engineered bioartificial muscle. *Hum Gene Ther.* 1998;9:2555–2564.
58. Chromiak J, Shansky J, Perrone CE, Vandenburgh HH. Bioreactor perfusion system for the long term maintenance of tissue-engineered skeletal muscle organoids. *In Vitro Cell Dev Biol.* 1998;34:695–703.
59. Guignandon A, Usson Y, Laroche N, et al. Effects of intermittent or continuous gravitational stresses on cell-matrix adhesion: quantitative analysis of focal contacts in osteoblastic ROS 17/2.8 cells. *Exp Cell Res.* 1997;236:66–75.
60. Angele P, Kujat R, Nerlich M, Yoo J, Goldberg V, Johnstone B. Engineering of osteochondral tissue with bone marrow mesenchymal progenitor cells in a derivatized hyaluronan-gelatin composite sponge. *Tissue Eng.* 1999;5:545–554.
61. Schoeters G, Leppens H, Van Gorp U, Van Den Heuvel R. Haemopoietic long-term bone marrow cultures from adult mice show osteogenic capacity in vitro on 3-dimensional collagen sponges. *Cell Prolif.* 1992;25:587–603.
62. Goodwin TJ, Prewett TL, Wolf DA, Spaulding GF. Reduced shear stress: a major component in the ability of mammalian tissues to form three-dimensional assemblies in simulated microgravity. *J Cell Biochem.* 1993;51:301–311.
63. Goodwin TJ, Schroeder WF, Wolf DA, Moyer MP. Rotating-wall vessel coculture of small intestine as a prelude to tissue modeling: aspects of simulated microgravity. *Proc Soc Exp Biol Med.* 1993;202:181–192.
64. Schwarz RP, Wolf DA, Trinh TT. Horizontally rotated cell culture system with a coaxial tubular oxygenator. US patent no 5,026,650; June 25, 1991.
65. Van Wezel AL. Microcarrier/cultures of animal cells. In: Paterson MK, ed. *Tissue Culture: Methods and Applications*. New York: Academic Press; 1973.
66. Glacken MW, Fleischaker RJ, Sinskey AJ. Mammalian cell culture: engineering principles and scale-up. *Trends Biotechnol.* 1983;1:102–108.
67. Goodwin TJ, Jessup JM, Wolf DA. Morphologic differentiation of colon carcinoma cell lines HT-29 and HT-29KM in rotating-wall vessels. *In Vitro Cell Dev Biol.* 1992;28A:47–60.
68. Croughan MS, Hamel JF, Wang DI. Hydrodynamic effects on animal cells grown in microcarrier cultures. *Biotechnol Bioeng.* 1987;29:130–141.
69. Cherry RS, Papoutsakis ET. Physical mechanisms of cell damage in microcarrier cell culture bioreactors. *Biotechnol Bioeng.* 1988;32:1001–1004.
70. Cherry RS, Hulle CT. Cell death in the thin films of bursting bubbles. *Biotechnol Prog.* 1992;8:11–18.
71. Croughan MS, Wang DI. Growth and death in over agitated microcarrier cell cultures. *Biotechnol Bioeng.* 1989;33:731–744.
72. Nilsson K, Buzsaky F, Mosbach K. Growth of anchorage-dependent cells on macroporous microcarriers. *Biotechnology.* 1986;4:989–990.
73. Croughan MS, Sayre SS, Wang DI. Viscous reduction of turbulent damage in animal cell culture. *Biotechnol Bioeng.* 1989;33: 862–872.
74. Thalmann E. Biological experiences in bubble-free aeration system. *Acta Biotechnol.* 1989;39:511–516.
75. Clark J, Hirstenstein H, Gebb C. Critical parameters in the microcarrier culture of animal cells. *Dev Biol Stand.* 1980;46: 117–124.
76. Feder J, Tolbert WR. The large-scale cultivation of mammalian cells. *Sci Am.* 1983;248:36–43.
77. Tschopp A, Cogoli A, Lewis ML, Morrison DR. Bioprocessing in space: human cells attach to beads in microgravity. *J Biotechnol.* 1984;1:287–293.
78. Gmunder FK, Cogoli A. Cultivation of single cells in space. *Appl Micrograv Technol.* 1988;3:115–122.
79. Schwarz RP, Goodwin TJ, Wolf DA. Cell culture for three-dimensional modeling in rotating-wall vessels: an application of simulated microgravity. *J Tissue Cult Methods.* 1992;14: 51–57.
80. Wolf DA, Schwarz RP. Analysis of Gravity-Induced Particle Motion and Fluid Perfusion Flow in the NASA-Designed Rotating Zero-Head-Space Tissue Culture Vessel. *NASA Technical Paper* 3143; 1991.

81. Baker TL, Goodwin TJ. Three-dimensional culture of bovine chondrocytes in rotating-wall vessels. *In Vitro Cell Dev Biol Anim.* 1997;33:358–365.
82. Haffen K, Kedinger M, Simon-Assmann P. Mesenchyme-dependent differentiation of epithelial progenitor cells in the gut. *J Pediatr Gastroenterol Nutr.* 1987;6:14–23.
83. Kedinger M, Haffen K, Simon-Assmann P. Intestinal tissue and cell cultures. *Differentiation.* 1987;36:71–85.
84. Kedinger M, Simon-Assmann PM, Lacroix B, Marxer A, Hauri HP, Haffen K. Fetal gut mesenchyme induces differentiation of cultured intestinal endodermal and crypt cells. *Dev Biol.* 1986;113: 474–483.
85. Stallmach A, Hahn U, Merker HJ, Hahn EG, Riecken EO. Differentiation of rat intestinal epithelial cells is induced by organotypic mesenchymal cells in vitro. *Gut.* 1989;30:959–970.
86. Quaroni A. Crypt cell development in newborn rat small intestine. *J Cell Biol.* 1985;100:1601–1610.
87. Quaroni A. Development of fetal rat intestine in organ and monolayer culture. *J Cell Biol.* 1985;100:1611–1622.
88. Hahn U. Extracellular matrix proteins in small-intestinal cell cultures. *Scand J Gastroenterol Suppl.* 1988;151:70–78.
89. Moyer MP, Huot RI, Ramirez A Jr, Joe S, Meltzer MS, Gendelman HE. Infection of human gastrointestinal cells by HIV-1. *AIDS Res Hum Retroviruses.* 1990;6:1409–1415.
90. Herlyn M, Kath R, Williams N, Valyi-Nagy I, Rodeck U. Growth-regulatory factors for normal, premalignant, and malignant human cells in vitro. *Adv Cancer Res.* 1990;54:213–234.
91. Shih IM, Herlyn M. Autocrine and paracrine roles for growth factors in melanoma. *In Vivo.* 1994;8:113–123.
92. Goodwin TJ, Prewett TL, Spaulding GF, Becker JL. Three-dimensional culture of a mixed mullerian tumor of the ovary: expression of in vivo characteristics. In Vitro Cell Dev Biol Anim. 1997;33:366–374.
93. Prewett TL, Goodwin TJ, Spaulding GF. Three-dimensional modeling of T-24 human bladder carcinoma cell line: a new simulated microgravity vessel. J Tissue Cult Methods. 1993;15: 29–36.
94. Kerbel RS, Rak J, Kobayashi H, Man MS, St Croix B, Graham CH. Multicellular resistance: a new paradigm to explain aspects of acquired drug resistance of solid tumors. Cold Spring Harbor Symp Quant Biol. 1994;59:661–672.
95. Li LH, Bhuyan BK, Wallace TL. Comparison of cytotoxicity of agents on monolayer and spheroid systems. Proc Am Assoc Cancer *Res.* 1989;30:2435A.
96. Smith KS, Badiner GJ, Adams FG, Wilson DK, Li LH, Bhuyan BK. Modified 2-tumour (L1210, colon 38) assay to screen for solid tumor selective agents. In: *Proceedings of the Symposium on Anticancer Drug Discovery and Development*. Detroit, MI; 2006.
97. Lawler EM, Miller FR, Heppner GH. Significance of three-dimensional growth patterns of mammary tissues in collagen gels. *In Vitro.* 1983;19:600–610.
98. Miller BE, Miller FR, Heppner GH. Assessing tumor drug sensitivity by a new in vitro assay which preserves tumor heterogeneity and subpopulation interactions. *J Cell Physiol Suppl.* 1984;3:105–116.
99. Miller BE, Miller FR, Heppner GH. Factors affecting growth and drug sensitivity of mouse mammary tumor lines in collagen gel cultures. *Cancer Res.* 1985;45:4200–4205.
100. Simon-Assmann P, Bouziges F, Vigny M, Kedinger M. Origin and deposition of basement membrane heparan sulfate proteoglycan in the developing intestine. *J Cell Biol.* 1989;109: 1837–1848.
101. Teicher BA, Herman TS, Holden SA, et al. Tumor resistance to alkylating agents conferred by mechanisms operative only in vivo. *Science.* 1990;247:1457–1461.
102. Kobayashi H, Man S, Graham CH, Kapitain SJ, Teicher BA, Kerbel RS. Acquired multicellular-mediated resistance to alkylating agents in cancer. *Proc Natl Acad Sci U S A.* 1993;90: 3294–298.
103. Margolis J, Li ZP. Heat sterilisation to inactivate AIDS virus in lyophilised factor VIII [letter]. *Aust N Z J Med.* 1986;16:413.
104. Ingram M, Techy GB, Saroufeem R, et al. Three-dimensional growth patterns of various human tumor cell lines in simulated microgravity of a NASA bioreactor. *In Vitro Cell Dev Biol Anim.* 1997;33:459–466.
105. Margolis L, Hatfill S, Chuaqui R, et al. Long term organ culture of human prostate tissue in a NASA-designed rotating wall bioreactor. *J Urol.* 1999;161:290–297.
106. Zhau HE, Goodwin TJ, Chang SM, Baker TL, Chung LW. Establishment of a three-dimensional human prostate organoid coculture under microgravity-simulated conditions: evaluation of androgen-induced growth and PSA expression. *In Vitro Cell Dev Biol Anim.* 1997;33:375–380.
107. Wang R, Xu J, Juliette L, et al. Three-dimensional co-culture models to study prostate cancer growth, progression, and metastasis to bone. *Semin Cancer Biol.* 2005;15:353–364.
108. Vertrees RA, Zwischenberger JB, Boor PJ, et al. Cellular differentiation in three-dimensional lung cell cultures. *Cancer Biol Ther.* 2008;7:404–412.
109. Nickerson CA, Goodwin TJ, Terlonge J, et al. Three-dimensional tissue assemblies: novel models for the study of *Salmonella enterica* serovar *Typhimurium* pathogenesis. *Infect Immun.* 2001;69:7106–7120.
110. Hiemstra PS, Bals R. Series introduction: innate host defense of the respiratory epithelium. *J Leukoc Biol.* 2004;75:3–4.
111. Gibson MC, Perrimon N. Apicobasal polarization: epithelial form and function. *Curr Opin Cell Biol.* 2003;15:747–752.
112. Knight DA, Holgate ST. The airway epithelium: structural and functional properties in health and disease. *Respirology.* 2003;8:432–446.
113. Cotran R, Kumar V, Collins T. *Robbins Infectious Diseases*. 6th ed. Philadelphia: Saunders; 1999.
114. Garofalo RP, Haeberle H. Epithelial regulation of innate immunity to respiratory syncytial virus. *Am J Respir Cell Mol Biol.* 2000;23:581–585.
115. Polito AJ, Proud D. Epithelia cells as regulators of airway inflammation. *J Allergy Clin Immunol.* 1998;102:714–718.
116. Ke Y, Reddel RR, Gerwin BI, et al. Human bronchial epithelial cells with integrated SV40 virus T antigen genes retain the ability to undergo squamous differentiation. *Differentiation.* 1988;38: 60–66.
117. Gray GD, Wickstrom E. Evaluation of anchorage-independent proliferation in tumorigenic cells using the redox dye alamar Blue. *Biotechniques.* 1996;21:780, 782.
118. Wu R, Sato GH, Whitcutt MJ. Developing differentiated epithelial cell cultures: airway epithelial cells. *Fundam Appl Toxicol.* 1986;6:580–590.

119. Stoner GD, Katoh Y, Foidart JM, Myers GA, Harris CC. Identification and culture of human bronchial epithelial cells. *Methods Cell Biol.* 1980;21A:15–35.
120. Adler KB, Li Y. Airway epithelium and mucus: intracellular signaling pathways for gene expression and secretion. *Am J Respir Cell Mol Biol.* 2001;25:397–400.
121. Fukamachi H. Disorganization of stroma alters epithelial differentiation of the glandular stomach in adult mice. *Cell Tissue Res.* 1986;243:65–68.
122. Sutherland AE, Calarco PG, Damsky CH. Expression and function of cell surface extracellular matrix receptors in mouse blastocyst attachment and outgrowth. *J Cell Biol.* 1988;106: 1331–1348.
123. Wiens D, Park CS, Stockdale FE. Milk protein expression and ductal morphogenesis in the mammary gland in vitro: hormone-dependent and -independent phases of adipocyte-mammary epithelial cell interaction. *Dev Biol.* 1987;120:245–258.
124. Buset M, Winawer S, Friedman E. Defining conditions to promote the attachment of adult human colonic epithelial cells. *In Vitro Cell Dev Biol.* 1987;23:403–412.
125. Daneker GW Jr, Mercurio AM, Guerra L, et al. Laminin expression in colorectal carcinomas varying in degree of differentiation. *Arch Surg.* 1987;122:1470–1474.
126. Durban EM, Knepper JE, Medina D, Butel JS. Influence of mammary cell differentiation on the expression of proteins encoded by endogenous BALB/c mouse mammary tumor virus genes. *Virus Res.* 1990;16:307–323.
127. Kaye GI, Siegel LF, Pascal RR. Cell replication of mesenchymal elements in adult tissues. I. The replication and migration of mesenchymal cells in the adult rabbit dermis. *Anat Rec.* 1971;169:593–611.
128. O'Brien LE, Zegers MM, Mostov KE. Opinion: Building epithelial architecture: insights from three-dimensional culture models. *Nat Rev Mol Cell Biol.* 2002;3:531–537.
129. Goodwin TJ, Deatly AM, Suderman MT. Three-dimensional engineered high fidelity normal human lung tissue-like assemblies (TLA) as targets for human respiratory virus infection. American Society for Virology 22nd Annual Meeting; 2003 (abstract).
130. Suderman MT, Mossel E, Watts DM, et al. Severe acute respiratory syndrome (SARS)-CoV infection in a three-dimensional human bronchial-tracheal (HBTE) tissue-like assembly. American Society for Virology 23rd Annual Meeting; 2004 (abstract).