PHENOTYPIC DIFFERENCES IN THE BEHAVIOR OF IMMORTALIZED HUMAN BRONCHIAL EPITHELIAL CELLS IN THREE DIMENSIONAL CULTURE SYSTEMS

by

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ABSTRACT

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Human Bronchial Epithelial Cells (HBECs) immortalized with viral oncoproteins E6/E7, are potentially malignant as they have numerous chromosomal aberrations. Recently a model system was established in which HBECs were immortalized by overexpressing ectopic Cyclin-dependent-kinase-4 (Cdk4) and human Telomerase Reverse Transcriptase (hTERT). These cells were shown to have an intact p53 checkpoint and had well expressed epithelial differentiation markers. HBECs are non-tumorigenic as they do not form colonies in soft agar or tumors in nude mice. The purpose of this research was to establish the phenotypic behavioral differences between HBECs immortalized with E6/E7 viral oncoproteins and HBECs immortalized with

Cdk4 and hTERT. A three dimensional culture model of a differentiated bronchial epithelium was constructed to study the differences between the HBECs immortalized by the two methods. The system consists of lung fibroblasts embedded in a type I collagen matrix on which the HBECs are plated. The collagen gels are raised to an air-liquid interface and the HBECs are induced to differentiate for 21 days, in media conducive to differentiation. Pairs of HBECs immortalized using E6/E7 (with/without hTERT) or Cdk4/hTERT obtained from four donors were tested. Normal lung fibroblasts from a 61 year old male (61ML), or a 35 year old female (35FL), were used in constructing the stromal compartment. Fibroblast cell line FBL 15 isolated from the bronchial tumor of a 59 year old male was also used.

Results indicate that HBECs immortalized with E6/E7 viral oncoproteins have an invasive phenotype regardless of culture conditions or fibroblasts used. The invasive behavior of HBECs immortalized with Cdk4/hTERT was found to be fibroblast specific. The 61ML fibroblasts derived from the aged donor's lung tissue were permissive to the invasion of HBEC KTs unlike the 35FL fibroblasts obtained from the younger donor's lung tissue. Aged donor fibroblasts were permissive to the invasion of immortalized HBECs, thereby implying a strong connection between aging and cancer.

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CHAPTER 1

INTRODUCTION

1.1 Normal Human Lung

1.1.1 Histological Structure of Bronchial Epithelium

The human bronchus is lined by a layer of epithelial tissue that is exposed to the external environment. The bronchial epithelial tissue is pseudostratified in structure, with a single layer of progenitor basal cells and columnar epithelial cells. The columnar epithelial cells are taller than they are wide and are polygonal in shape. These cells have their nuclei at two or more levels, which gives them a stratified appearance and hence the term 'pseudo-stratified'. The columnar bronchial epithelial cells are polarized with the basal surface attached to the basal lamina (basement membrane) and the apical surface exposed to atmospheric air. The basal lamina components are contributed by the epithelial cells and the cells of the underlying connective tissue. The basement membrane contributes to cellular adhesion, cell shape maintenance and tissue integrity. The basal surface of the columnar epithelial cells is rich in integrins and other extracellular matrix receptors which attach to the protein structures that make up the basement membrane. During an organism's developmental phase, the bronchial epithelial cells differentiate to form a mucocilliary epithelium. The apical surface of the cells are rich in cilia that are membrane covered extensions of the cell surface, typically

occurring in tufts or covering the entire surface of the cells. Some of the columnar epithelial cells are specialized secretory cells called goblet cells that secrete mucous.

The bronchial epithelium is separated from the stromal compartment or lamina propia by the basement membrane. The stroma here is the connective tissue composed of a mesh of collagen (types I and III) fibers and lung fibroblasts embedded in the collagen, together forming the extracellular matrix of the epithelium.

1.1.2 Functions of the Bronchial Epithelium

The epithelium lines and protects organs such as the lungs that are exposed to the external atmosphere. The mucous secreted by the goblet cells of the bronchial epithelium chiefly consists of glycoproteins called mucins or mucin precursors called mucinogens. The foreign particles that enter the lung are trapped in the mucous. The mucous precludes the respiratory tract from drying up, thus protecting the tract from soreness, inflammation and other mechanical and chemical injuries. The cilia on the apical cell surface beat in waves and move the mucous along with entrapped foreign particles towards the esophagus to be swallowed and removed from the lungs. Defects in the mucociliary system lead to diseases. For example, in a disease like asthma the respiratory passage narrows and thickens thereby blocking the mucus from being moved. This causes accumulation of mucus in the respiratory passageway, leading to difficulty in breathing.

The cross talk between epithelial cells and the mesenchymal fibroblasts together helps maintain tissue integrity. If any of the signaling pathways/mechanisms between

these two components become faulty, disease sets in. The epithelium which forms the protective barrier for any internal organ is also the tissue from which most cancers arise.

1.2 Lung Cancer

1.2.1 Prevalence

Of all cancers in humans, lung cancer has the highest rate of incidence. It is the number one cancer killer of men and women in the United States of America and the world over. Diagnosis at early stages of the cancer poses an enormous challenge. The disease is rarely symptomatic until the cancer is in a highly advanced stage. Treatment options include radiation therapy or chemotherapy or a combination of both.

Smoking is the number one cause of lung cancer. Tobacco smoke contains numerous carcinogenic particles that bombard the respiratory tract and wound it over time. Radon gas is the second leading cause of lung cancer and it finds its way into houses from the soil below, through cracks in the foundation or other openings. Asbestos dust, chromium, uranium, and certain petroleum products are other known carcinogens. These carcinogens cause genetic and epigenetic changes to lung tissues exposed to them, eventually leading to cancer. The molecular pathogenesis of lung cancer is being studied in great depth. Improvements in technology such as microarray analysis have given us better clues to solve the puzzle. However, the five-year survival rate for lung cancer patients is very poor. Most patients suffering from lung cancer succumb to the disease within a year of diagnosis. The 5-year survival rate for lung cancer patients diagnosed with clinical stage 1A is about 60% and it ranges anywhere between 2%-40% for patients diagnosed with clinical stage II-IV lung cancer (Wistuba,

& Gazdar, 2006). A lot more research needs to be carried out to make possible earlier detection and improved treatment efficacies. It is necessary to find more therapeutic agents to better the quality of life of lung cancer patients and to extend their life spans.

1.2.2 Oncogenes and Tumor Suppressors

Mutations in a gene alter the production and activity of its related proteins. Mutations accruing in a cell eventually instigate tumor development and cancer progression. Proto-oncogenes encourage cellular growth whereas tumor suppressor genes inhibit it. Mutated forms of proto-oncogenes transform them into oncogenes which result in an uninhibited proliferation of cells. Common oncogenes involved in lung cancer are erb-B1 (codes for Epidermal Growth Factor Receptor), erb-B2 (codes for the her2/neu receptor family), K-ras (involved in growth stimulatory signaling pathways), c-myc (transcription factor that stimulates activation of growth-promoting genes), Bcl-2 (codes for cell-suicide blocking protein), MDM2 (antagonist of p53 checkpoint) etc. The ras family of oncogenes is one of the commonly activated +oncogenes in lung cancer (Wistuba and Gazdar, 2006).

The tumor suppressor genes (TSG) most often encountered in lung cancer are p53, $p16^{INK4A}$, retinoblastoma (RB), APC, etc. TSG p53 codes for protein p53 which maintains genomic integrity by bringing about apoptosis of cells who's DNA have been damaged. Protein p53 is a transcription factor which induces expression of downstream genes such as p21, HDM2, and GADD45. Such genes regulate checkpoints in the cell cycle signaling pathways that lead to G1 cell cycle arrest. Gene RB codes for protein pRB that breaks the progression of the cell cycle. Mutations or loss of TSGs leads to

proliferation of cells with abnormal cellular DNA, unrestrained cell cycle progression, and evasion of cellular senescence.

Proto-oncogenes and tumor suppressors work in tandem in normal tissues to keep cancer at bay. But when their signaling pathways, and/or check points go awry, everything goes haywire. Cells lose contact inhibition, evade normal cell cycle arrest, proliferate incessantly and ultimately lose tissue integrity and gross function. The cells become cancerous. Eventually cancer cells begin to invade and metastasize to other locations in the body. Lung cancer is no exception to this disease process. The most common oncogenes involved in lung cancer are RAS and genes that encode protein kinase enzymes. The primary tumor suppressors that are often lost or mutated in most cancers are p53 and RB. Numerous research studies have been conducted to understand the signaling pathways these molecules are involved in. Currently, cancer therapeutics are engineered to target both oncogenes and tumor suppressors.

1.2.3 Lung Cancer Pathogenesis

Histologically and clinically, lung cancer is classified into small-cell lung cancer (SCLC) which is a neuro-endocrine subtype, and non-small cell lung cancer (NSCLC) which encompasses adenocarcinome, squamous cell carcinoma and large cell carcinoma. Modern comparative genomic hybridization techniques can be utilized to detect chromosomal imbalances that are present in lung cancer tissues.

Squamous cell type carcinomas usually arise centrally whereas adenocarcinoma and large cell carcinomas usually arise peripherally. Researchers believe lung cancers

arise after a series of progressive pathological changes known as preneoplastic or premalignant lesions (Colby, et al., 1998).

One of the first features that a cancer cell acquires is immortality. This allows a cell to divide and replicate endlessly. DNA segments at the ends of chromosome function as the cellular clock and are called telomeres. Each time a cell replicates, its telomere shortens in length. Shortening of telomeres ultimately leads to a breakage in the chromosomes and brings about genetic chaos in the cell. This results in cellular suicide. But most cancer cells evade the telomere shortening senescence mechanism but activating the enzyme telomerase that is necessary for the elongation of the telomeres. They are now able to replicate ceaselessly.

Genetic mutations in proto-oncogenes or TSG in cells bring about transformations of the tissues they are a part of. Additionally if the epithelial tissue is subject to environmental carcinogens, more mutations begin to accrue in the genome of the abnormal cells. Hyperplasia, metaplasia, squamous dysplasia and carcinoma in situ (CIS) are the stepwise histo-pathological sequence of events leading to invasive squamous cell carcinoma in the respiratory mucosa. The basal and mucous producing respiratory cells begin to divide and grow in an uninhibited fashion. This is called hyperplasia. Hyperplasia of the cells is often accompanied by squamous metaplasia. Integrity of a pseudo-stratified bronchial epithelium is compromised. The bronchial epithelial cells become squamous and change in function. This stage is therefore referred to as squamous metaplasia. Change in size and shape of cells is termed dysplasia. Squamous dysplasia characterized by cytological disturbances or irregularity

is a lesion that is considered truly neoplastic. Cytological aberrations, an intact basement membrane, an almost completely disrupted tissue architecture and nonexistence of stromal invasion are characteristics of CIS. As soon as the nutrients such as oxygen are in short supply for these abnormal overactive premalignant epithelial cells, they begin disintegrating parts of the basement membrane and start migrating into the stromal compartment in search of further nourishment. This process is called invasion. Ultimately, the invading cells metastasize through the lymphatic system or blood to other distant sites in the body, attach themselves to the tissues there and begin to form tumors.

1.2.4 The War against Lung Cancer

Research in lung cancer is centered on two main areas: anticancer therapies that include drug development and testing and cancer biology. Molecular cancer biology is a subsection of cancer biology in which the role of oncogenes and TSGs in disease development and progression are studied. Mouse models are the current standard for testing effect of therapeutic drugs. Mouse models are difficult to construct. Additionally, they consume considerable amount of money and time to maintain. Obtaining data from a single mouse can take up to even six months. As new drugs cannot enter clinical trials unless proven fairly safe in animal models, mouse models are an absolute necessity. Two dimensional cell cultures are used for testing the role of various oncogenes, TSGs and their interplay. Drugs are first tested on two dimensional cell cultures in the laboratories. Moving from two dimensional cell cultures in tissue culture flasks to mice is indeed a huge step. Cells in the body are organized into tissues

that have specific functions. *In vitro* two dimensional cell cultures are far from representing the *in vivo* tissue organization of the cells. By developing three dimensional culture models, one can hope to narrow the huge gaps that exist in current research methods that utilize two dimensional cultures and animal models. Hence there arises a need for the development of such a culture system.

1.3 Three Dimensional Culture Systems

Organotypic cultures have come under the spotlight in the tissue engineering arena in recent years. Studies using three dimensional organotypic systems are very useful as such systems are modeled to better mimic an organism's *in vivo* conditions as opposed to two dimensional systems. In this study, an effort has been made to construct a three dimensional model of the bronchial epithelium. Such a system is expected to be immensely useful in understanding lung cancer pathogenesis and evaluating the potential effectiveness of therapeutic drugs as it can accommodate experimental intervention. Essentially, one can specifically alter any of the important components of this system: epithelial, fibroblast, media, or matrix.

1.3.1 Usability of the system

Behavior of cancer cells in two dimensional tissue culture plastic is very different from that in three dimensions, so much so that Weaver, et al., stated in 2002, "Suddenly, the study of cancer cells in two dimension seems quaint, if not completely archaic!". Tissue structure and integrity needs to be maintained to prevent the onset of cancer. There exists a close connection between defects in terminal differentiation and neoplastic transformation of bronchial epithelial cells (Lechner, et al., 1983), (Pfeifer, et

al., 1989). Two dimensional tissue culture systems may not provide the right answers while studying the molecular pathogenesis of cancer. Tissue integrity can be achieved by using structures such as floating collagen gels. On a similar note, drug sensitivity studies on cancer cells might be more effective both cost-wise and treatment-wise if new drugs could be tested on structures that represent *in vivo* features more closely. Normally testing in mice can take up to 6 months or more. However, three dimensional culture assays can be completed in one month's time which essentially translates into savings in time. It is also difficult to solely monitor the cells initially injected into mice. Such observations can be made clearly in organotypic culture systems.

The three dimensional culture system can be used to study and evaluate the effect of growth factors, hormones and vitamins. One can also understand the transformation of normal epithelial cells to a malignant invasive phenotype.

1.3.2 Organotypic Cultures of the Bronchial Epithelium

Potentially, immortalization of normal cells could spontaneously result from downregulation of a differentiation gene (Prasad, N. et al., 2001). A majority of lung cancers are bronchial carcinomas and therefore there arises the need to establish normal and malignant culture models of bronchial epithelial cells. Differentiation of epithelial cells has been found to be dependent on factors derived from the mesenchymal components of the tissue (Ghalbzouri et al.., 2004).

CHAPTER 2

LITERATURE REVIEW

2.1 Components

Three dimensional cocultivation models of epithelial and mesenchymal cells have been used as an alternative to monolayer cultures due to ease of manipulation and preservation of architecture of cellular complexes (Al-Batran, et al., 1999). Skin models have been constructed using de-epithelialized basement membrane tissue and seeding keratinocytes onto them. One other commonly used model utilizes a collagen gel with or without fibroblasts (Auger, et. al., 1998). One of the problems with growing epithelial cells is the loss of specialized properties that the cells possessed when in vivo. This problem is commonly known as dedifferentiation and it is believed that the most likely cause is the propagation of cells from the early, proliferative, undifferentiated stage of the correct lineage. This brings us to the fact that the epithelial cells would need the right environment for induction of differentiation. This environment can be provided by mimicking the architecture of the tissue in vivo. There are four main parameters that are permissive to the induction of differentiation: 1) Soluble inducers (like hormones and growth factors), 2) cell-cell interaction, 3) cell-matrix interaction, 4) polarity and cell shape (Freshney, et al., 2002). Provision of a microenvironment replete with all these factors will be highly conducive to the differentiation of epithelial cells.In this research study, such a model was adopted. The four main components of this model are:

collagen, fibroblasts, epithelial cells, and cell culture media. A three dimensional gel of reconstituted type I collagen fibrils served as a matrix for lung fibroblasts. Epithelial cells were plated atop the collagen gels which are contracted by the lung fibroblasts. The cultures were maintained in transwell plates in an air-liquid interface. The air-liquid liquid interface and the media used aid the differentiation of the epithelial cells.

Human skin models have been developed with successful differentiation of keratinocytes including development of a stratum corneum (Vaughan et. al., 2004). However much success has not been reported as far as a differentiated bronchial epithelium is concerned. An effort was made in this study to establish a differentiated model of the bronchial epithelium, using the methods described by Vaughan, et al, 2004. Bronchial epithelial cells are exposed directly to the external atmosphere just like the skin keratinocytes. Hence, as a starting point, the lung model was set up using the same components that were used in the skin model. All the components and the air-liquid interface setup were meant to aid the process. The keratinocytes were however replaced by bronchial epithelial cells. Fundamentally, this model is considered to mimic *in vivo* lung conditions as the bronchial epithelial cells are exposed to an air-liquid interface and the collagen along with the fibroblasts represents the connective tissue in the bronchus.

2.1.1 Collagen

A floating collagen gel is prepared using liquid type I collagen extracted from the tendon of rat tails, in this case with dilute acetic acid. The collagen serves as a matrix in which the fibroblasts are embedded. Together with the fibroblasts the collagen

matrix serves as the stroma to the bronchial epithelial cells. This collagen matrix can contract depending on factors secreted by epithelial cells as they change their cell shape during the process of differentiation. Providing a collagen matrix that can be reshaped and remodeled by the epithelial cells, allows the epithelial cells to achieve a cell shape that resembles their in vivo histology. Therefore for achieving maximum expression of differentiation, it is very important to grow the epithelial cells on a collagen raft.

2.1.2 Fibroblasts

Stromal interaction has been shown to play an important role in the differentiation of epithelial cells. Fibroblasts in the stroma release a number of factors that are active in the differentiation and morphogenesis of epithelial cells. They are typically cytokines and growth factors such as keratinocyte growth factor, hepatocyte growth factor etc. Fibroblasts control the complete differentiation of many epithelial tissues. Epidermal homeostasis and differentiation is influenced by the mesenchymal compartment. The mesenchymal fibroblasts in the collagen gel eventually reorganize the collagen matrix by secreting extracellular matrix components that contracts the collagen gel. The fibroblasts that may have initially proliferated in the matrix attain a resting state and alter protein synthesis comparable to that in the dermis (Couloumb, et al., 1984). The collagen gel is contracted to a densely structured lattice that serves as a dermal equivalent in the organotypic model of the skin. When collagen lattices without fibroblasts are used, only a thin layer of incompletely differentiated epithelia arise (Maas-Szabowski, et al., 2000).

It is believed that the cross talk between the epithelial cells and the stromal fibroblasts aid cancer pathogenesis and progression. One of the theories regarding this concept gaining attention is that, tumorigenic epithelial cells recruit fibroblasts in the stroma and convert them into myofibroblasts or 'Cancer-Associated Fibroblasts' (CAFs), which then aid the epithelial cell migration by producing collagenases to digest the extracellular matrix proteins (Micke and Ostman, 2005), (Kiaris, et al., 2004), (Mueller and Fusenig, 2004). There is thought to be heavy crosstalk and signaling between the stromal fibroblasts and the epithelial cells in any epithelial tissue. This signaling brings about the proper organization and differentiation of the epithelial cells during development. In the collagen matrix, the fibroblasts pull on the collagen fibrils to contract the gel and form a well knit strong lattice for the epithelial cells to attach to, proliferate and differentiate on. This way the collagen fibrils are taut and form a strong mesh. The fibroblasts organize themselves in such a way that they are not in contact with one another. A number of studies have been conducted to study the contraction of collagen gels by fibroblasts.

2.1.3 Human Bronchial Epithelial Cells (HBECs)

The bronchial epithelial cells are the most important component of the system as it is their behavior that we are trying to characterize. Human bronchial epithelial cells obtained from various patients were immortalized in two ways: 1) using viral oncoproteins E6/E7 from HPV 16 and, 2) using CDK4/HTERT (Ramirez, et al., 2004). The HBECs immortalized with viral oncoproteins are deemed transformed in nature as they have lost their p53 checkpoint and have numerous other chromosomal aberrations.

In comparison, microarray data for HBECs immortalized by overexpression of CDK4 and HTERT indicates that these cells clustered together with non-immortalized bronchial cell lines. They also had an intact p53 checkpoint in contrast to cells immortalized with E6/E7.

Differences in the phenotypic behavior of Human Bronchial Epithelial Cells (HBECs) immortalized by the two different methods were evaluated using the organotypic culture model. To that effect, the two strains of HBEC lines from the same patient immortalized with or without viral oncoproteins were seeded atop the collagen gels. The epithelial cells can polarize completely only in three dimension. The collagen offers a three dimensional surface for the epithelial cells. In two dimensional cultures, they are partially polarized and most cells tend to achieve a squamous formation in two dimension. To reproduce the pseudostratified columnar formation like *in vivo* bronchial epithelium, the HBECs require a three dimensional environment.

2.1.4 Media

The bronchial epithelial cells were grown in culture regularly using Keratinocyte-Serum Free Media (K-SFM). When plated on a three dimensional matrix, the cells do not undergo differentiation automatically. A three dimensional environment alone is insufficient to bring about differentiation. It is necessary to provide the essential nutrients, growth factors and differentiation stimulating factors, thereby creating a microenvironment suitable for HBECs to differentiate. It is a widely accepted fact that proliferating and differentiating cells in epithelial culture populations are two independent mutually exclusive events. Epithelial cells need hormonal factors and other

chemical stimulants to trigger the onset of differentiation *in vitro*. The factors required are supplied to cells through cell culture media.

Soluble inducers include hormones, cytokines, growth factors and vitamins (A, D, E and K). Cell-cell interaction between adjacent cells allows the transfer of signal transducers such as cAMP. The interaction also provides for contact inhibition and hence a decrease in proliferation. Since the bronchial model was initially constructed using the components of the skin model except for the epithelial cells, the Rheinwald & Green media (RG) which is regularly utilized in skin models, (Wu., et. al, 1982), was adopted. RG media has DMEM and F12 in the ratio of 1:3 and 5% serum as shown in table 2.1. The other factors such as Epidermal Growth Factor (EGF), Cholera Toxin (CT), and insulin are considered as factors that aid proliferation. Hydrocortisone, Tri-iodothyronine (T3), transferrin, adenine, and ascorbic acid are the differentiation promoting factors are used in the ratios shown in table 2.2.

Hydrocortisone is essential to maintain growth rate, colony morphology and differentiation (Rheinwald and Green, 1975). Cholera toxin raises cyclic AMP levels and seems to prevent epithelial cells from growing in size, thereby antagonizing the onset of terminal differentiation (Sun and Green, 1976; Barrandon and Green, 1985). EGF aids epithelial cell migration, increases the size of the clonogenic populations thereby opposing terminal differentiation (Rheinwald and Green, 1977). All these factors increase the clonogenic population, their growth rate and lifespan. Insulin, transferrin and T3 reduce the dependency of keratinocytes on serum from 20% to 10% and help improve growth rate (Rheinwald, 1980).

Table 2.1 Major Components of Rheinwald Green Medium

Components	**Volume in 500ml of Media (ml)**
DMEM	356.25
F-12	118.75
Fetal Calf Serum	25.0
Antibiotic/Antimycotic	5.0

Table 2.2 Detailed Composition of Rheinwald Green Medium

Other Components	**Concentration**	**Purpose**
Hydrocortisone	4µg/ml	Increase proliferation
EGF	0.02µg/ml	decrease senescence, increase migration
Transferrin	5µg/ml	
Insulin	5µg/ml	Increase proliferation
Cholera Toxin	$1x10^{-4}$µM	Increase proliferation
Tri-iodothyronine	$2x10^{-5}$µM	
Adenine	180µM	Increase proliferation

As the study progressed, it was learned that serum induced squamous differentiation in bronchial epithelial cells (Lechner, et al., 1984). When bronchial cells undergo squamous differentiation they are considered metaplastic. RG media induced squamous differentiation of the HBECs. The Laboratory of Human Carcinogenesis (LHC) medium developed by Lechner and Laveck is serum free and supports clonal growth of human bronchial epithelial cells (Lechner, et al., 1982). This medium contains supplements that induce the differentiation of un-immortalized bronchial epithelial cells (Matsui, et al., 1998). Major components in RG and Matsui media are compared in table 2.3. This media has Epidermal Growth Factor (EGF), insulin, T3, epinephrine and Bovine Serum Albumin (BSA) as factors that promote proliferation. In addition to these factors, the media also had Bovine Pituitary Extract (BPE), Ethanolamine (ET) and Phospho-Ethanolamine (PET), transferrin, hydrocortisone,

adenine and al-trans Retinoic Acid (RA) as differentiation inducing agents. This media is referred to as Matsui media henceforth. The effects of RG and Matsui media on the behavior of HBECs in three dimensional cultures were compared.

Table 2.3 Comparison of Components in Rheinwald Green & Matsui Media

Factor	**RG Media**	**Matsui Media**
Hydrocortisone	0.4 µg/ml	0.2 µM
EGF	20 ng/ml	0.5 ng/ml
Transferrin	5 µg/ml	10 µg/ml
Tri-iodothyronine	0.02 nM	10 nM
Insulin	5 µg/ml	5 µg/ml
Adenine	180 µM	178 µM
Cholera Toxin	10^{-10} M	-
Ascorbic Acid	50 µg/ml	-
Ethanolamine	-	0.5 µM
Phospho-Ethanolamine	-	0.5 µM
Bovine Serum Albumin	-	0.5 mg/ml
Epinephrine	-	3.3 µM
Retinoic Acid	-	50 nM
Bovine Pituitary Extract	-	0.22 mg/ml

CHAPTER 3

MATERIALS AND METHODS

3.1 Cell Lines

3.1.1 Immortalization of HBECs

Tissue samples were obtained with informed consent from patients undergoing surgical bronchial resections or bronchoscopies at St. Paul Hospitals affiliated to the University of Texas Southwestern Medical Center at Dallas (UTSWMCD). The samples were transported in media immediately after surgery. As a first step, each tissue is minced up into small pieces. The tissue explants are allowed to grow in partially defined Keratinocyte Serum Free Medium (K-SFM, Catalog# 17005, Gibco, Gaithersburg, MD) supplemented with BPE and EGF (Catalog# 37000, Gibco, Gaithersburg, MD), and antibiotic/antimycotic solution [penicillin (10,000 IU/mL), streptomycin (10,000 mg/ mL), amphotericin B (5,000 IU/mL); Gibco, Gaithersburg, MD]. Serum free medium is used since serum has a differentiating effect on the HBECs. They are left undisturbed in a low oxygen incubator (flushed gas mixture of 2% O_2, 5% CO_2 and 93% N_2) at 37°C for about 10 days. Unimmortalized primary HBECs are maintained in low oxygen in order to alleviate the damage resulting from of oxidative stress, due to $p16^{INK4A}$ induction. If the media looks turbid or spent at the end of a week, the culture is checked under the microscope to see if the HBECs have attached. When there are cell outgrowths in many tight colonies, the cells are ready to

be trypsinized. The cells are first washed with 1X Solution A (10X stock formulation is 71.5g HEPES, 4ml 10N NaOH, 7.2g glucose, 2.24g KCl, 71.3g NaCl, 1.42g Na_2HPO_4, 10ml phenol red solution and 700ml water, pH 7.5). Since the HBECs are not as robust as fibroblasts or cancer cells, they are trypsinized using a mild trypsin (Catalog# CC 5012, Cambrex, Walkersville, MD). After the cells have all detached, trypsin neutralizing solution (TNS) (Catalog# CC 5002, Cambrex, Walkersville, MD) is added. The population doubling (PD) when the cells are first split is recorded as zero. Cell PDs are kept track of every time the cells are trypsinized. The PD is calculated using the formula shown in appendix A. Unimmortalized HBECs grow up to about 4 or 5 passages after which they senesce completely. So it is important to immortalize these cells as quickly as possible before they begin to senesce. After the third passage, when the cells are at 50%-70% confluence, pLXSN retroviral constructs from HPV 16 are used to infect HBECs with E6/E7 viral oncoproteins. The time frame for viral infection is a critical one and should be observed carefully in order to successfully immortalize the HBECs. For the infection, 60µl of hexa-di-methionine polybrene solution is added to two milliliters of K-SFM media. Two milliliters of the retroviral construct solution is added to this mixture. The final viral construct mixture is then added to the cells. No more than 18 hours following infection, the viral infected media is replaced with 20ml of fresh K-SFM. The retroviral vectors for Cdk4, and hTERT were constructed using pSRαMSU ($G418^+$) and pBabe ($puromycin^+$) parent vectors respectively. If Cdk4 is introduced into the cells and after a 72 hour recovery period, the infected cells are selected for with media containing 30µg/ml G418. After a few passages when the cells

are healthy and at about 50%-70% confluency, hTERT is introduced and successfully infected cells are selected for after a 72 hour recovery period using 250ng/ml puromycin. The cells are passaged and grown to over a 100 PDs to verify immortalization. The cell line thus immortalized is given the suffix 'KT' which stands for Cdk4-hTERT. The cells that are infected with E6/E7 viral oncoproteins are also passaged serially. After being grown up to a 100 PDs, they are confirmed immortalized. These cell lines are given the subscript E for E6/E7. On the other hand, if they senesce within a 100 PDs, they are infected with hTERT. This helps them recover from senescence. These cell lines are given the subscript ET which stands for E6/E7-hTERT (Ramirez, et al., 2004).

Table 3.1 Human Bronchial Epithelial Cell Lines

No.	HBEC Pairs		Patient Details		Cancer History	Smoking History
	ET/E	KT	Gender	Age		
1	3ET	3KT	Female	65	No Cancer	Smoker
2	12ET	12KT	Female	55	NSCLC	Ex-Smoker
3	15E	15KT	Male	59	Squamous	Ex-Smoker (100 packs/year)
4	24E	24KT	Female	74	NSCLC	Ex-Smoker (40 packs/year)

3.1.2 Generation of Oncogenic Variants of HBECs

Immortalized HBEC cell lines with mutant K-RAS^{V12} and/or p53 knockdowns were created using cell line HBEC 3KT that was immortalized using Cdk4/hTERT (Sato et al., 2006). Briefly, the pSUPER vector was transfected to generate the small interfering RNA to achieve stable p53 knockdown. The pBabe-hyg vector was transfected to achieve mutant K-RAS^{V12} expression. The oncogenic variants of HBEC 3KT were generated by retroviral infection of the cell line with the viral constructions

described. Three cell lines HBEC 3KT-$p53_{kd}$, HBEC 3KT-k $RAS^{V12}{}_{mut}$ and HBEC 3KT-$p53_{kd}$-k $RAS^{V12}{}_{mut}$ were used in this study. The subscript 'kd' stands for knockdown and the subscript 'mut' stands for mutated.

Table 3.2 HBEC Oncogenic Variants

No.	Cell Line	Oncogenic Variation
1	HBEC 3KT-$p53_{kd}$	p53 Knockdown
2	HBEC 3KT-k $RAS^{V12}{}_{mut}$	K-RAS Mutation
3	HBEC 3KT-$p53_{kd}$-k $RAS^{V12}{}_{mut}$	p53 Knockdown & K-RAS Mutation

3.1.3 Fibroblasts

Three normal lung fibroblast cell lines (see table 3.3), two adult normal fibroblasts 35FL (Coriell Institute for Medical Research (CIMR), AG02603) and 61ML (CIMR, AG02262A) and the fetal fibroblast IMR-90 (ATCC, CCL-186) were obtained as a kind gift from the Shay-Wright laboratories. The fibroblast cell line FBL15 was isolated from biopsies of bronchial tumors (see table 3.3). All fibroblasts were grown in XS medium which is a prepared mixture of Medium 199 (Catalog #31100-035, Invitrogen, Carlsbad, CA) and Dulbecco's Modified Eagle Medium (DMEM) (Catalog #12100-046, Invitrogen, Carlsbad, CA) in the ratio of 1:3, 17g sodium bicarbonate, 0.44g sodium pyruvate (Catalog# P5208, Sigma-Aldrich, St. Louis, MO) supplemented with 10% Fetal Bovine Serum (Catalog# 100-500, Gemini Bio-Products, Sacramento, CA). Cell lines were incubated in a humidified environment at 37°C, 5% CO_2. The culture medium was replaced once every four days. They media was thus replenished twice a week and the cultures when confluent were trypsinized with trypsin (Catalog #25300-054, Gibco, Gaithersburg, MD). XS media is used as the trypsin neutralizer.

The fibroblasts were unimmortalized and hence cells with young PDs were used in the experiments.

Table 3.3 Lung Fibroblasts

No.	Cell Line	Patient Details		Tissue Sample Description
		Gender	Age	
1	35FL	Female	65	Apparently Normal
2	61ML	Male	55	Apparently Normal
3	FBL15	Male	59	Squamous Carcinoma
3	IMR-90	Female	Fetal	Apparently Normal

3.1.4 Lung Cancer Cells

Details of cancer cell lines generated from patients with bronchial tumors used in the study are shown in table 3.4. These cell lines are grown in RPMI-1640 supplemented with 5% FBS. All the cells were incubated in a humidified environment at 37○C, 5% CO2.

Table 3.4 Lung Cancer Cells

No.	Cancer Cell Lines	Patient Details	Phenotype
1	Calu3	25 year old Male lung	Adenocarcinoma
2	A549	58 year old Female lung	Alveolar Carcinoma
3	H460	Male lung	Large Cell

3.2 Differentiation Assays

3.2.1 Preparation of Collagen Gels

Type I collagen extracted from rat tail tendon (Catalog# 08-115, Upstate Biotech, NY) was used at a concentration of 4mg/ml. A mixture called the 'premix', was prepared using 10X MEM (Catalog #11430, Gibco, Gaithersburg, MD)**,** Sodium Bicarbonate (71.5mg/ml), Gentamycin Sulfate (Catalog# G1397, Sigma-Aldrich, St.

Louis, MO), New Born Calf Serum (Catalog# 16010-167, Gibco, Gaithersburg, MD), and L-Glutamine (Catalog# G7513, Sigma-Aldrich, St. Louis, MO) (see table 3.5).

Table 3.5 Collagen Premix Composition

Components	**Volume Required for a 12-Well Plate (ml)**
10X MEM	2.2
L-glutamine	0.2
Gentamicin sulfate	0.025
Sodium bicarbonate	0.7
New Born Calf Serum	2.5

The fibroblasts are washed with solution A and trypsinized. Two million cells are re-suspended in 2ml of XS medium. The cell suspension is then placed on ice. Filtered distilled water is added to the collagen I solution and the mixture is pipetted up and down. The total volume of water and collagen I solution should equal 18.5ml. The ratio of collagen to water is determined based on the concentration of the batch of collagen I received. For example, if the concentration of the collagen I batch is 4mg/ml, then 7.0ml of collagen is added to 11.5ml of water. A total of 5.7ml of the premix is then added to the mixture of distilled water and collagen I solution. This solution is neutralized using sodium hydroxide in increments of 50µl, to pH balance the premix-collagen solution. If the premix was freshly prepared, this solution is yellow in color. Sodium hydroxide is added to the premix-collagen solution until the color of the solution turns into a salmon pink. As the premix ages, the solution becomes more basic in nature and hence this step can be omitted. The mixture is pipetted several times to thoroughly mix all the ingredients and 0.5 ml of this solution is pipetted into each well of a 12 well plate and incubated for 6 minutes. About 4 minutes into the incubation, the

fibroblast cell suspension is added to the remaining mixture of collagen, water and premix, and pipetted several times. Care should be taken to avoid formation of bubbles. At the end of 6 minutes of incubation, the collagen in the well plate must have become a gel. The well plate is removed from the incubator and 1.5 ml of the remaining cell mixture is added to each of the 12 wells. The plate is then incubated for 1 hour and 20 minutes at the end of which the collagen becomes a gel and appears milky and translucent. Approximately 2ml of XS media is then added to each well. Using a 10µl micropipette tip, the gels are released from the sides of the wells. The gels might have to be lifted from under in order to detach the collagen gels from the bottom of the wells. At the end of the first 24 hours, the gels usually contract by approximately 50% of their original diameter as shown in figure 3.1.

The gels are allowed to contract completely over a period of 4-10 days at the end of which they are roughly around 4mm in diameter. The media in the wells is not replaced during the gel-contraction phase. At the end of 8 to 10 days, the collagen gels usually contract by about 80%.

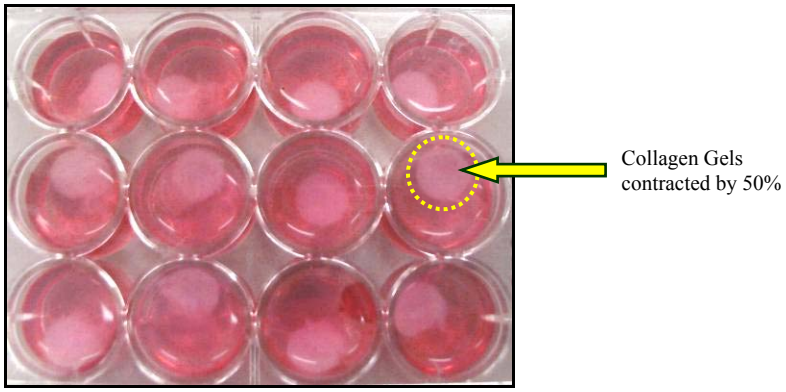

Figure 3.1 Collagen Gel Contraction after 24 hours

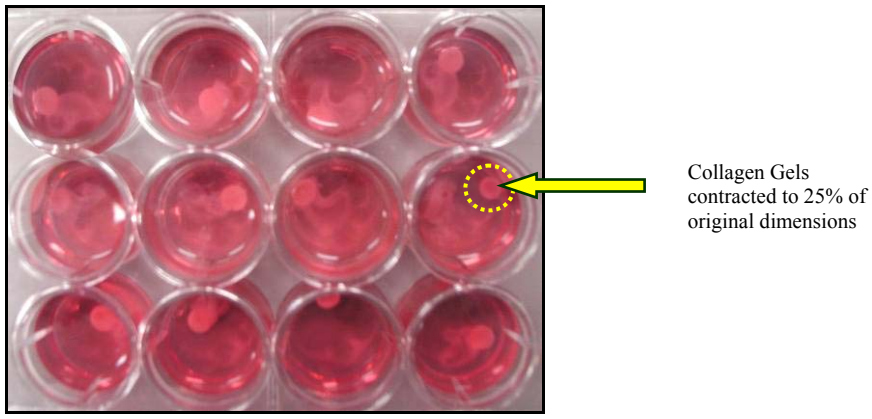

Figure 3.2 Collagen Gel Contraction after 8 days

3.2.2 Plating of Epithelial Cells on Collagen Gels

At the end of the contraction phase, the collagen gels are ready to serve as substrates for the HBECs. The HBECs are trypsinized and re-suspended in differentiation media such that each ml of media has about 520,000 cells. The cell suspensions are then placed on ice. The collagen gels are lifted carefully from the 12 well plates using forceps and placed in the upper wells of a 6 well transwell plate. Up to four plugs can be placed in one well but usually three gels are placed in one well so there is not too much competition among cultures for nutrients supplied by the media between the cells. Glass cloning cylinders are placed atop each collagen gel and 50µl of the HBEC cell suspension that was placed on ice is added to each cloning cylinder. This volume has approximately 26,000 epithelial cells.

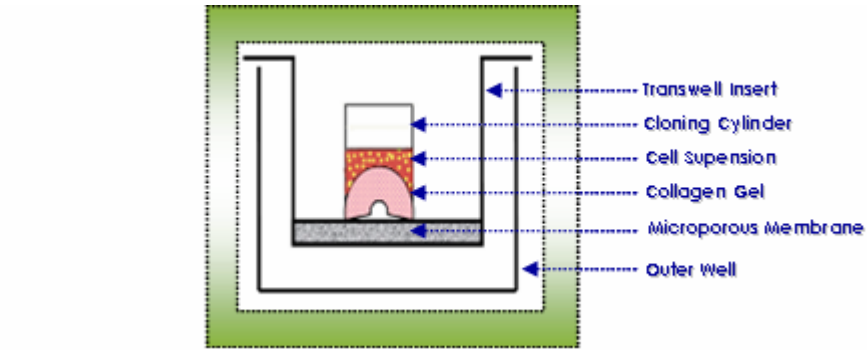

Figure 3.3 Schematic Representation: Plating HBECs on collagen gels

Figure 3.4 Photograph: Plating HBECs on collagen gels

The cylinders were initially removed after 5.5 hours of incubation at a 37°C, 5% CO_2 as suggested in the skin model protocol. The HBECs take approximately 12 hours after plating, to attach to collagen coated tissue culture plastic. Hence the protocol was modified to remove the cylinders after overnight incubation. The cultures are left undisturbed for a period of 4 days after which the media from the upper well is removed and the plugs are raised to the air-liquid interface. The media in the lower well is also changed. In the initial set of experiments the media in the lower well was replaced every

2 days. The cells were however using the media extensively as was apparent by the change in media color from red to yellow in two days. Thereafter the media was replaced everyday.

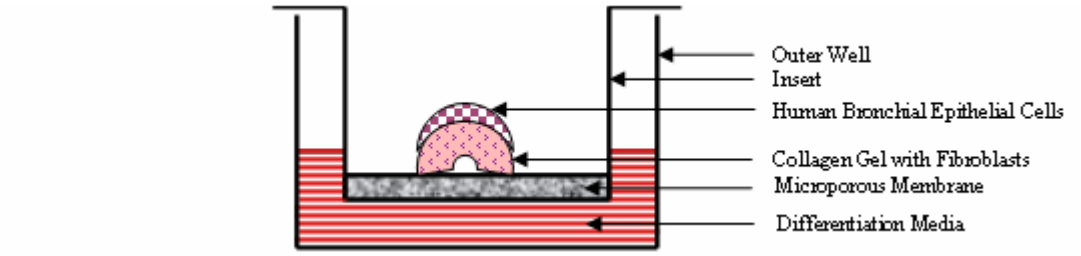

Figure 3.5 Schematic Representation of Three Dimensional Culture System

3.2.3 Fixing the Collagen Gels

On the 21st day, counting from the day the collagen gels are raised to the air-liquid interface, the media is removed from both wells and the collagen gels are fixed in 10% Neutral Buffered Formalin (NBF). The formalin is left on the collagen gels overnight at 4ºC, after which the formalin replaced with Phosphate Buffered Saline (PBS). The fixed cultures are now ready for processing.

3.2.4 Processing, Sectioning and Staining of Collagen Gel Cultures

The fixed collagen gels are then processed, sectioned and stained. Briefly, the gels are removed from the PBS and cut in two semi-circular halves. They are then embedded in cassettes that are filled with paraffin. After they are paraffin embedded, they are processed in a processor. They are then sectioned using a microsectioning sectioning device and then mounted on slides. The sections are then stained with Hematoxylin and Eosin dyes.

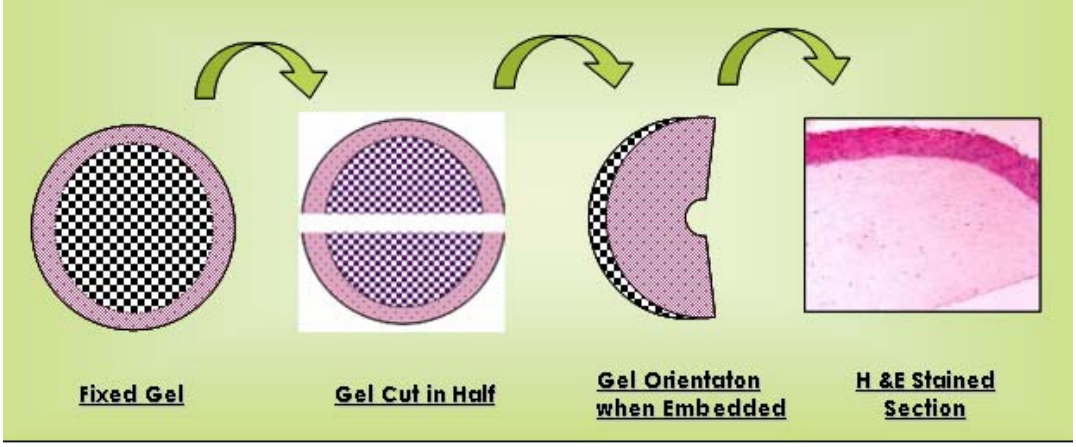

Figure 3.6 Schematic Representation of Three Dimensional Culture System

Table 3.6 Timeline of an Experiment

Step No.	Experimental Phase	No. of Days
1	Growing cells to 90% confluence	03
2	Pouring collagen gels	01
3	Plugs contracting	10
4	Seeding cells on gels	01
5	Incubating gels submerged in media	04
6	Incubating gels in air-liquid interface	21
7	Fixing gels and sending to histology	01
8	Getting result of staining	10
Total no. of days to complete one experiment		51

3.3 Zymogram Assay

The procedure for zymography of proteases in denaturing polyacrylamide gels was followed (Quesada, et al., 1996). The zymography was performed in an SDS-polyacrylamide gel containing gelatin (1mg/ml). Samples to be tested were mixed with one part Tris-Glycine SDS sample buffer (2X) and allowed to stand for 10 minutes at room temperature. The samples are not heated. Samples are applied (typically 10ul) and the gel is run with 1X Tris-Glycine SDS running buffer at standard running conditions

(~125V, constant voltage) for 60-120 minutes. The run time depends on the gel percentage, and running buffer concentration and pH. When the bromophenol blue tracking dye reaches the bottom of the gel, the run is stopped. A 10X stock of the zymogram renaturing buffer (2.5% Triton X-100 in 50mM Tris-HCl, pH 7.5) is diluted 1:9 with deionized water. The gel is incubated with 100ml of the renaturing buffer with gentle agitation for 30 minutes at room temperature. The denaturing buffer is decanted and replaced with 100 ml of 1X developing buffer (50mM Tris base, 50mM Tris-HCl, 0.2M NaCl, 5mM CaCl2, 0.02% Brij 35, distilled water). The gel is equilibrated at room temperature for 30 minutes with gentle agitation. The gel is then incubated at 37°C with fresh 1X zymogram developing buffer for at least four hours. For maximum sensitivity, the gels are incubated overnight at 37°C. The gels are then stained with Coomassie blue R-250 for 30 minutes, to detect the presence of metalloproteases. The gels are then destained with appropriate Coomassie R-250 destaining solution (methanol, acetic acid, water in the ratio of 50:10:40). Areas of protease activity where the protease has digested the gelatin substrate appear as a clear band against a dark blue background.

CHAPTER 4

RESULTS

4.1 Effect of Media on Differentiation

The K-SFM, in which two-dimensional cultures of HBECs are maintained, is a proliferation inducing medium. The EGF and BPE supplements in K-SFM aid clonal growth of HBECs. Media has an undeniably significant role to play in the differentiation of epithelial cells. K-SFM does not contain factors necessary for inducing differentiation of bronchial epithelial cells. RG media has been used commonly to maintain organotypic cultures of the skin in which keratinocytes have been shown to undergo complete stratification and differentiation. RG media was therefore considered more appropriate for inducing differentiation of the HBECs compared to K-SFM. Preliminary results confirmed that HBECs aligned themselves better on the collagen gel when cultures were maintained in RG media. The results are as shown in figure 4.1. In cultures maintained in the K-SFM media, the epithelial cells did not show good organization or polarization. As expected however, they showed much better alignment and a somewhat organized epithelial layer in the RG media. Hence RG media was used in subsequent experiments.

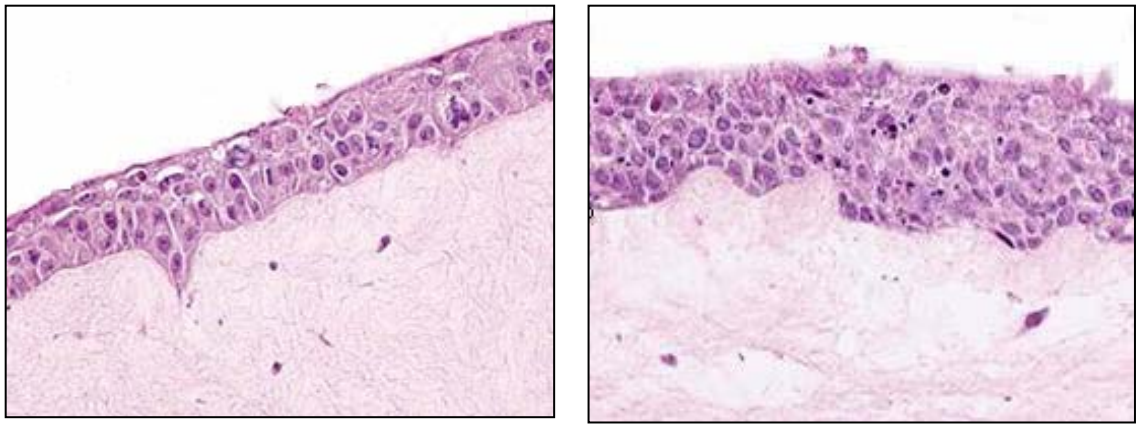

Figure 4.1 Rheinwald Green media is superior to KSFM for HBEC differentiation
HBEC culture in RG media to the left and K-SFM to the right, 20X Magnification

In time it was discovered that RG media only induced squamous metaplasia or dysplasia in the HBECs. Sachs et al., 2003 had used a media that was a 50-50 composition of LHC medium and DMEM to bring about pseudo-stratification of the tracheal epithelium. The inorganic components 'Stock4', 'Stock11' and 'Trace Elements' are no longer available at Biosource (now a part of Invitrogen Corp., Carlsbad, CA) and were omitted from the media preparation. The next step was to compare the effect of the Mastui media and the Rheinwald Green media on the HBECs in the 3D system.

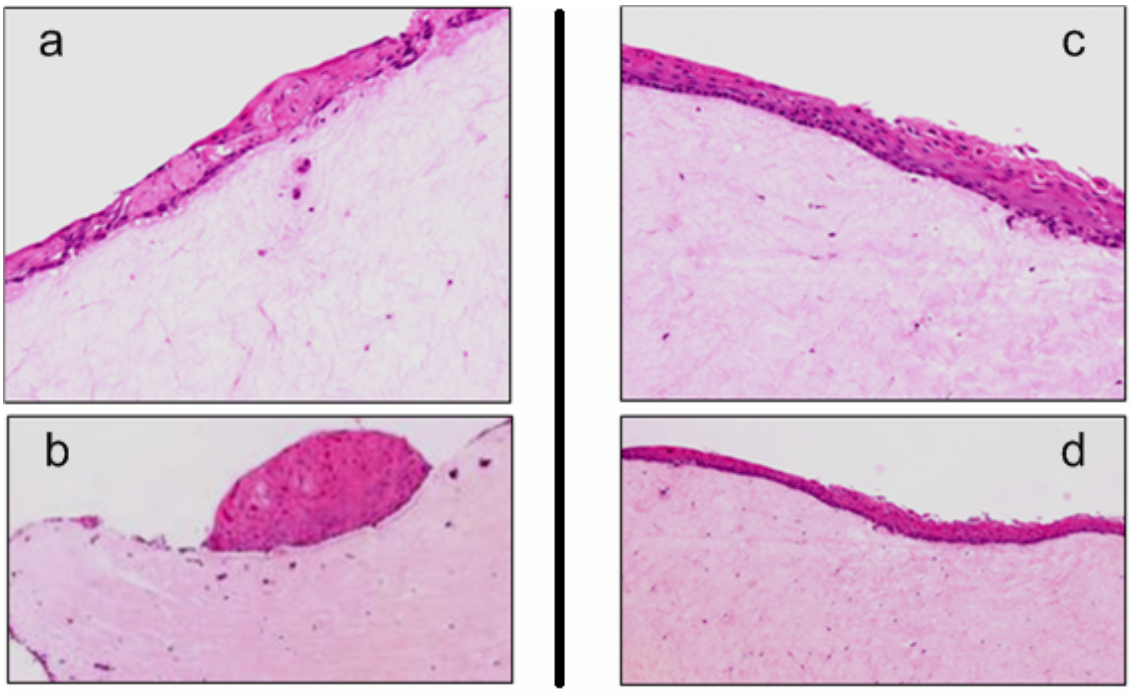

Figure 4.2 (a, b) show HBEC 12KT on 35FL fibroblasts in RG media at 10X and 4X magnifications. 4.2 (c, d) show HBEC 12KT on 35FL fibroblasts in Matsui media at 10X and 4X magnifications.

Figure 4.2 illustrates better differentiation of HBECs in Matsui media. After a series of experiments, it was concluded that the Matsui medium induced squamous stratification but failed to produce the desired pseudo-stratification of HBECs. Although both the RG and Matsui media induced squamous differentiation of the HBECs, there were other phenotypic effects that these media had on the cells. The HBECs were more malignant-like and invaded into the collagen gel to a greater depth, when maintained in RG media than when maintained in Matsui media. Often, the cells looked dysplastic and abnormal in RG media. Therefore it was concluded that the Matsui media induced a more normal phenotype in HBECs compared to RG media.

On delving into the reasons behind the failure of HBEC pseudo-stratification in Matsui media, it was discovered that Sachs et al., 2003 had left out the calcium chloride that Matsui et al., 1998, had originally used in their media. Calcium chloride has been documented to be important for inducing differentiation. This was tested by growing cultures Matsui medium with 0.3mM calcium chloride.

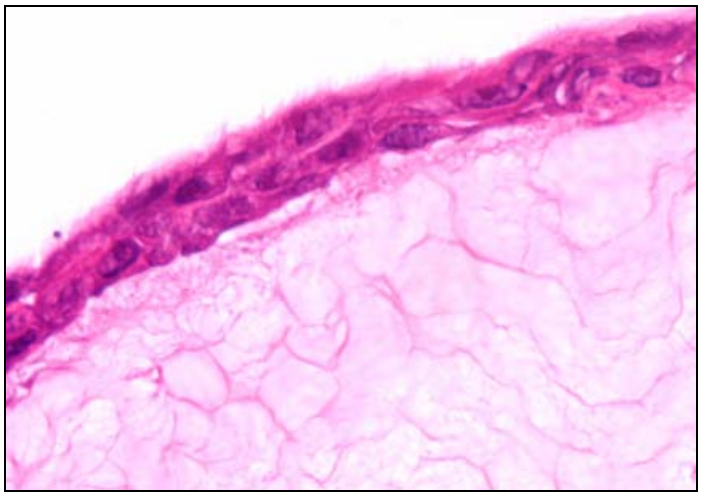

Figure 4.3 shows HBEC 12KT on 35FL fibroblasts in Matsui media with added calcium chloride; possible presence of cilia, basement membrane and goblet cells, 60X magnification

4.2 Number of Weeks Needed to Maintain Organotypic Cultures

The organotypic cultures of the skin keratinocytes were maintained in the air-liquid interface for a period of four weeks. Initially the HBEC 3D cultures were also maintained for four weeks. Any time saved would appreciably improve the feasibility of an assay that takes more than a month to complete. Therefore, growth of cultures was compared at the end of each week of a four week period. The results (data not shown) indicated that a period of three weeks was sufficient for the development of the three

dimensional culture as there were not any significant differences between cultures maintained for three and four weeks.

4.3 HBECs Immortalized with E6/E7 Oncoproteins are Invasive

It is believed that the HBECs that were immortalized using the HPV 16's E6/E7 oncoproteins are transformed in nature compared to the HBECs that were immortalized with Cdk4/hTERT (Ramirez, et al., 2004). One of the main aims of this project was to study the differences between the effects of the two different immortalization methods on HBEC phenotypic behavior, if any. The first comparison experiments were conducted using HBEC 3ET and HBEC 3KT in RG media. HBEC 3ET immortalized with E6/E7 dramatically invaded into the collagen gels whereas the HBEC 3KT immortalized with Cdk4/hTERT formed a layer of squamous epithelial cells on top of the collagen gel and did not invade in almost any of the cultures (data not shown).

Behavior of one cell line, HBEC 3 alone, cannot be used to establish the trend in invasive and non-invasive behavior of ET and KT cell strains respectively. Therefore more HBEC cell line pairs were tested, to detect distinct differences that might exist between ET and KT strains. The HBEC pairs 3ET/KT, 12ET/KT, 15E/15KT and 24E/24KT were chosen from the panel of HBEC cell lines. Figure 4.3 shows the phenotypic differences in invasive behavior between the ET/KT HBEC pairs.

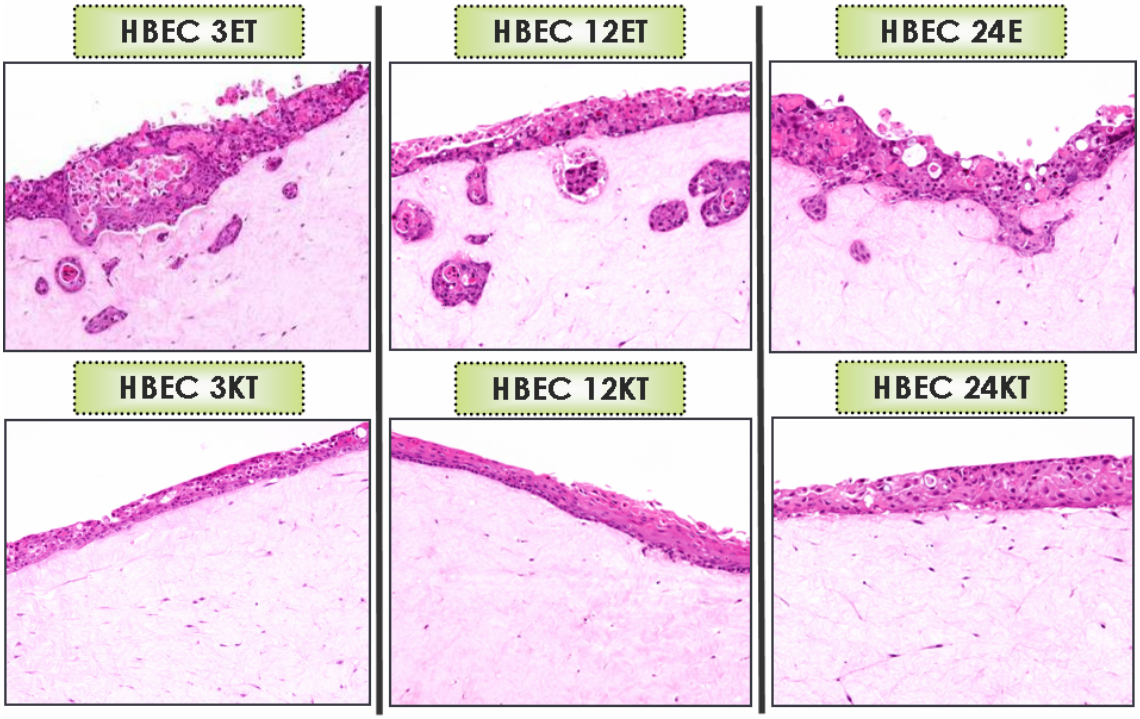

Figure 4.4 the difference in invasive behavior between HBEC ETs and HBEC KTs with 35FL fibroblasts in Matsui media, 10X magnification.

The ET cell lines were expected to exhibit an abnormal phenotypic behavior compared to the KT cell lines. As seen from figure 4.3, HBEC 3ET, HBEC 12ET and HBEC 24ET behaved in a similar fashion, invading extensively through the collagen gel. HBEC 3KT, HBEC 12KT and HBEC 24KT on the other hand, showed almost no invasion into the collagen gels. HBEC15 was an exception in that both HBEC 15E and HBEC 15KT showed extensive invasion through the collagen gels as seen in figure 4.4.

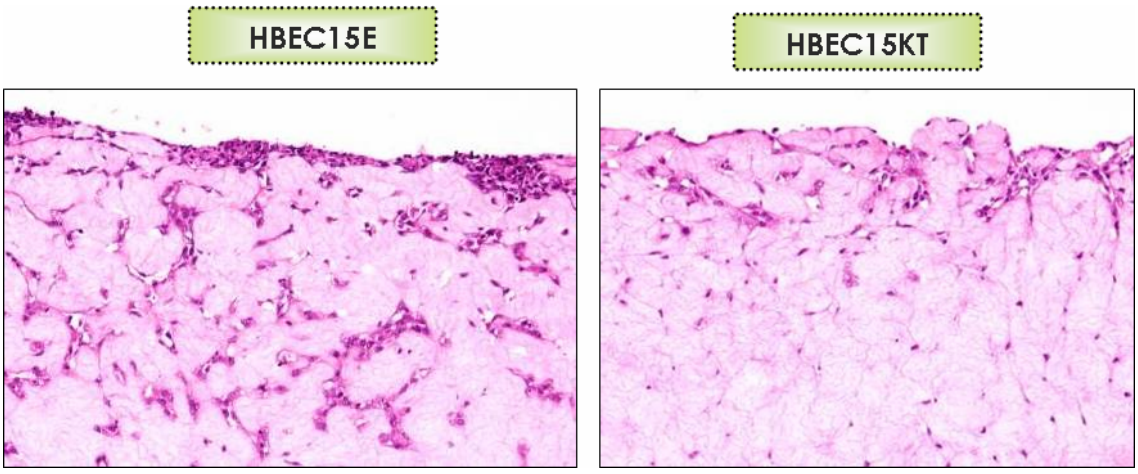

Figure 4.5 shows HBEC 15E and 15KT on 35FL fibroblasts in Matsui media, 10X magnification

It is to be noted that HBEC 15 and 24 did not receive any hTERT when immortalized with E6/E7 oncoproteins. HBEC 15E and HBEC 15KT repeatedly behaved in the same fashion as shown in figure 4.4 irregardless of fibroblast type or media conditions.

The lung cancer cell lines H460, A549 and Calu3 however did not invade through the collagen gels (data not shown). These were not further explored for lack of sufficient time.

4.4 Fibroblasts Influence HBEC Invasion

The HBEC KT cell lines which were not invasive in cultures with 35FL fibroblasts were invasive in cultures with 61ML fibroblasts. The HBEC ET cell lines were invasive when using both fibroblasts but the extent of invasion was more in cultures with 61ML fibroblasts.

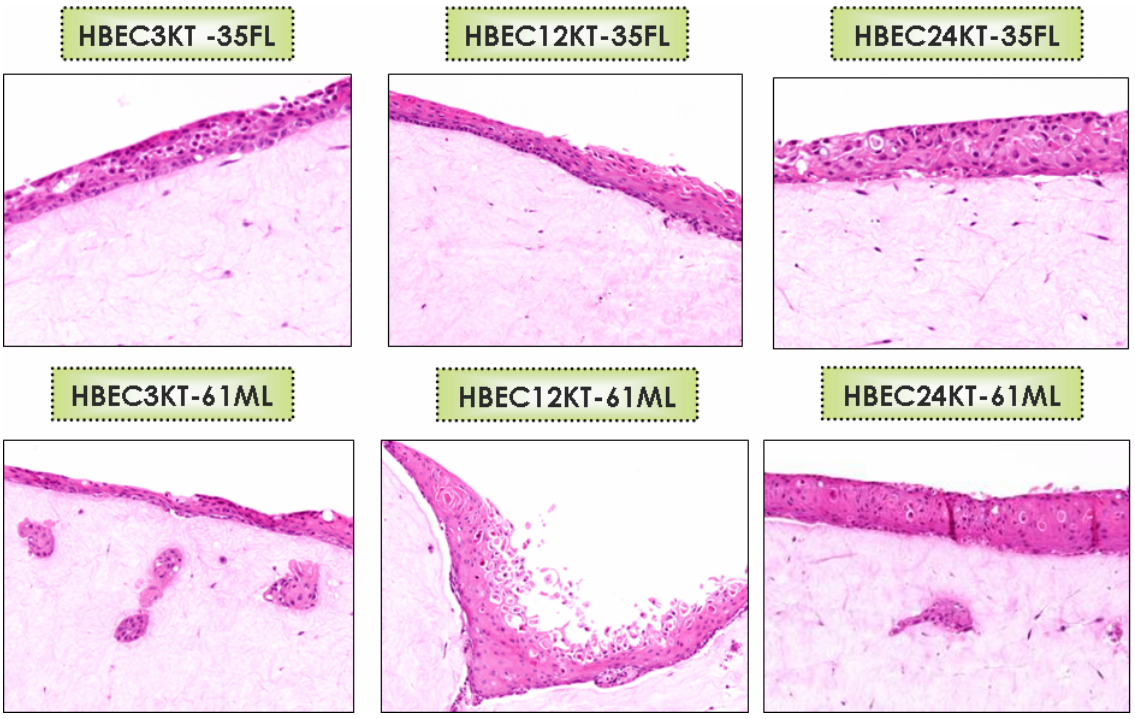

Figure 4.6 shows the difference in invasive behavior of HBEC KTs on collagen gels with 35FL and 61ML fibroblasts in Matsui media, 10X magnification.

The immortalized HBECs were maintained in cultures for long periods of time and used in the organotypic cultures. As the PD of the HBECs increased due to continued culturing, they began exhibiting an abnormal phenotype. HBEC 3KT which was initially non-invasive for the most part in RG media, began invading the collagen gels. This process progressed over time and eventually reached a stage where HBEC 3KT began producing collagenase to digest the entire collagen gel, when cultured with 61ML fibroblasts. Although HBEC 3KT was extensively invasive in cultures using the 35FL, it did not digest the collagen gel as shown in figure 4.6. The collagenase activity was detected by a zymogram assay that showed the presence of active collagenases. The collagenase activity correlated with the difference in HBEC behavior when using 35FL

and 61ML fibroblasts. The cross-talk between HBEC 3KT and the fibroblasts differed between 35FL and 61ML as can be seen from figure 4.7.

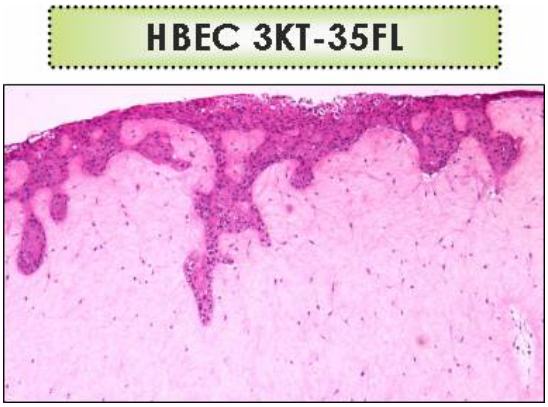

Figure 4.7 shows the invasion of HBEC 3KT of older PDs, in cultures with 35FL fibroblasts under 4X magnification

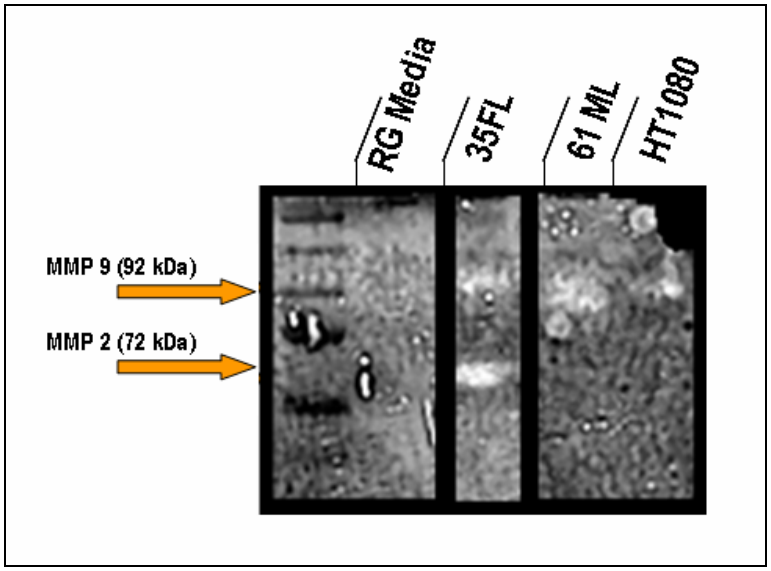

Figure 4.8 shows the difference in MMP activation in cultures of HBEC 3KTs on collagen gels with 35FL and 61ML fibroblasts in RG media.

RG media was used as the negative control and supernatant from HT1080 fibrosarcoma cells was used as a positive control. The media collected from 3D cultures

of HBEC 3KT-35FL had both MMP9 (collagenase I) and MMP2 (collagenase IV), although MMP2 was the activated form present, as can be seen from figure 4.7. The media collected from 3D cultures of HBEC 3KT-61ML had very high concentrations of activated MMP9 as seen from the 92kDa band in figure 4.7. This explained the complete degradation of the type I collagen gel culture.

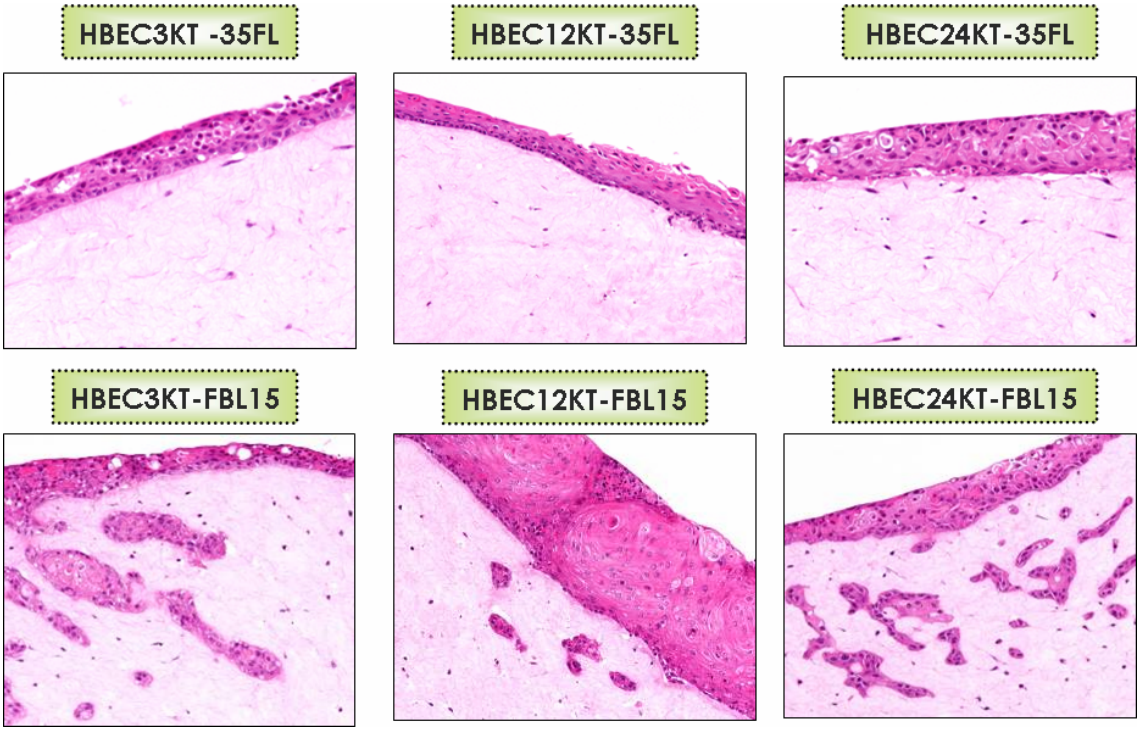

Figure 4.9 shows the difference in invasive behavior of HBEC KTs in collagen gels with 35FL and FBL15 fibroblasts in Matsui media, 10X magnification.

The fibroblast FBL15 was derived from a squamous cell lung cancer tissue sample. When used in the organotypic cultures, these fibroblasts induced invasive behavior of the HBECs as seen in figure 4.8. The invasion exhibited by HBECs with FBL15 embedded in the collagen gel was greater than the invasion induced by 61ML fibroblasts.

4.5 Fetal Fibroblasts Induce HBEC Invasiveness

The fetal fibroblast IMR99 induced invasive behavior in the all the HBECs to some degree (data not shown). The fetal fibroblasts were in vitro aged in the cultures.

4.6 Invasiveness of Oncogenic Variants of HBECs

Oncogenic variants of HBEC 3KT were shown to invade into the three dimensional collagen gel cultures (Sato, M. et al., 2006). Three of the oncogenic variants of HBECs included in this study proved to be invasive and conformed with published data. The degree or depth of invasion varied among the three cell lines. HBEC 3KT-$p53_{kd}$ cell line showed the least invasive behavior and the cell line with the combined hits, HBEC 3KT-$p53_{kd}$-K- $RAS^{V12}{}_{mut}$ exhibited the most invasive behavior with the HBEC 3KT-K- $RAS^{V12}{}_{mut}$ cell line falling somewhere in between the other two cell lines as show in figure 4.8. It is to be noted that these cell lines did not form tumors in nude mice as shown by Sato M. et al., 2004. HBEC 3KT in the same experiment, formed a non-invasive monolayer (data not shown).

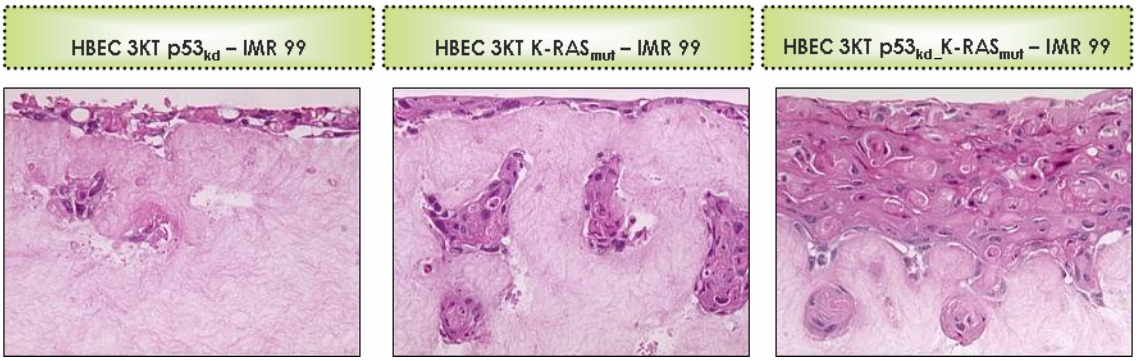

Figure 4.10 shows the difference in invasive behavior of oncogenic variants of HBECs on IMR 99 fetal fibroblasts, 20X magnification.

CHAPTER 5

DISCUSSION AND CONCLUSIONS

5.1 Discussion

Differentiation of epithelial cells has been difficult to establish in culture. Studies conducted with different culture conditions for 3D models, indicate that culture media is one of the important components in bringing about differentiation in vitro. Documented evidence indicates that Rheinwald Green media brings about squamous stratification of human keratinocytes. Bronchial epithelial cells are not supposed to be squamous stratified, but columnar pseudo-stratified. HBECs most often exhibited stratified squamous metaplasia in the 3D collagen gel cultures. Epithelial cell populations are mixed populations of differentiating and propagating cells that are widely exclusive of each other. Cultures of selected HBECs are non-homogenous, with populations of undifferentiated cells and other cells at various stages of differentiation. The Rheinwald Green media which helps keratinocytes to proliferate and differentiate has high concentrations of various proliferation inducing factors. The media described by Matsui was able to induce pseudo-stratification and ciliogenesis of unimmortalized tracheal epithelial cells (Matsui, et al., 1998). EGF and cholera toxin are proliferation inducing factors in RG media. The Matsui media has no cholera toxin but the RG media has. Matsui media has an EGF concentration that is 100 fold less than that in RG media. Tri-iodothyronine (T3) which is a known differentiation inducing factor is however at a

10nm concentration in the Matsui media which is 500 times greater than the concentration of T3 in RG media. The Matsui media which brought about better differentiation of HBECs in the 3D collagen gels is superior in comparison to RG media as far as bronchial epithelial differentiation is concerned.

Sachs et al., 2003, had left out calcium chloride from the formulation of media described by Matsui. Low concentrations of calcium chloride < 0.1mM are known to cause proliferation and high concentrations of calcium chloride > 0.3mM, is known to be important for inducing differentiation. Addition of 0.3mM calcium chloride to Matsui media induced better differentiation of HBECs evidenced by an organized layer of basal cells and presence of ciliation. Calcium chloride dose curves need to be studied to determine the appropriate concentration to be administered to the cells via media. All these results indicate that the media used to culture organotypic models of the bronchial epithelium plays a critical role influencing differentiation and phenotypic behavior.

HBECs immortalized with E6/E7 oncoproteins have the potential to become oncogenic when growing in conditions conducive to tumorigenicity (Coursen et al., 1997). HBECs immortalized with E6/E7 viral oncoproteins have been shown to have an unstable genome and a number of chromosomal aberrations (Ramirez et al., 2004). These cell lines have lost their p53 checkpoint. They have a number of other mutations and pathways that have gone askew such as the $p16^{INK4A}$-Cyclin D1-Cdk4-RB one. These genetic aberrations endow the E6/E7 immortalized HBECs with a phenotype that could potentially lead to malignancy.

In organotypic cultures of human keratinocytes, E6 oncoprotein of HPV 8 has been shown to cause invasion of keratinocytes into the dermis (Blanton, et al., 1991), (Akgul, et al., 2005). Therefore it was no surprise that all HBECs 3ET, 12ET, 15E and 24E immortalized with E6/E7 oncoproteins and/or hTERT are highly invasive in the three dimensional culture models (see figure 4.5). The HBEC ETs invaded no matter which fibroblasts were used. They invade no matter which media is used.

The HBECs immortalized with Cdk4/hTERT on the other hand, showed significantly better epithelial morphology and a non-invasive phenotype when fibroblast cell line 35FL is used (refer figure 4.5). Human keratinocytes enforced to express hTERT and bypass $p16^{INK4A}$ attain immortality yet retain normal differentiation characteristics (Dickson, et al., 2000). When in RG media, the HBECs occasionally invaded even when 35FL fibroblasts were used. However they did not invade when Matsui media was used. This further proves that the Matsui Media induced a more normal phenotypic behavior in the HBEC KTs.

HBEC 3KT, 12KT and 24KT, did not invade when fibroblasts 35FL was used in the collagen gels maintained Matsui media. The 35FL bronchial fibroblasts were derived from a young 35 year old female donor. These same HBECs invaded when fibroblasts 61ML were used in the collagen gels. These fibroblasts were obtained from an older 61 year old male donor. HBEC 15KT stood out from the rest of the HBEC KTs, invading regardless of the culture conditions or the fibroblasts used.

HBEC 3 cell line was established from the bronchial tissue of a donor who did not have any cancer at the time of donation. HBECs 12 and 24 were generated from

patients who had NSCLC. The HBEC 15's were generated from the bronchus of a 59 year old male with squamous cell cancer who had a smoking history of a 100 packs a year until he quit. It is premature to conclude anything based on these facts but experiments conducted in future could be used to explore any possible differences in phenotypes of HBECs from patients with squamous cell cancer or NSCLC.

Technically, tumor cells should be invasive in order to be malignant but often, cells isolated from a patently malignant human tumor form tumors in nude mice but do not invade or metastasize. The oncogenic variants of HBECs all invaded the collagen matrices and to different depths. Oncogenic variants of immortalized HBECs exhibit significantly greater anchorage independent growth in soft agar than normal immortalized HBECs (Sato et al., 2006). Transformed cell lines also exhibit higher proteolytic activity compared to untransformed cell lines (Mahdavi & Hynes, 1979).

Epithelial-Mesenchymal interaction is believed to play a critical role in carcinogenesis. It is theorized that aging fibroblasts secrete factors that support epithelial invasion into the stroma that eventually leads to metastasis (Parinello, et al., 2005). The aging fibroblasts stimulate epithelial cell invasion by secreting matrix metalloproteases (MMPs) and other proteases that modify the extracellular environment by degrading collagen, basement membrane proteins etc. Senescent fibroblasts were shown to promote epithelial cell growth and tumorigenesis (Krtolica, et al., 2001). Senescent fibroblasts were also shown to alter mammary epithelial cell differentiation (Parrinello, et al., 2004). In these studies, the senescent fibroblasts induced tumorigenesis of preneoplastic and neoplastic epithelial cells. However they had very

little impact on normal epithelial cells. The HBECs used in this study were however immortalized and hence not completely normal as immortalization is one of the earliest events that occur in carcinogenesis. Also, Cdk4 gene expression was found to be at a high level in 23% of lung tumors (Wikman, et al., 2005). The HBECs were immortalized with Cdk4 and hTERT which are both known to be active in most cancer cells.

The HBEC KT cell lines which were not invasive in cultures with 35FL fibroblasts were invasive in cultures with 61ML fibroblasts. Among the adult fibroblast cell lines used in the organotypic cultures, fibroblasts derived from the 61 year old male and not the ones generated from the 35 year old female, influenced HBEC KTs to behave abnormally and were permissive to HBEC invasion. The HBEC ET cell lines were invasive when using both fibroblasts, but the extent of invasion was greater in cultures with 61ML fibroblasts.

Experiments performed initially with HBEC 3KT maintained in RG media also pointed to the fact that in vitro aged fibroblasts from the aged 61 year old donor were permissive to invasion (figure 4.5). The depth of invasion progressed with time and ultimately reached a stage wherein the entire collagen gel was degraded. Zymogram assay results confirmed the presence of activated MMP9 which degrades type I collagen, the type of collagen the 3D gel is made up of. In vitro aged 35FL fibroblasts derived from the 35 year old donor were permissive for HBEC 3KT to invade under the same conditions but the cultures remained intact and the collagen gel was not degraded. It is evident from these results that significant differences exist between the fibroblasts

and also the interaction between epithelial and stromal fibroblasts. The donor's age seems to be an obvious factor contributing to invasion since both fibroblasts were derived from patients with no apparent disease. The data indicates that stromal invasion of bronchial epithelial cells in three dimensional cultures is connected with events occurring both in the epithelial and also the stromal layer.

Aging fibroblasts contribute to malignant transformation of epithelial cells (Krotilca et al., 2001). It is not yet clear whether such effects are initiated by the fibroblasts themselves or if they are the results of recruitment of mesenchymal fibroblasts called myofibroblasts by the epithelial cells (Micke et al., 2004). EGF concentrations that induced stromal invasion of keratinocytes in organ cultures resulted in an upregulation of MMP-9 as well (Moon et al., 2001). C-met expression of myofibroblasts is thought to constitute an autocrine loop in cancer-stromal myofibroblasts that might play a role in invasion and metastasis of lung adenocarcinoma (Tokunou et al., 2001). Some researchers speculate that epithelial-mesenchymal transition occurs when epithelial cells becoming cancerous (Micke et al., 2004).

Cancer Associated Fibroblasts (CAFs) or myofibroblasts have been shown to be permissive to the invasion of pre-malignant and malignant epithelial cells. Investigators are exploring the possibility of using CAFs as targets in anti-cancer therapy (Micke et al., 2004). The fibroblast FBL15 was derived from a 59 year old male patient with squamous cell cancer. In organotypic cultures maintained in Matsui media, these fibroblasts induced invasive behavior of the HBECs to a greater degree when compared

to 61ML fibroblasts. The invasive behavior observed could be related to the fact that these fibroblasts were from an older donor. However since these tissues were derived from tumor tissue samples, this cell line cannot be included in the same category as the 61ML fibroblasts. It would be interesting to characterize these cell lines in future experiments and see if the association of these fibroblasts with lung cancer had any corresponding influence on the invasion of the HBEC KTs

The oncogenic variants were considered to be advanced in the stages leading to malignancy (Sato et al., 2004). These cell lines did not produce tumors in nude mice and hence cannot be considered to be oncogenic. The cell lines HBEC 3KT-$p53_{kd}$, HBEC 3KT-K-$RAS^{V12}{}_{mut}$, and HBEC 3KT-$p53_{kd}$-K-$RAS^{V12}{}_{mut}$ were used in preliminary experiments on the organotypic cultures. Invasive behavior was expected out of all the three oncogenic variants and results were in conformance with predictions. The degree or depth of invasion varied among the three oncogenic variant lines. HBEC 3KT-p53kd cell line showed the least invasive behavior and the cell line with the combined hits, HBEC 3KT-p53kd-K-$RAS^{V12}{}_{mut}$ exhibited the most invasive behavior with the HBEC 3KT-K-$RAS^{V12}{}_{mut}$ cell line falling somewhere in between the other two cell lines. Loss of p53 combined with mutations in K-RAS is a very common phenomenon that is frequently observed in lung cancer. These preliminary results are highly encouraging and could be pursued in greater depth in the future.

5.2 Conclusions

The organotypic model of the bronchial epithelium used in this project has proven to be an assay that is appropriate for studying invasive behavior of bronchial

epithelial cells. Media used to induce differentiation of HBECs in organotypic cultures clearly plays a very important part in the establishment of a proper three dimensional model. The Matsui medium has been shown to be superior for permitting normal bronchial epithelial cell phenotype and differentiation. The Rheinwald Green medium has been proven to be unsuitable for inducing a normal phenotype and differentiation of HBECs. HBECs immortalized with E6/E7 viral oncoproteins exhibit a highly invasive phenotype with cells looking malignant, thus establishing the fact that E6/E7 oncoproteins when used to immortalize bronchial epithelial cells impart an abnormal phenotype. Possible presence of cilia, basement membrane and basal cell layer obtained in cultures grown in Matsui media with calcium chloride point toward the fact that HBECs immortalized with Cdk4/hTERT retain their ability to differentiate. The phenotypic differences between HBEC ETs and HBEC KTs are clearly distinguishable with some exceptions such as HBEC 15. HBEC KTs are more normal in comparison to HBEC ETs. The mesenchymal fibroblast cells included in the stromal compartment of the organotypic cultures have been shown to play a crucial role in influencing epithelial phenotype, differentiation and invasion. Thus epithelial-mesenchymal interaction has been shown to be of great importance in recapitulating phenotypic behavior of epithelial cells. The fibroblast lines generated from older donors are more permissive to the invasion of HBECs but fibroblast cell lines derived from younger donors are not. Appropriate culture conditions such as using the right media and air-liquid interface to stimulate differentiation of HBECs has a critical role in establishing the nature of epithelial-mesenchymal interactions. Thus differences in the phenotypic behavior of

immortalized human bronchial epithelial cells have been established using three dimensional culture models.

5.3 Future Work

The HBEC KTs most often exhibited squamous stratification, squamous metaplasia or dysplasia in the culture conditions used. The media used for inducing differentiation of HBECs needs to be fine-tuned to induce pseudo-stratification. An optimal number of fibroblasts need to be used in the organotypic models as too many or too few fibroblasts will affect the differentiation of epithelial cells in an inappropriate fashion. Different fibroblasts contract the collagen gels at different rates. The fibroblasts from older donors contract the gels much slower than the fibroblasts from younger donors. This implies that the number of fibroblasts per square centimeter of the collagen gel would be different. The epithelial-fibroblast cross talk could be altered due to this factor. Hence this might be a variable that needs to be controlled. Contraction of collagen gels by varying number of fibroblasts used could be studied to solve this issue.

More HBEC ET/KT pairs could be studied especially taking into account the patient's cancer history to see if it adds to existing knowledge. HBECs and fibroblasts used in this study could be characterized using microarrays or other assays to compare and contrast gene expression, protein expression etc. Close attention needs to be given to HBEC 15 which stood out as an exception from the other HBECs. Influence of age and gender of fibroblast cell line donors on the phenotypic behavior of HBECs needs to be studied in greater detail to come to elaborate conclusions. The relevance of cancer-associated-fibroblasts on epithelial cell invasion is an interesting area of research that

looks very promising. There are thus a number of areas in this project that have the potential to shed greater light on lung cancer pathogenesis.

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