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Branching Morphogenesis of Immortalized Human Bronchial Epithelial Cells in Three-Dimensional Culture

Aadil Kaisani^a, Oliver Delgado^a, Gail Fasciani^a, Sang Bum Kim^a, Woodring E. Wright^a, John D. Minna^{b,c,d}, and Jerry W. Shay^{a,e}

^aDepartment of Cell Biology; UT Southwestern Medical Center at Dallas; Dallas, Texas, 75390; U.S.A

^bHamon Center for Therapeutic Oncology; UT Southwestern Medical Center at Dallas; Dallas, Texas, 75390; U.S.A

^cDepartment of Internal Medicine; UT Southwestern Medical Center at Dallas; Dallas, Texas, 75390; U.S.A

^dDepartment of Pharmacology; UT Southwestern Medical Center at Dallas; Dallas, Texas, 75390; U.S.A

^eCenter for Excellence in Genomics Medicine Research, King Abdulaziz University, Jeddah, Saudi Arabia

Abstract

While mouse models have contributed in our understanding of lung development, repair and regeneration, inherent differences between the murine and human airways requires the development of new models using human airway epithelial cells. In this study, we describe a three-dimensional model system using human bronchial epithelial cells (HBECs) cultured on reconstituted basement membrane. HBECs form complex budding and branching structures on reconstituted basement membrane when co-cultured with human lung fetal fibroblasts. These structures are reminiscent of the branching epithelia during lung development. The HBECs also retain markers indicative of epithelial cell types from both the central and distal airways suggesting their multipotent potential. In addition, we illustrate how the model can be utilized to understand respiratory diseases such as lung cancer. The 3D novel cell culture system recapitulates stromal-epithelial interactions *in vitro* that can be utilized to understand important aspects of lung development and diseases.

Keywords

Fibroblasts; Distal airways; Bronchial epithelial cells; Branching; Differentiation

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Corresponding Author: Jerry W. Shay, Department of Cell Biology, UT Southwestern Medical Center at Dallas, Dallas, Texas, 75390, U.S.A., 1- 214-633-1994, Jerry.Shay@UTSouthwestern.edu.

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Introduction

Due to lack of good *in vitro* models, our understanding of development and stem cell biology of the human lung remains limited. Important aspects of human lung development, repair and regeneration have been studied through the use of mouse models (Morrisey and Hogan, 2010). Although these murine models have provided valuable insights into lung homeostasis and regeneration, there are intrinsic differences between the human and mouse airway epithelia (Rock and Hogan, 2011; Rock et al., 2010). Basal cells are limited to the trachea in mice while they are present throughout the human airways (Boers et al., 1998; Evans et al., 2001). Clara cells (also referred to as club cells) are found throughout the murine airways but are enriched only in the distal bronchioles of the human lung (Boers et al., 1999; Rawlins et al., 2009). Conversely, variant Clara cells have been demonstrated in the murine lung to self-renew and give rise to differentiated progeny in an event of injury, but there is no evidence of these cells in the human lung (Hong et al., 2001; Rackley and Stripp, 2012). These differences support the importance of developing *in vitro* model systems using human airway epithelial cells.

In an effort to recapitulate the native airway architecture and differentiation, different *in vitro* model systems using human bronchial epithelial cells (HBECs) have previously been established (Bals et al., 2004; Fessart et al., 2013; Franzdottir et al., 2010; Pageau et al., 2011). When primary HBECs are cultured on a contracted fibroblast matrix and raised to air-liquid interface (ALI), the HBECs are able to differentiate into ciliated and goblet cells (Vaughan et al., 2006). Although the ALI culture system demonstrates the ability of HBECs to differentiate into lung epithelial cells lining the central lung, it does not address differentiation in the distal airways. Recent studies have also described culturing HBECs in reconstituted basement membrane (Matrigel®) to reproduce a more physiologically relevant microenvironment for cell differentiation (McQualter et al., 2010; Rock et al., 2009). HBECs cultured in three-dimensional (3D) Matrigel® models differentiate into distinct lung epithelial lineages however, these studies did not address the importance of stromal epithelial interactions (Wu et al., 2011). Signaling from the mesenchyme plays a critical role in lung development. Cues from stromal cell types such as fibroblasts, endothelial cells and smooth muscle cells are important in determining epithelial cell fate (Kimura and Deutsch, 2007). These interactions also contribute to respiratory disease such as lung cancer where the stroma plays a critical role in cancer progression and metastasis (Mueller and Fusenig, 2004). Current *in vitro* models also fail to recapitulate phenotypic features such as branching morphogenesis of the distal lung airway during development. These phenotypes have mostly been mimicked using primary tissues from embryonic human and mouse lungs (Miura and Shiota, 2000; Weaver et al., 2000).

Previously, it has also been suggested that ectopically introduced telomerase and cyclin-dependent kinase 4 immortalized HBECs display characteristics of multipotent stem cells of the lung (Delgado et al., 2011). These HBECs express markers indicative of several epithelial cell types from both central and distal airway lineages in two-dimensional culture (2D). When cultured in different types of 3D systems, subtle changes in the microenvironment result in the ability of HBECs to differentiate into multiple central and

distal lung epithelial cell types. In ALI conditions, these HBECs can differentiate into ciliated and goblet cells (Vaughan et al., 2006). When embedded in Matrigel®, HBECs form cyst like structures resembling and expressing markers indicative of cells from the distal lung airways (Delgado et al., 2011). These observations are consistent with the hypothesis that the adult human lung may contain a multipotent stem-like cell capable of differentiating into multiple cell lineages in different microenvironments.

In the present study, we describe a novel HBEC 3D culture system that displays the phenotype reminiscent of lung branching morphogenesis during development. Branching of the lung buds terminating into alveoli, the site of gas exchange during lung development, signifies differentiation of lung epithelium into distal airway cell types such as Clara and Type II pneumocytes (Warburton et al., 2010). We demonstrate that when HBECs are seeded on top of Matrigel®, overlaying fetal lung fibroblasts (IMR90 cells), the HBECs form an aggregate structure that buds and branches (resembling the canalicular/saccular period of lung development). HBECs in this culture system maintain markers of multiple cell types in the airway epithelium indicating their multipotent stem-like characteristics. We also describe how this new 3D system can be utilized to not only understand lung development, but also lung diseases such as lung cancer.

Material and Methods

Cell Culture

Immortalized HBEC3, HBEC13, and the experimentally transformed HBEC3 cells were cultured in Keratinocyte Serum Free media (KSFM) (Gibco) at 37°C and 5 % CO₂. The KSFM media contained 50 µg/mL of bovine pituitary extract and 5 µg/mL of epidermal growth factor. These cells were also cultured on porcine gelatin coated tissue culture dishes (Sigma Aldrich) as described previously (Ramirez et al., 2004). For 3D culture studies, HBECs were cultured in air liquid interface media (Vaughan et al., 2006). IMR90s and other primary fibroblasts derived from normal lung tissues were cultured in DMEM media (Thermo Scientific) supplemented with 10% Cosmic Calf Serum (Thermo Scientific) at 37°C in 5% CO₂ and 2% O₂.

3D Matrigel® Culture

HBECs 3D cultures were performed as previously described (Lee et al., 2007) with minor modifications. IMR90 fibroblasts were seeded as a feeder layer at a density of 1.25×10^5 cells/cm² 48 hours prior to seeding HBECs. Pre-thawed growth factor reduced and phenol-red free Matrigel® (BD Biosciences) was layered on top of the fibroblasts (75 µL/cm²) and then allowed to gel at 37°C for 15 minutes. The Matrigel® plated was at 100% concentration and was not diluted with media. 1.0×10^5 /cm² HBECs were suspended in ALI media and seeded on top of the Matrigel®. Approximately 24 hours after plating the HBECs, ALI medium was removed and replaced with ALI medium containing 10% Matrigel. There is no difference between the phenotype of HBECs cultured on top of Matrigel® with or without the 10% overlay (Supplementary Figure 1). Adding 10% Matrigel aids in extending the integrity of the 3D culture. The 3D cultures were done in

triplicates and repeated multiple times. Cultures were grown at 37°C and 5 % CO₂ for up to 15 days with media being changed every other day.

Immunofluorescence Staining

For 3D immunofluorescence analysis of the HBEC Matrigel® cultures, the aggregated lung budding structures were washed twice with cold phosphate buffer saline (PBS) and fixed for 30 minutes at RT with 4% paraformaldehyde. Following fixation, the structures were washed twice with PBS and 2% agarose was poured directly into the well containing the 3D HBEC structures to create a plug. The agarose was allowed to cool down at RT and the plugs containing the 3D HBEC structures were placed in histology cassettes. The samples were then processed, embedded and 5µm thick sections were cut for immunofluorescence analysis. The sections were de-paraffinized through three different xylenes, rehydrated in a series of ethanol and washed in de-ionized water for 5 minutes. Following heat induced antigen retrieval, sections were blocked at RT with 10% goat serum albumin (Zymed) in PBS containing 1% bovine serum albumin (Sigma Aldrich) and 0.025% Triton X-100 (Sigma Aldrich) for 2 hours. Sections were rinsed with PBS and incubated with primary antibodies overnight at 4°C in a humidified chamber. The following primary antibodies were used for 3D immunofluorescence analysis: mouse monoclonal anti-p63 (Millipore; clone 4A4), mouse monoclonal anti-Keratin 14 (Thermo Scientific; Cat. #MS-115-P), rabbit polyclonal anti-CCSP (Santa Cruz; sc-25554), rabbit polyclonal anti-SP-A (gift from Dr. Carole Mendelson, University of Texas Southwestern Medical Center, Dallas, TX)(Odom et al., 1987), mouse monoclonal anti-MUC5AC (Abcam; ab24070), and mouse monoclonal anti-TTF1 (Santa Cruz; 8G7G3/1). The antibodies used have been tested for immunofluorescence analysis of paraffin-embedded samples.

Sections were washed three times in PBS after overnight primary antibody incubation and stained with secondary antibodies. A secondary antibody deletion control to check the specificity was performed with each primary antibody. The sections were incubated at a dilution of 1:500 for an hour at RT in a dark humidified chamber (Alexa Fluor 488 goat anti-rabbit (Invitrogen; A-11008) or Alexa Fluor 488 goat anti-mouse (Invitrogen; A-11001)). Sections were then washed three times with PBS and mounted with Vecta Shield mounting medium with DAPI (Vector Labs) cover-slipped, and imaged. (Axiovert 200 M fluorescence microscope). Each staining was performed on multiple sections in triplicates.

Electron Microscopy

3D structures were fixed in 2.5 % glutaraldehyde (in 0.1 M cacodylate) solution overnight at 4°C. The samples were then placed in a 2% agarose plug and processed according to standard electron microscopy procedures. The final samples were then imaged using a Tecnai G2 Spirit 120KV TEM.

RESULTS

IMR90 Fibroblasts Stimulate Branching Morphogenesis of HBEC3s when Cultured on Reconstituted Basement Membrane Matrix

HBEC3s used in the current study were obtained from a central lung bronchi and immortalized using ectopic expression of human telomerase reverse transcriptase (hTERT; T) and cyclin-dependent kinase 4 (CDK4; K) as described previously (Ramirez et al., 2004). Immortalized HBEC3 KTs retain a normal/non-malignant phenotype in cell culture thus allowing the use of an isogenic human bronchial cell population to characterize aspects of lung development and differentiation *in vitro* (Ramirez et al., 2004; Sato et al., 2006). When cultured on gelatin-coated dishes, HBEC3 KTs proliferate into a two-dimensional confluent monolayer that has a cobblestone appearance (Figure 1A). However, when HBEC3 KTs are cultured on top of reconstituted basement (Matrigel®) in the presence of IMR90 fetal lung fibroblasts seeded as feeder layer underneath (Figure 1B), HBEC3 KTs form aggregate structures that develop into complex branching structures by ten days (Figure 1C). In the absence of an IMR90 monolayer, HBEC3 KTs fail to form any self-aggregating structures and do not further develop into any budding and branching structures (Figure 1D). The monolayer of IMR90 cells under the Matrigel® culture remains viable for the duration of the culture and does not form any structures (Figure 1E). HBEC3 KTs cultured with other lung fibroblast cell lines seeded as monolayer under Matrigel® also form branching structures (Figure 1F), indicating that the phenotype of HBEC3 KTs is not limited to one type of feeder layer.

To further investigate the formation of branching structures by HBEC3 KT cells on Matrigel®, the cultures were observed over multiple days. When HBEC3 KTs are seeded on top of Matrigel®, the cells self-aggregated into tubule-like structures after one day (Figure 2A). This phenotype is very similar to what is observed in endothelial cell organotypic cultures (Madri and Williams, 1983). Small bud-like structures emerge alongside of the tubules by the third day; as indicated by the arrows (Figure 2B). The budding structures form complex branching structures by the sixth day (Figure 2C) and continue to increase in size and complexity by day ten (Figure 2D). The complex branching structures are reminiscent of bronchoalveolar units in the lung during the canalicular and saccular stages of development. Since the aggregation and formation of branching structures by HBEC3 KTs is dependent on the presence of IMR90s seeded under the Matrigel®, we tested the effects of varying IMR90 monolayer densities during the Matrigel® culture (Supplementary Figure 2). Significant differences are observed in the phenotype of HBEC3 KTs in 3D culture depending on the density of IMR90s. Lower densities of these fibroblasts are not able to induce complex branching in HBEC3 KTs. The fibroblasts also remain as a monolayer on the bottom of the culture dish and do not make direct contact with the HBEC3 KTs above the Matrigel® layer (Supplementary Figure 3). These observations emphasize the importance of cues from the microenvironment that are necessary for lung development and differentiation (Kimura and Deutsch, 2007; Warburton et al., 2010).

The branching structures that formed on top of Matrigel® are organized at the buds as indicated by the expression of E-cadherin at the cell-cell junctions (Figure 2E). Hematoxylin

and eosin staining of the structures after eight days in culture also demonstrate polarity and organization within the budding structures (Figure 2F). Cells within the buds display a cuboidal to low columnar morphology. HBEC3 KT cells along the tubular structure seem to form a cavity with cells organized along the edge (Figure 2G). This may signify the formation of a lumen within the aggregated budding structure.

HBEC3 KTs Express Basal Cell Markers when Cultured in 3D

We analyzed the expression of several epithelial cell markers in the branching 3D structures on Matrigel®. Mature cell-types in the adult lung can be identified by the expression of unique differentiation markers or a combination of a few markers (Wuensche et al., 1996). Previously, we have shown that HBEC3 KTs under 2D conditions express the basal cell marker p63 (Delgado et al., 2011). HBEC 3KTs retain expression of p63 in 3D culture by day 8 as observed by immunofluorescence analysis (Figure 3A). Both the buds and the tubules within the branching structures express p63 which is localized within the nuclei. HBEC3 KTs also express Keratin 14 (K14), another marker of basal cells in 3D culture (Figure 3B) (Rock et al., 2010). K14 is expressed within the cytoplasm of the branching structures. Immunostaining for mature goblet cells with Muc5AC was negative (data not shown). Basal cells in the human airways are present throughout but enriched in the central airways (Rackley and Stripp, 2012; Rock et al., 2010). Even though the branching phenotype of HBEC3 KTs resembles the morphology observed in the distal lung, all the cells retained basal cell expression. These observations suggest that further microenvironmental cues from the human lung stroma may be necessary to completely differentiate HBEC3 KTs into distal lung lineages.

Differentiation Markers from Distal Lung Cell Types are Expressed by HBEC3 KTs in 3D Culture

Examining the expression of different cell type markers in 3D culture suggests HBEC3 KTs also retain the potential to differentiate into cell types originating in the distal lung. HBEC 3KTs express Clara cell secretory protein (CCSP) when cultured on Matrigel® (Figure 4A). CCSP is a marker of Clara cells with limited expression in Type II pneumocytes (Rawlins et al., 2009). CCSP was uniformly expressed in the cytoplasm of HBEC3 KT cells in 3D culture (Day 8). No difference in the expression is observed between cells that are present within the buds or tubules. CCSP expression has not been previously reported in cells from the central airways (e.g. basal cells) and is restricted to cell types in the distal airway (Rock et al., 2010). Expression of Surfactant Protein-A (SP-A) is observed in HBEC3 KTs (Day 8) cultured on Matrigel® (Figure 4B) which is expressed in both Type II pneumocytes and Clara cells (Madsen et al., 2003). Although SP-A is expressed in HBEC3 KTs in 2D culture, expression of SP-A in 3D is more representative of its expression and secretion *in vivo*. SP-A expression is more prominent within the buds of the branching structures with more distinct expression towards the apical side of HBEC3 KTs. The expression of SP-A within the branching structure resembled its secretion into the alveolar space where it facilitates air exchange (Warburton et al., 2010). Transmission electron microscopy on HBEC3 KTs branching structures after 8 days of culture identified structures that resemble lamellar bodies within individual HBEC3 KT cells (Figure 4D–E) SP-A is generally associated with lamellar bodies which are surrounded by lipid vesicles. Lamellar bodies secrete surfactant to

reduce surface area tension at the site of air-exchange within the alveolar sacs (Fehrenbach, 2001). HBEC 3KTs also express aquaporin 5, a marker of alveolar type I cells that is potentially expressed in many other cell types of the lung including basal cells (Krane et al., 2001). HBEC 3KTs express AQP5 under both 2D (Delgado et al., 2011) and 3D culture conditions (Supplementary Figure 4).

Thyroid-transcription factor (TTF-1) is a multifunctional transcription factor expressed by the multipotent progenitor cells of the airway during development (Boggaram, 2009). TTF-1 is expressed in Clara cells and Type II pneumocytes in the adult lung. TTF-1 drives the transcription of CCSP and other surfactant protein expressed by Clara cells and Type II pneumocytes (Maeda et al., 2007). TTF-1 expression is observed in the HBEC3 KT branching structures on Matrigel® (Figure 4C) by day 8. Although TTF-1 is a transcription factor, a majority of the protein is expressed within the cytoplasm. TTF-1 is variably detected in HBEC3 KT under 2D conditions while it's constitutively expressed in cells cultured on Matrigel®. These observations indicate that HBEC3 KT when cultured on Matrigel® in the presence of IMR90 cells contain the capacity to differentiate into cell types originating in the distal lung.

Analyzing Partially/Fully Transformed HBEC3 KT and Other HBECs in 3D Culture Model

To investigate if other HBEC cell lines can recapitulate the phenotype of HBEC3 KT in 3D, we cultured another normal HBEC immortalized cell line (HBEC13 KT) on Matrigel®. Similar to HBEC3 KT, HBEC13 KT form a monolayer with cobblestone appearance under 2D conditions (Figure 5A). When cultured on top of Matrigel®, HBEC13 KT reproduces the branching phenotype observed in HBEC3 KT (Figure 5B). In the absence of IMR90 as a feeder layer under the 3D culture, HBEC13 KT also fail to form complex branching structures (data not shown). This observation suggests that the 3D culture system can be used to study multiple HBEC cell lines and that the phenotype observed is not limited to HBEC3 KT. The importance of signaling from the stroma during branching process is also confirmed in HBEC13 KT.

To evaluate if the HBEC 3D model could be utilized to study lung cancer progression we cultured partially or completely transformed HBEC3 KT on Matrigel®. HBEC3 KT with ectopic expression of mutant K-ras v12 (Figure 5C) or HBEC3 KT with ectopic expression of mutant K-rasv12 and shRNA TP53 stably knocked down (Figure 5E) exhibit normal HBEC phenotype in 2D. These cell lines also produce branching structures similar to normal HBEC3 KT when cultured in 3D with IMR90s (Figure 5 D, F). The observations suggest that introduction of oncogenic K-ras v12 and significant down regulation of the tumor suppressor TP53 is not sufficient to cause loss of branching phenotype in 3D. HBEC3 KT cells with over-expression of K-ras v12, c-myc and knocked down TP53 are completely transformed and do not display normal HBEC cell morphology in 2D (Figure 5G). The cells do not display a cobblestone epithelial appearance; rather the cells convert to a mesenchymal phenotype in 2D. When cultured on Matrigel®, the cells aggregate and form tubular structures after day 1 but fail to progress into branching structures (Figure 5H). Instead, the cells form invasive structures within the Matrigel® (indicated by the arrows in

Figure 5H) correlating to their phenotype in 2D. These results demonstrate how this *in vitro* 3D system can be utilized in studying lung cancer progression.

Discussion

Development of human cell culture models is an important complement to animal models in understanding basic developmental, differentiation, and diseases processes. In the present study, we have described a novel *in vitro* lung epithelial cell culture system that is reminiscent in part to the process of lung branching morphogenesis during development. In our model, we also discovered that interaction between fibroblasts and the HBECs were critical to reproduce the branching phenotype *in vitro*. This is analogous to human lung development where critical signaling from the mesenchyme leads to the branching and differentiation of the airway epithelia. Developmental processes such as branching morphogenesis of the lung have primarily been studied using embryonic human or mouse tissue extracts (Miura and Shiotani, 2000; Weaver et al., 2000). This model allows the use of immortalized HBECs to study such process *in vitro* and also recapitulates important stromal epithelial interactions. While our understanding of the lung epithelium homeostasis, repair and regeneration has been greatly enhanced by the use of mouse models, translating them to the human lung has been hindered due to the inherent anatomical differences between the two species (Rackley and Stripp, 2012; Rock and Hogan, 2011; Warburton et al., 2010). The present study demonstrates a new cell-based model to understand these processes within the human lung.

A critical role in organogenesis and tissue maintenance is performed through the crosstalk between the epithelial and mesenchymal cells. This signaling is critical for many tissues including mammary glands and prostate (Cunha, 2008; Kimura and Deutsch, 2007; Ronnov-Jessen and Bissell, 2009). The initial lung emerges from the ventral foregut endoderm and requires a coordinated sequence of processes to develop (Kimura and Deutsch, 2007). The mesenchyme originates from the mesoderm which includes fibroblasts, endothelial cells and other connective tissue and these cell types progress in parallel to the surrounding developing airways. Induction of transcription factors and growth-factor signaling in a temporal-spatial manner orchestrate migration and branching of the lung airway epithelium (Kimura and Deutsch, 2007). The signaling also plays a role in specification of cell fate once the airway has developed (Boggaram, 2009; Maeda et al., 2007; Rock and Hogan, 2011). While several reports have described the formation of spheroid structures when human or mouse airway epithelial cells are cultured in Matrigel®, these models do not account for the epithelial-mesenchymal interactions (McQualter et al., 2010; Rock et al., 2009; Wu et al., 2011). The lack of such interaction may explain why HBECs in these models do not differentiate into cells associated with the distal airway. Interaction between stromal components such as fibroblasts and endothelial cells is much more intimate in the distal airway. Septation of terminal sacs in the branching lung epithelia involves interactions between airway epithelial cells, myofibroblasts and endothelial cells (Warburton et al., 2010). Fibroblast growth factor (FGF) signaling in particular FGF-10 from the mesenchyme also plays a critical role in differentiation and branching in the developing distal lung (Nyeng et al., 2008). Furthermore, fibroblasts adjacent to Type II pneumocytes in adult human tissue are responsible for maintaining communication between epithelia and

migrating leukocytes (Sirianni et al., 2003). Branching morphology of HBECs in our 3D model support the notion that co-culturing mesenchymal and epithelial cells may be required to differentiate HBECs into cells enriched in the distal airways.

Most prior studies involving cell culture models have also failed to recapitulate the branching phenotype *in vitro* using immortalized HBECs. Branching morphogenesis of HBECs in 3D culture was recently described (Franzdottir et al., 2010). There are distinct differences under which cells are cultured in our versus the Franzdottir et al (2010) studies. Franzdottir and coworkers embedded HBECs immortalized with E6/E7 viral oncogenes in Matrigel®. The HBECs were co-cultured with human umbilical vein endothelial cells (HUVECs) resulting in the formation of branching structures (Asgrimsson et al., 2006). In our model, HBECs immortalized with CDK4 and TERT are not embedded but overlaid on top of Matrigel® with fetal lung fibroblasts below the Matrigel as a monolayer. Under these conditions the HBECs aggregated to form budding and branching structures. When HBEC3 KTs are cultured on Matrigel® with HUVECs seeded as the feeder layer, HBEC 3KTs did not form any tubule-like structures after day 1 but aggregated and formed cyst-like structures (Supplementary Figure 5A). When HUVECs are used a branching phenotype was rarely observed, while the vast majority of structures failed to demonstrate branching phenotypes by day 10 (Supplementary Figure 5B). Interestingly, the endothelial cells seeded under the Matrigel® formed tubule-like structures (Supplementary Figure 5C). The results demonstrate that while signaling from multiple stromal components may be required for development and differentiation of the lung epithelium, factors secreted from fibroblasts are crucial in our 3D cell culture system for HBEC3 KTs. Although HBECs grown with endothelial cells or fibroblasts under certain conditions may have similar phenotypes, the effects of different immortalization processes on differentiation are also not completely understood and certain subtle variations may exist between different HBEC lines.

HBEC3 KTs encompass multipotent capacity *in vitro* as described in the current study and by us previously (Delgado et al., 2011). The cells were derived from the central airway of the lung but in 2D culture, express markers of both central and distal lung. The cells express basal cell markers p63 and keratin 14 (central) while also expressing Surfactant protein A, C, D and CCSP (distal). When placed in different 3D culture conditions, HBEC3 KTs demonstrate the capacity to differentiate into both central and distal lineages of the human airways (Delgado et al., 2011; Vaughan et al., 2006). These observations support the suggestion that there may be a human lung stem-like cell type capable of multipotent differentiation. When cultured on top of Matrigel® HBEC3 KTs form branching structures that retain these markers from multiple cell lineages. Immunofluorescence analysis of the branching structures demonstrate that HBEC3 KTs during branching continue to express basal cell, Clara cell and Type II pneumocytes cell markers. This indicates that signaling from other cell types beside fibroblasts or further changes in the microenvironment may be necessary to entirely differentiate the HBEC3 KTs into distal airway. TTF-1 a transcription factor which regulates surfactant proteins and CCSP expression in the distal lung was also expressed in 3D (Maeda et al., 2007). Expression of TTF-1 in HBEC3 KTs is variable in 2D, but TTF-1 is constitutively expressed evenly in the cytoplasm of HBEC3 KTs in the

branching structures. This indicates that HBEC3 KT in the branching structures are primed to differentiate into distal lineages but additional signaling from the stroma may be required.

We also tested the utility of this novel 3D model to analyze lung cancer progression. HBEC3 KT has previously been utilized to understand lung cancer progression. Partially transformed HBEC3 KT with the addition of oncogenic mutant K-ras v12, knockdown of TP53 or both do not form xenograft tumors in immuno-compromised mice at a high frequency (Sato et al., 2006). Our 3D branching model was able to predict this *in vitro*, as these cells were able to branch and bud and appeared similar to unprogressed HBEC3 KT. Fully transformed HBEC3 KT with oncogenic K-ras v12, c-myc and knocked down TP53 are able to form tumors in mice at a higher frequency compared to partially transformed HBEC3 KT (Sato et al., 2013) and when cultured in 3D on top of Matrigel® failed to branch and bud. The cells formed invasive structures in Matrigel® indicating their malignant transformation and their capacity to invade the basement membrane. The 3D model was able to distinguish between partial and fully transformed cell lines *in vitro* and predict their behavior *in vivo*. Therefore, the culture system may provide a physiologically relevant system that may help predict tumorigenic behavior *in vivo*. Loss of differentiation is a critical event in cancer progression and *in vitro* models such as the one described in the present studies may assist in understanding certain aspects of lung cancer progression and may be a useful system for testing therapeutic regimens. The 3D model can also help understand the epithelial-mesenchymal interactions in lung cancer progression.

Overall, our results characterize a novel model of branching morphogenesis using hTERT and Cdk4 immortalized human bronchial epithelial cells. We demonstrate that signaling from mesenchymal cells is critical for HBEC3 KT to branch and bud when cultured on top of Matrigel®. The current model can also be extended further to recapitulate complex signaling between epithelium and stroma by including other stromal factors. Conversely, the model can also be simplified to understand the role of individual factors in lung development and diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

HBECs	Human bronchial epithelial cells
3D	Three-dimensional
2D	Two-dimensional

CDK4	Cyclin-dependent kinase 4 (K)
hTERT	Human telomerase reverse transcriptase (T)
ALI	Air liquid interface
CCSP	Clara cell secretory protein
HUVECs	Human umbilical vein endothelial cells

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Highlights

- We describe a novel culture system using human bronchial epithelial cells (HBECs)
- HBECs form complex budding and branching structures when cultured on Matrigel®
- The branching phenotype is dependent on signaling from IMR90 fibroblasts
- HBECs retain their multipotent characteristics in Matrigel® culture
- The system can be used to study diseases such as lung cancer

Figure 1. HBEC3 KT cells exhibit different morphologies under different culture conditions

HBEC3 KT cells cultured in 2D and 3D conditions exhibit different morphologies. (A) Under 2D culture conditions on gelatin coated plates, HBEC3 KT cells grow in a monolayer with a cobblestone appearance. (B) When seeded on top of Matrigel® in the presence of IMR90s seeded as a monolayer, (C) HBEC3 KT cells aggregate into tubular structures which bud and branch (Day 10). (D) In the absence of IMR90s, HBEC3 KT cells fail to form complex branching structures when cultured on Matrigel®. (E) IMR90s seeded as a monolayer under the Matrigel® culture do not form any structures or invade the Matrigel®. (F) The phenotype of HBEC3 KT cells on Matrigel® is not limited to IMR90s and can be recapitulated with other primary lung fibroblast cells. Scale bar 100 μ m.

Figure 2. HBEC3 KT cells form complex branching structures when co-cultured with IMR90s on Matrigel®

When co-cultured with IMR90s on Matrigel®, (A) HBEC3 KTs aggregate and form tubule-like structures after 1 day. This phenotype is very similar to what is observed in 3D cultures of endothelial cells. (B) Small budding structures emerge from the initial tubule-like structure by day 3. (C) Initial budding structures start to branch by day 6. The aggregated structures continue to grow and (D) develop more complex branching by day 10. (E) E-cadherin immunostaining shows formation of cell-cell junctions and organization within the HBEC3 KT structures. (F) HBEC3 KT cells in the buds are organized and exhibit a columnar morphology by day 8 as indicated by the H&E stain. (G) Cells in the tubule part of the structure are also organized along the length of the structure by day 8. Scale bar 100µm (A–D, F, G), 10µm (E).

Figure 3. Branching HBEC3 KT structures express basal cell markers

HBEC3 KT cells cultured on Matrigel® were immunostained for expression of basal cell markers (Day 8). (A) Nuclear staining with DAPI is shown in blue. p63 expression is seen in all the nuclei within the branching structures. (B) Keratin 14, another basal cell marker is expressed in the cytoplasm of all the cells within the branching structure. Scale bar 10 μ m

Figure 4. HBEC3 KTs express markers of distal lung lineages when cultured on Matrigel® HBEC 3KT cells cultured on Matrigel® were immunostained for markers of cell types present in the distal lung (Day 8). (A) Nuclear stain with DAPI is shown in blue. CCSP (red) is expressed in HBEC3 KTs in 3D culture. The protein is localized within the cytoplasm of the cells in the branching structure. (B) Immunostaining with antibody against SP-A (red), a marker for type II pneumocytes cells. The expression of the protein is localized towards the apical side of the buds of the branching structures. (C) TTF1 is expressed in HBEC3 KTs cultured on top of Matrigel®. The TTF1 protein is expressed in the cytoplasm in a majority of the cells with weak nuclear expression within a few cells. Scale bar 10µm (A–C). (D–E) Transmission EM analysis on branching structures after 8 days in culture show the presence of lamellar-like bodies within the HBEC3 KT cells in 3D culture. Scale bar 0.5µm (D–E).

Figure 5. The *in vitro* 3D culture system can be utilized for understanding lung cancer progression

The 3D system can be used to understand different respiratory diseases by analyzing the branching phenotype on Matrigel®. The branching characteristic that HBEC 3KTs demonstrate on Matrigel® can be recapitulated by other HBECs. (A) HBEC13 KT exhibits cobblestone monolayer appearance in 2D. (B) When cultured on top of Matrigel® in the presence of IMR90s, HBEC13 KT forms complex branching structures. (C) Mutant K-rasv12 or (E) both K-rasv12 and stable TP53 knockdown HBECs retain normal epithelial morphology in 2D. (D, F) These partially transformed cells also form branching structures when cultured on Matrigel®. (G) HBEC3 KT expressing K-rasv12, c-myc and knockdown TP53 displays a mesenchymal morphology in 2D culture. (H) These HBECs fail to form branching structures when cultured on top of Matrigel®. The cells invade through the reconstituted basement membrane (pointed by the arrows). Scale bar 100µm