Exploring the differentiation pattern of bronchial epithelial cells in culture

Hildur Sigurgrímsdóttir

Thesis for the degree of Master of Science
University of Iceland
Faculty of Medicine
Department of Biomedical Science
School of Health Sciences

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Hildur Sigurgrímsdóttir

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Umsjónarkennarar: Þórarinn Guðjónsson, Ph.D

Magnús Karl Magnússon, MD

Meistaranámsnefnd: Þórarinn Guðjónsson, Ph.D

Magnús Karl Magnússon, MD

Tómas Guðbjartsson, Ph.D, MD

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Supervisors: Þórarinn Guðjónsson, Ph. D

Magnús Karl Magnússon, MD

Masters committee: Þórarinn Guðjónsson, Ph. D

Magnús Karl Magnússon, MD

Tómas Guðbjartsson, Ph. D, MD

Faculty of Medicine

Department of Biomedical Science

School of Health Sciences

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Ágrip

Öndunarvegur þ.m.t. berkjur og lungu eru samsett úr fjölda mismunandi þekjufrumugerða. Í efri öndunarvegi fyrirfinnst sýndarmarglaga stuðlaþekja sem þynnist eftir sem neðar dregur og endar í einfrumulagi í lungnablöðrunum þar sem loftskiptin fara fram. Efri öndunarvegir eru samsettir úr mismunandi frumum einsog bifhærðum frumum, bikarfrumum og basal frumum. Basal frumur eru taldar vera stofnfrumur í öndunarvegi og þar með ábyrgar fyrir því að mynda hinar sérhæfðu frumur þekjuvefjarins. Basal frumur bregðast við skemmdum á þekjunni með því að skipta sér ósamhverft og gefa af sér dótturfrumur sem geta sérhæfst og endurmyndað skemmda vefinn. Mikilvægt er að rannsaka eiginleika þekjufrumna úr öndunarvegi í frumurækt þar sem það getur gefið vísbendingu um hvernig frumur þroskast í berkjum og lungum. Í þessu samhengi er mikilvægt að geta rannsakað bæði ferskar frumur beint úr vef sem og vel skilgreindar frumulínur.

Ferskar frumur eru ræktaðar beint úr líkamsvef og þykja líkjast upprunafrumum betur heldur en frumur úr þekktum frumulínum sem er búið að erfðabreyta. Þekktar frumulínur hafa verið ræktaðar lengi í tilbúnu umhverfi, í frumuæti og í tvívíðu ræktunarumhverfi. Ferskar frumur eru teknar úr lífveru og endurspegla að hluta til ástand líkamans sem þær eru upprunnar frá. Ferskar frumur hafa takmarkað æviskeið þar sem ekki er búið að gera þær ódauðlegar líkt og frumulínurnar. Þetta gerir það að verkum að oft er erfitt að endurgera niðurstöður sem fengnar eru úr ferskum frumum frá einum einstaklingi því mikill munur getur verið milli sýna.

Í þessarri rannsókn voru ferskar þekjuvefsfrumur ræktaðar úr 19 berkjusýnum. Það var munur á tjáningu markpróteina milli sýna og það virtist verða breyting á tjáningu ákveðinna próteina miðað við tíma í rækt og hversu oft frumunum hafði verið skipt. Hlutfall p63 jákvæðra frumna virðist aukast eftir fyrstu skiptingu og slímseytandi frumur virðast ekki finnast eftir fyrstu skiptingu.

VA10 frumulínan er þekjuvefsfrumulína upprunnin úr berkju og hún tjáir sömu prótein og basal frumur. VA10 frumur eru p63 jákvæðar, mynda greinótta formgerð í þrívíðri ræktun og sýndarlagskipta þekju í loft-vökva rækt (air-liquid interface). VA10 frumur eru misleitur frumuhópur og hafa ekki allar sömu próteintjáningu. Hluti verkefnisins var að einangra undirhóp úr VA10 frumulínunni sem hefði fleiri eiginleika basal frumna heldur en upprunalega frumulínan. Það var gert með því að einangra frumur sem höfðu sterka tjáningu á yfirborðspróteinum tengdum basal frumum. Undirhópar sem tjáðu NGFR og β4 integrin í miklu mæli voru einangraðir. Undirhóparnir uxu ekki eins hratt og upprunlega frumulínan en markpróteinatjáning þeirra var mjög lík VA10.

VA10 frumulínan var gerð ódauðleg með *E6* og *E7* genunum en próteinafurðir þeirra hamla virkni og brjóta niður p53 og retinoblastoma prótein. Upprunalega VA10 frumulínan myndar greinótta formgerð í þrívíðri ræktun með litlum holrýmum en almenn holmyndun þeirra er ófullkomin. Einn hluti verkefnisins sneri að því að láta VA10 frumur sýna betri holmyndun þegar þær vaxa greinótt í þrívíðri ræktun. Tjáning *E6* og *E7* genanna var slegin niður með shRNA en þegar shRNAið var virkjað þá minnkaði greinótt formgerð frumnanna í þrívíðri ræktun verulega

Niðurstöður þessarar rannsóknar skila aukinni þekkingu á svipgerðarstöðugleika og eiginleikum ferskra lungnafruma. Niðurstöðurnar undirstrika mikilvægi þess að frumur úr mismunandi einstaklingum

séu notaðar við rannsóknir vegna þess hve breytileiki þeirra getur verið mikill. Ennfremur benda niðurstöðurnar til þess að ferskar frumur breyti markpróteintjáningu sinni eftir skiptingum. Mikilvægt er að auka skilning okkar á eiginleikum ferskra frumna úr mannavef til þess að auka gagnsemi þeirra í rannsóknum. Samhliða því er mikilvægt að skilgreina betur styrk- og veikleika frumulínanna því best væri að sameina notkun þekktra frumulína og ferskra frumna til að fá sem bestar og áreiðanlegastar niðurstöður.

Abstract

The respiratory system epithelium, including the bronchi and lungs, consists of several different types of epithelial cells. The upper respiratory tract has a pseudostratified epithelium and the epithelium grows thinner in the more distal respiratory tract ending in a simple epithelium in the alveoli where the gas exchange occurs. The epithelium of the upper respiratory tract is composed of various types of epithelial cells such as ciliated, goblet and basal cells. The basal cells are thought to be stem cells in the airways and the more differentiated cells of the epithelium arise from them. The basal cells serve as a reserve cell population that divides asymmetrically in response to injury. Research on the attributes of epithelial cells *in vitro* is important as the results can give information on how the cells of the bronchi and lungs develop. In these studies it is beneficial to use both primary cells and established cell lines.

Primary cells are cultured from donor tissue and are thought to represent the cell of origin better than established cell lines that have been genetically altered. Established cell lines have been grown for a long time in artificial environments, both in cell culture media and in two dimensional culture. Primary cells have been a part of a living being and reflect in part the state of the body they are taken from. However, they have a limited lifespan, as they have not been immortalized like the established cell lines and reproducing results can be difficult because of variations between donors.

In this study, primary epithelial cells were cultured from 19 bronchial tissue samples. The marker expression showed considerable donor variation and indicated a change in marker expression by time and passage. There seems to be a tendency for enrichment of p63 positive cells after first passage and mucin expression that identifies goblet cells does not seem to be present in passages after the initial one.

The VA10 cell line is a bronchial epithelial cell line that has a marker expression corresponding to basal cells. The VA10 cells are p63 positive, a basal cell marker, generating branching bronchioalveolar-like structures and pseudostratified epithelium in 3D culture and air-liquid culture, respectively. The VA10 cells are a heterogeneous group with a broad surface marker expression and a part of the project was to test if the cell line could be enriched for more basal cell properties by using a cell sorting technique based on antibodies against surface proteins expressed on basal cells. Subgroups of cells that had a high expression of basal cell markers NGFR and β4 integrin respectively were isolated. They showed a reduction in growth and a marker expression profile similar to that of the parental VA10 cells.

The VA10 cells were immortalized with the *E6* and *E7* genes, which degrade and bind p53 and retinoblastoma protein respectively. The original VA10 cell line forms branching structures with small cavities but their lumen formation is flawed. The *E6* and *E7* genes were knocked down with a shRNA construct in an effort to make VA10 cells that would have an improved lumen formation in branching structures in Matrigel. The knockdown of *E6* and *E7* was successful but when the shRNA was activated, the branching formation in Matrigel was severely reduced.

This study describes the characterization of freshly isolated primary cells from the human bronchus. The results underline the importance of using primary cells from multiple donors because of donor variance. Additionally, the cells are quite dynamic when it comes to surface marker expression, potentially changing as time passes. It is important to try and further our understanding of human primary cells to make them more useful as research tools. At the same time it would be beneficial to understand the strengths and limitations of both primary cells and cells lines since the best experiments would undoubtedly be conducted by combining the strengths of both for the most accurate conclusions.

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3D	Three-dimensional
ALI	Air-liquid interface
BrdU	Bromodeoxyuridine
cDNA	Complementary DNA
CK	Cytokeratin
EP bridges	Epithelial bridges
EpCAM	Epithelial cell adhesion molecule
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
HPV 16	Human papillomavirus 16
HUVECs	Human umbilical vein endothelial cells
IGR	Integrin
NGFR	Nerve growth factor receptor
MACS	Magnetic-activated cell sorting
PBS	Phosphate buffered saline
pRb	Retinoblastoma protein
qRT-PCR	Quantitative real time polymerase chain reaction
rBM	Reconstituted basement membrane
shRNA	Small hairpin RNA
TET	Tetracycline
TGF-β	Transforming growth factor-β
TNT	Tunneling nanotubes

1 Introduction

1.1 Anatomy and physiology of the human lung

The human lung is a complex tubular organ that facilitates gas exchange in and out of the body. The lungs are lobular and divided into two parts, right and left lung. The right lung is divided into three lobes and the left lung is divided into two lobes. The trachea divides into two large primary bronchi which in turn divide into secondary bronchi when entering the lung. Each lobe is entered with its own secondary bronchus that branches on, forming numerous smaller bronchi and then bronchioles that give rise to millions of alveoli that have a vast surface for gas exchange (Tortora & Derrickson, 2009). An overview of the respiratory system can be seen in figure 1. In total, the bronchial tree of the human lung consists of over 10^5 conducting airways and 10^7 respiratory airways (Metzger et al., 2008). The lung is highly vascularized as a close contact to vasculature is necessary for successful gas exchange (Tortora & Derrickson, 2009).

Oxygenized air is inhaled from the atmosphere into the trachea through the mouth or nose, into the primary bronchi which divide into smaller bronchioles. Finally oxygen flows to the alveoli where it diffuses over the thin alveolar membrane into the blood in exchange for the carbon dioxide (CO_2) brought by the deoxygenated blood from the heart (Tortora & Derrickson, 2009).

1.2 The embryonic development of the lung

Around the fourth week of embryonic development, the respiratory system begins to grow out of the foregut. The first indicator of the lung bud is a localized expression of the transcription factor Nkx2.1 in the foregut at embryonic day 9.5 (E9.5) in mice and approximately at day 28 in humans. A crucial player in murine lung development is fibroblast growth factor 10 (Fgf10), because the formation of the lung buds is entirely dependent on the localized expression of the Fgf10 in the mesenchyme overlaying the buds and fibroblast growth factor receptor 2 (Fgfr2) in the endoderm (Morrisey & Hogan, 2010).

The lung bud elongates and forms the trachea, which branches and forms the bronchi and bronchioles. During weeks 6-16 of human embryonic development, most of the airways are formed. After week 16 the tissue becomes vascularized and the gas exchange area, including respiratory bronchioles, alveolar ducts and primitive alveoli containing alveolar cells of type I and type II are formed. The mature alveoli start to form in week 30 and continue to form after birth for the first 8 years of life (Tortora & Derrickson, 2009; Daniely et al., 2004). At birth, only 15% of the alveoli that a fully formed lung is composed of have been formed (Hsia et al., 2004).

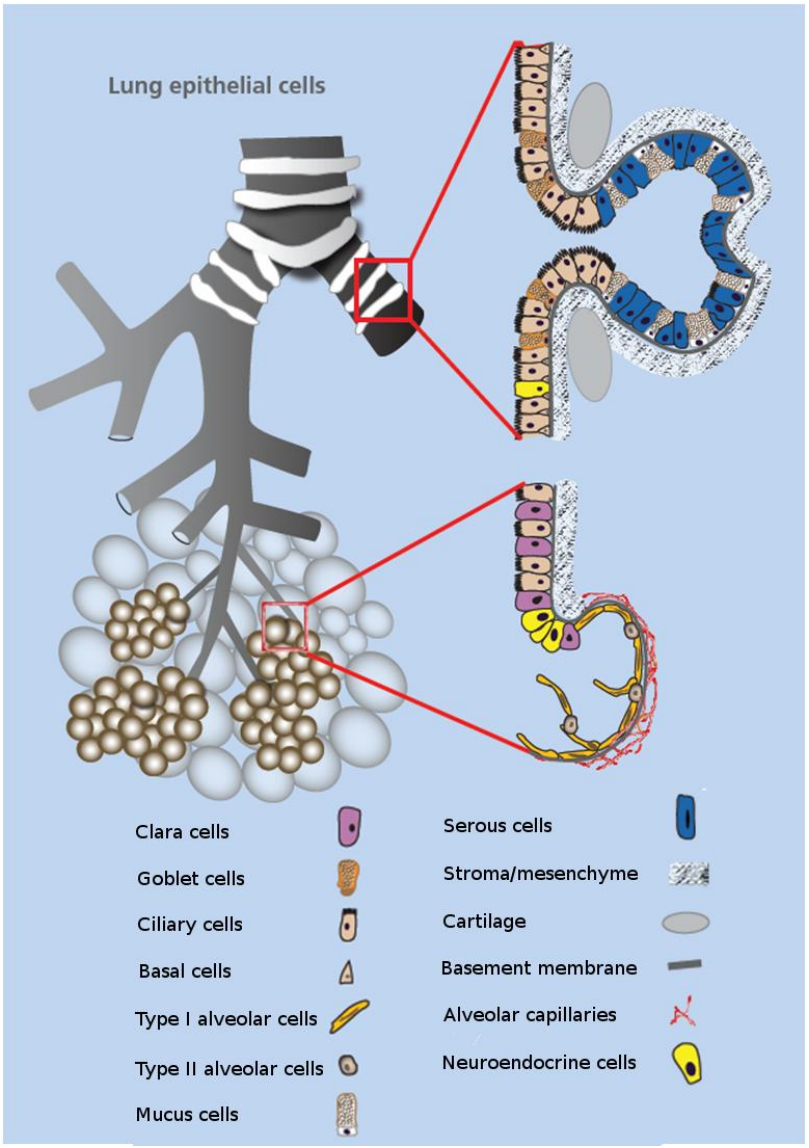

Figure 1: Overview of the anatomy of the human lung and the histology of the bronchial epithelium and the alveolar epithelium.

On the left is a drawing of the anatomy of the lung, starting with the trachea and ending in the alveolar tissue. On the right, a closer look is taken at the epithelium of the bronchi (upper) with the submucosal glands, and at the more distal alveolar epithelium (lower) (Magnusson & Gudjonsson, 2011).

1.2.1 Branching morphogenesis

Branching morphogenesis is an essential part of the development of several organs, such as the lung (Warburton et al., 2010), prostate (Bergthorsson et al., 2013), salivary gland (Sequeira et al., 2013) and mammary gland (Fata et al., 2003). During embryonic development, airway branching starts with a small lung bud that grows and branches into the surrounding mesenchyme. In the lung, the branching of the intrapulmonary airways is very intricate and complicated as the numbers of branches rises. Distal airway branching is thought to heed a simple set of rules encoded in the genome (Warburton et al., 2010). Modeling the branching of the airway has been difficult due to the complexity of the branching tree and lack of appropriate cellular models. Metzger et al. (2008) examined

hundreds of fixed murine lungs on E11 to E15 and proposed that branching can be divided into three simple geometric forms; domain branching, planar bifurcation and orthogonal bifurcation. These forms occur repeatedly to form different branching arrangements (Metzger et al., 2008; Warburton et al., 2010).

Domain branching occurs when daughter branches form in longitudinal rows on a parent branch. These daughter branches form at different positions around the circumference of the parent branch like the rows of bristles on a bottle-brush. Both planar and orthogonal bifurcation are initiated in the same way, with the division of the parental branch. Planar bifurcation occurs when the tip bifurcates more than once, and the airway branches all stay on the same plane, like the teeth of a comb. Orthogonal bifurcation is identical except that the axis of bifurcation rotates 90° between bifurcations. Therefore, instead of the branches all staying in the same plane they form a bouquet of branches called rosettes. This is visualized in figure 2 where each of the branching processes is drawn from two vantage points to show their three dimensional (3D) construction (Metzger et al., 2008).

Metzger and colleagues propose that each of the branching modes has a relatively simple, genetically encoded control (see figure 2). The domain branching needs two controls; one to tell how far apart on the longitudinal axis of the parent branch the daughter branches should be (periodicity generator) and another one to control where on the circumference of the parent branch the daughter branches should form (domain specifier). The bifurcation forms need a control that divides the tip of the branch (bifurcator). In addition to the bifurcator the orthogonal bifurcation needs a control to rotate the axis of the bifurcation (rotator). Figure 2 shows each of these controls with the branching forms that they generate (Metzger et al., 2008).

Although the airway tree seems arbitrary at first glance, its form is stereotypical, though slightly variable between individuals. The lung development and branching morphogenesis is under complex genetic control that includes transcription factors from at least four groups; forkhead box (FOXA1, FOXA2, HFH8, and HFH4), Nkx homeodomain (NKX2.1), RA receptors, and the Gli family. Various growth factor pathways are involved, e.g., FGF, FGFR, Sprouty and the transforming growth factor-β family (TGF-β), which influences the SMAD proteins. Those examples are only a few of the multiple genes and proteins involved in the lung development (Warburton et al., 2010). Additionally, many important signals that drive epithelial growth, differentiation and branching morphogenesis come from the surrounding mesenchyme (Morrisey & Hogan, 2010).

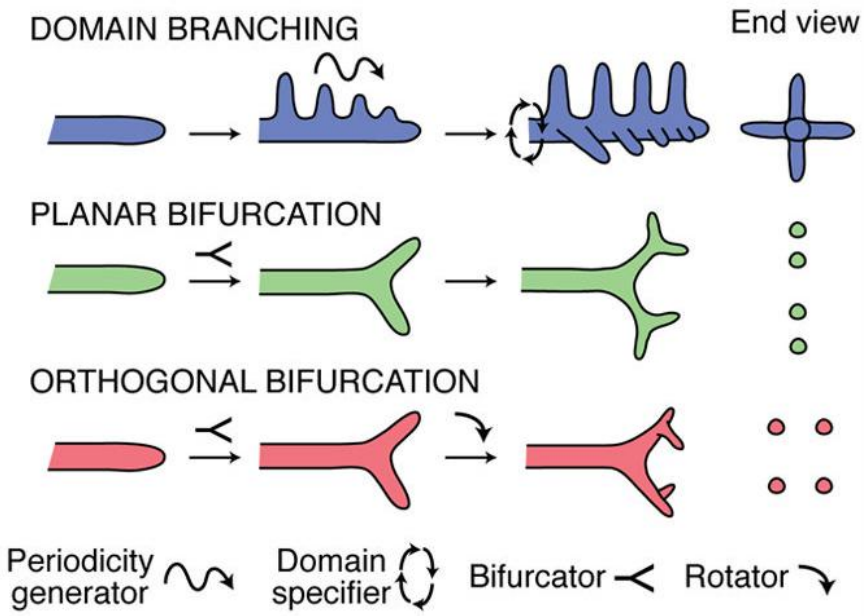

Figure 2: Three distinct geometric forms that when combined form the airway branching tree

Domain branching (depicted in blue), planar bifurcation (depicted in green) and orthogonal bifurcation (depicted in red) are shown with their end view and the genetic controls that control each (Metzger et al., 2008).

1.3 Lung histology

The histology of the respiratory system, i.e., the lung and the air passages to them, is complex due to its long anatomical distance and intricate function. The trachea is easily identified by the C-shaped cartilage that keeps the tracheal lumen from collapsing (see figure 1). The trachea has four layers, the mucosa, the submucosa, a cartilaginous layer and the adventitia. The mucosa consists of the epithelium, the basement membrane, which is made of densely packed collagenous fibers and loose connective tissue of the lamina propria. The basement membrane marks the boundary between epithelium and the lamina propria, which is highly cellular and home to diverse types of cells such as lymphocytes, plasma cells, mast cells, eosinophils and fibroblasts. An elastic membrane separates the submucosa from the mucosa. This membrane is relatively loose connective tissue that contains the larger distributing blood vessels and lymphatics of the tracheal wall, as well as mucous glands that have ducts reaching to the surface of the epithelium. The C-shaped cartilage is between the submucosa and the adventitia that binds the trachea to the adjacent structures in the neck and the mediastinum (Ross & Pawlina, 2011; Tortora & Derrickson, 2009).

The anatomy of the primary bronchi is very similar to that of the trachea but as the secondary bronchi form, the shape of the cartilage changes. This coincides with the entrance of the bronchi into the lung. The C-shaped cartilage rings are replaced by irregularly shaped cartilage plates, which are distributed around the entire circumference of the bronchi. This gives the secondary bronchi a round shape, as opposed to the flattened ovoid shape of the trachea and the primary bronchi. As the secondary bronchi branch and get narrower, the cartilage plates become fewer and smaller. When the diameter of the bronchus is around 1 mm, the cartilage plates are no longer present. At that point the bronchus is called a bronchiole (Ross & Pawlina, 2011).

Another significant change in the anatomy of bronchi as they enter the lung is the addition of a smooth muscle layer between the mucosa and the submucosa. This smooth muscle forms a continuous layer around the bronchi and as they narrow and divide further the muscle layer becomes more conspicuous. In the smaller bronchi, the smooth muscle layer may appear discontinuous because of its spiral course around the bronchi (Ross & Pawlina, 2011).

1.4 Epithelial cells in the respiratory system

The tracheal epithelium is a pseudostratified ciliated columnar epithelium consisting of cells that can be categorized into three main groups: ciliated, secretory and basal. Figure 1 illustrates the cellular composition of the tracheal epithelium. All cells have contact with the basement membrane. Ciliated cells are the most abundant cells of the tracheal epithelium. They extend through the full thickness of the epithelium and have short cilia on the luminal side (pale pink in figure 1). The cilia are directed towards the lumen where they are responsible for clearing of mucus and debris. The goblet cells are secretory and reach out into the lumen (orange in figure 1). They are non-ciliated and generate mucus that they secrete into the lumen. There are secretory cells in the submucosal glands as well (blue and gray in figure 1). Basal cells are small, round cells located near the basement membrane. They are stem cells and serve as a reserve cell population that can replenish the bronchial epithelium after injury (Ross & Pawlina, 2011; Tortora & Derrickson, 2009; Knight & Holgate, 2003).

1.5 The basal cells

The basal cells are thought to comprise around 6-30% of the epithelial cells in the airway (Rock et al., 2010). Their role as stem cells is to replace the differentiated cells of the epithelium by asymmetric division after injury. Basal cells maintain homeostasis in the epithelium by dividing and differentiating. If that homeostasis is interrupted, it can in extreme cases lead to basal cell hyperplasia or epithelial hypoplasia. Because the basal cells have stem cell abilities and are long-lived, mutations of their genome or epigenetic modifications may affect the individual, making them more susceptible to respiratory diseases (Rock et al., 2010).

Basal cells with stem cell abilities that lie close to the basement membrane are common in stratified and pseudostratified epithelia in the human body and exist, for example, in the hair follicle (Zhang et al., 2009) and in the epidermis (Clayton et al., 2007). Basal cells are not the only group of cells with stem cell abilities in the lung. For example, the alveolar type II cells can differentiate into alveolar type I cells, which are the more plentiful epithelial cells of the alveolar region (Ardhanareeswaran & Mirotsou, 2013). Clara cells are non-ciliated secretory cells in the simple epithelium of the distal bronchioles. They can divide and proliferate in response to injury to the epithelium and then return to a quiescent state (Stripp & Reynolds, 2008; Ardhanareeswaran & Mirotsou, 2013; Weiss et al., 2011; Kajstura et al., 2011).

The epithelial cells in the adult trachea normally have a low turnover rate. When lung injury occurs, the epithelial cells, except for the ciliated ones, proliferate to repair the tissue (Rock et al., 2009; Rawlins et al., 2007). Lineage tracing experiments have been conducted to see what cells proliferate the most after lung injuries (Hong et al., 2003; Hong et al., 2004). Rock et al. (2009) did a lineage

tracing experiment where at the beginning most of the labeled cells were scored as basal cells (~98%). As time passed the percentage of labeled basal cells declined but secretory and ciliated cells with a label were more numerous. In this study, basal cells gave rise to twice as many Clara cells as ciliated cells over the chase period (Rock et al., 2009).

These results can be explained in two ways. Firstly, ciliated cells might have a longer lifespan and thus not need to be replaced as often as Clara cells. Secondly, it is possible that basal cells first give rise to Clara cells, which then transition into ciliated cells. After lung injury, the basal cells give rise to opposite ratios of secretory and ciliated cells compared to the steady state (Rock et al., 2009). In the same study, a gene expression profiling was performed on basal cells and 627 genes were found that were upregulated in basal cells compared to non-basal cells. The upregulated genes had various functions; a number of them were involved in cell adhesion, e.g. *α6β4* integrins (IGR), some coded for transcription factors such as *Snai2,* signaling ligands like *Bmp7* and receptors such as *nerve growth factor receptor* (NGFR) (Rock et al. 2009).

Older studies showed that rat basal airway cells showed colony forming abilities in culture and can regenerate a differentiated mucociliary airway epithelium when seeded in rat tracheas that have had their epithelium removed and are planted subcutaneously in nude rats (Randell et al., 1991).

Basal cells may be a heterogeneous group: in a study done by Borthwick and colleagues, mice were subjected lung injury and injections of bromodeoxyuridine (BrdU). At time-points right after the injury period, 3 and 6 days after, BrdU-retaining cells were present along the entire tracheal length in both basal and luminal cell positions. Later on, at 20 and 95 days after the injury period, BrdU retaining cells were only found at gland ducts in the upper airway and at foci in the lower airways, systematically arranged at the intercartilage regions (Borthwick et al., 2001).

Basal cells commonly express the transcription factor p63, cytokeratins (CK) 5 and 14, α6/β4 IGR and NGFR (Rock et al., 2009; Rock et al., 2010; Ardhanareeswaran & Mirotsou, 2013). In this study the well known basal cell markers p63, β4 IGR and NGFR were used and are therefore shortly described.

1.5.1 p63

p63 is the marker most commonly associated with basal cells in the airway. It is a transcription factor with various functions, e.g., in cell proliferation, survival, apoptosis, differentiation, senescence and aging (Bergholz & Xiao, 2012). p63 belongs to the p53 family, same as p53 and p73. The importance of p63 in stratified epithelium has been established using transgenic mice. These mice die soon after birth and show developmental defects in many tissues, for example, the absence of a stratified epidermis, hair follicles, teeth, prostate and mammary glands and limbs which are either absent or truncated (Daniely et al., 2004; Mills et al., 1999). p63 null mice develop a trachea during embryonic period but the histology of the epithelia is abnormal. The epithelium is not pseudostratified like it should be but columnar, ciliated and seems to lack basal cells. Based on these observations, the main role of p63 seems to be in regulating the differentiation of stem cells into basal cells and/or the maintenance of tissue specific basal cells (Daniely et al., 2004). Overexpression of p63 occurs in various types of lung cancer (Graziano & De Laurenzi, 2011).

1.5.2 NGFR

Despite its name, NGFR has been described as a basal cell marker (Rock et al., 2009). Additionally it is found in a number of normal and cancerous cell types where it functions either as an oncogene or as a tumor suppressor (Tsang et al., 2012). It is a transmembrane low affinity protein receptor for the neurotrophin family. Unlike high affinity neurotrophin receptors, NGFR does not have an intrinsic enzymatic activity that is turned on when NGFR is activated. The response NGFR triggers upon activation is dependent on the recruitment of a variety of adaptor proteins to the intracellular domain of NGFR. Therefore, the cellular response NGFR triggers upon activation is varied, and can be as contrasting as one response promoting cell survival and one response promoting cell death. Currently NGFR is better known for its role in promoting apoptosis in nervous tissue (Tsang et al., 2012; Charalampopoulos et al., 2012; Roux & Barker, 2002; Underwood & Coulson, 2008).

1.5.3 β4 IGR

Basal cells are dependent on forming connections to the cells around them and the basement membrane. IGRs promote cell adhesion to the extracellular matrix and have a transmembrane connection to the cytoskeleton. IGRs can activate various intracellular pathways, which regulate a number of cellular processes, such as cell migration, proliferation and differentiation. IGRs are transmembrane laminin receptors that are active in dimers. β4 IGR forms a dimer with α6 IGR but α6 can form dimers with β1 IGR as well (Hynes, 2002; LaFlamme et al., 2008). Expression of the β4α6 IGR dimer has been linked to basal cells (Rock et al., 2009) and airway epithelial progenitor cells (Chapman et al., 2011).

The β4α6 IGR dimer is a part of the hemidesmosomes that promote the stable adhesion of basal epithelial cells to the underlying basement membrane. The dimer binds to plectin and this interaction is a critical part of the integrity of the hemidesmosome. The hemidesmosome is deactivated when the bond between the β4α6 IGR dimer and plectin is destabilized. This happens when, for instance, keratinocytes have to migrate to heal a wound (Margadant et al., 2008). Mutations of either α6 or β4 IGR are serious; individuals with mutations have a blistering skin phenotype and the severity of the condition corresponds to the loss of IGR function (Chapman et al., 2011).

1.6 Need for new respiratory cell culture models

Although rodents and in particular mouse models have been very useful to study lung development and lung stem cells there are various differences in histology of the epithelium (Rock et al., 2010; Rock et al., 2009; De Langhe & Reynolds, 2008). For example there is a variation in the epithelial stratification of the airways. In humans the pseudostratified epithelium that contains the basal cells covers the airways down to the terminal bronchioles and only the respiratory bronchioles are covered with a simple cuboidal epithelium. In mice this switch from pseudostratified epithelium to cuboidal occurs in the mainstem bronchi. This leaves only a small segment of the mouse airway epithelium that is comparable to most of the human airway epithelium (Rock et al., 2010, Rock et al., 2009).

The dissimilarity between the human and the murine lung makes the latter hard to use as an *in vivo* model under some circumstances. This dissimilarity could affect the responses to pathological

conditions, such as injury and inflammation, and emphasizes the need for good *in vitro* models of the airway epithelium representative of the human respiratory system (Morrisey & Hogan, 2010). However, *in vitro* models of human lung development are lacking. Several epithelial cell lines exist that portray the phenotypic traits of the proximal airway such as the trachea and the bronchi but there is a need for cell lines that mimic normal histological and developmental features of the lung, including branching morphogenesis of the distal airways (Franzdóttir et al., 2010).

An immortalized cell line with pulmonary origin can serve as a good research tool, given that it has been well characterized in terms of marker expression and cellular origin. The concern is that many established cell lines have not been properly characterized and that cell lines in general often do not retain the morphology and function of the cell of origin due to their immortalization (Zabner et al., 2003; Franzdóttir et al., 2010). Primary cells can be isolated from fresh tissue and they are thought to represent the cell of origin well. However, they are often hard to come by and show considerable variation between donors (Zabner et al., 2003).

1.7 The VA10 cell line as a respiratory cell culture model

Due to the complexity of the human airway and the diverse cell types in the airway epithelium, it is important to have a well defined cell line for each cell type in the normal airway epithelium. The cell line must correspond to normal cells *in vivo*. The VA10 cell line originates from primary bronchial epithelial cells. They were transfected with retroviral constructs containing the *E6* and *E7* oncogenes from human papillomavirus 16 (HPV16) to obtain immortality. The VA10 cell line has a marker expression pattern that corresponds to the basal cell. It expresses CK 5, 13, 14 and 17 and the α6β4 IGR. It also expresses the transcription factor p63, which is associated with basal cells (Halldorsson et al., 2007).

When cultured in an air-liquid interface (ALI) culture system, where the basolateral side of the cells is exposed to growth medium and the apical side is exposed to air, the VA10 cell line grows a pseudostratified layer where the expression of p63 is restricted to the basal layer. VA10 cells also generated high transepithelial electrical resistance in ALI culture. This indicates that the VA10 cells form functionally active tight junctions. When cultured in reconstituted basement membrane (rBM) VA10 cells produce round polarized colonies. That shows that the cells have, when grown in rBM, the potential to mimic the bronchial epithelium *in vivo* (Halldorsson et al., 2007).

When VA10 cells are grown in rBM with human umbilical vein endothelial cells (HUVEC), the VA10 cells form colonies that proliferate and form complex branching structures reminiscent of the bronchioalveolar units of the developing lung. Most of the branching structures are densely packed with nuclei but some show minor hollow cavities. These cavities are usually small and few, around 1-2 per colony (Franzdóttir et al., 2010).

1.7.1 Knockdown of *E6/E7* by shRNA

The *E6* and *E7* genes achieve immortality in cell lines by coding for proteins that bind and degrade the tumor suppressors p53 and retinoblastoma protein (pRb) respectively (Rampias et al., 2009). Gu et al. (2006) made a short hairpin RNA (shRNA) that targeted the E6 mRNA of HPV18 and transfected it to

HeLa cells with a lentiviral vector. They showed that a low dose lentiviral infection of the shRNA caused growth reduction in the HeLa cells and induced senescence. When the transfected HeLa cells were injected into Rag-/- mice, the weight of the tumor was lower than tumor weight from untransfected HeLa cells. A high dose infection of the lentiviral vector caused specific cell death via apoptosis in HeLa cells and Rag-/- mice did not form tumors upon injection (Gu et al., 2006).

1.8 Primary cell culture

Primary cells are isolated directly from fresh tissue of any sort. Primary cells are thought to represent the cell of origin better than an established cell line for several reasons, but mainly because primary cells have not been transfected with genes to make them immortal, as most established cell lines have been (Halldorsson et al., 2007; Alberts et al. 2004; Forbes, 2000). When isolating primary cells from the airway, the tissue can be removed from a live patient during lung surgery, from human donor lungs that have been rejected for transplantation, or post mortem. Getting cells from an autopsy is problematic, as there usually is a delay between death and harvesting the tissue that affects the viability of the cells and there is a greater probability of bacterial or yeast infections (Karp et al., 2002).

The primary cells have a limited lifespan and the cells go into replicative senescence after a few passages. The cells are then irreversibly growth-arrested and can not replicate DNA and therefore do not enter into the S-phase of the cell cycle, the phase of DNA duplication. Primary cells in replicative senescence continue to have metabolic activity and do not die. If they are fed regularly they can remain viable for some time (Lundberg et al., 2002; Wei & Sedivy, 1999; Dimri et al., 1995). One of the reasons for replicative senescence in primary cells is the shortening and eventual dysfunction of the telomeres (Beauséjour et al., 2003; Rodier & Campisi, 2011).

Cell in replicative senescence have increased activity of β -galactosidase activity. It can be used as a biomarker for cell in replicative senescence as quiescent and terminally differentiated cells do not express β-galactosidase under normal circumstances (Dimri et al., 1995).

Fresh human tissue for cell isolation can be difficult to acquire. Doing so requires close cooperation with a surgeon and/or a pathologist and is heavily regulated in Iceland. After obtaining permits, the tissue is often sparse and a limited amount of cells can be extracted from it. The primary cells also show donor variance and the viability of the cells obtained depends on the donor. The current isolation protocols are inconsistent, so getting a pure cell population can be problematic and time consuming. Lastly, there is always a chance that handling fresh tissue can pose a health risk (Forbes, 2000; Halldorsson et al., 2007; Karp et al., 2002; Fulcher et al., 2005).

Primary epithelial cells show a noticeable cell connection named epithelial bridges (EP bridges) in cell culture. These bridges are tubular and there are thought to have a function in cell communication and migration. There are two distinct types of EP bridges, type I connects two cells and type II connects many cells together. The composition of EP bridges makes them different from other cellular bridges such as cytonemes and tunneling nanotubes (TNT). All bridges contain F-actin but EP bridges contain microtubules as well. Cytonemes and TNTs do not transport genetic material but EP bridges have been shown to transport whole cells. The cells travel trough the tubular bridges

and because of their elasticity the EP bridges return to their normal shape. The EP bridges are E-cadherin and epithelial cell adhesion molecule (EpCAM) positive (Zani et al., 2010).

2 Aims

Establishment and long term cultivation of primary lung epithelial cells is a challenging task due to the number of different cell types found within the lung and because of their short lifespan, which limits their use in long term studies. In my opinion, it is important to isolate basal cells from the primary culture, since these cells represent a stem cell population that can generate other more differentiated cells. Most *in vitro* studies on human lung biology rely on immortalized lung epithelial cell lines. Although these cell lines are very useful in many studies, most of them can not be used to answer questions about developmental biology, stem cell properties and morphogenesis. The general aim of this study was to improve cell culture of primary lung epithelial cells with a focus on basal cells and to characterize basal cell properties of the VA10 lung epithelial cell line.

Specific goals:

1. Establish bronchial primary culture and characterize different cell types found within these cultures.
2. Sorting the VA10 cell line based on marker expression to isolate a subgroup that has a stronger basal phenotype and marker expression.
3. Silencing *E6* and *E7* in VA10 cells and analyzing the resulting phenotype.

3 Materials and methods

3.1 Cell culture

All cell cultures were maintained at 37°C Celsius and 5% CO_2 concentration.

3.1.1 VA10 cells

VA10 cells were maintained in T25 flasks (BD Biosciences, Franklin Lakes, New Jersey, USA) in commercially bought LHC-9 serum-free medium (Gibco, Carlsbad, California, USA), supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco). LHC-9 medium (Gibco) is specially made for bronchial epithelial cells. Culture medium was changed three times a week and cells were split 1:4-1:6.

3.1.2 VA10 cells with shRNA *E6/E7*

VA10 cells with shRNA were maintained the same way normal VA10 cells were. As the shRNA is on a conditional vector, i.e., the gene expression is turned on with the antibiotic tetracycline (TET), it is necessary to supplement the growth medium with TET for the shRNA to have any effect. For activation the culture medium was supplemented with 1.0 µg TET per ml of culture medium. The shRNA was activated for at least 3 days before any experiments were carried out.

3.1.3 HUVECs

HUVECs were kindly provided by Dr. Haraldur Halldórsson. They were maintained in T25 flasks (BD Biosciences) in commercially bought Vasculife medium (Lifeline Cell technology, Fredrick, Maryland, USA), supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin (Gibco) and 5% fetal bovine serum (FBS) (Gibco). Culture medium was changed three times a week.

3.1.4 Primary cell culture

The pulmonary biopsies were obtained from living patients that underwent lobectomy (most often for lung cancer), at the Department of Cardiothoracic Surgery at Landspitali. Permits from Landspítali ethical committee (Vísindasiðanefnd LSH 88374 – 96345) and informed consent were obtained. Parts of the lobe were removed by the surgeon, Tómas Guðbjartsson, together with a piece of the proximal lobar bronchus. All samples were collected in the surgery room within minutes of the tissue being removed from the body's blood supply, thus ensuring the freshness of the sample. The samples were immediately submerged in DMEM cell culture medium (Gibco), supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco). The DMEM (Gibco) was kept at 4°C Celsius and the sample kept on ice. The alveolar tissue was cut into smaller bits, frozen in liquid nitrogen for 30 seconds-2 minutes and then kept at -80°C Celsius for future research.

The bronchial segment was digested to isolate primary epithelial bronchial cells. The segment was rinsed in phosphate buffered saline (PBS) supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco) and all excess connective tissue removed from the specimen. The bronchus was then transferred into an enzyme digestive solution. Both trypsin (Gibco) and protease XIV (Sigma-Aldrich, St. Louis, Missouri, USA) were used. Trypsin (Gibco) was mixed with either PBS or DMEM

(Gibco) to form a 0.1% trypsin solution. Protease XIV (Sigma-Aldrich) was mixed with DMEM (Gibco) to form a 1 mg/ml solution. The bronchus was digested at 4°C Celsius for 48 hours. After the incubation period, the bronchus segment was scraped manually with a sterile scalpel and the resulting organoids were transferred to a collagen-coated (Advanced Biomatrix, San Diego, California, USA) T25 culture flask (BD Biosciences) and grown in LHC-9 medium (Gibco). The culture medium was changed daily for the first 2 days and the flask rinsed with PBS for removal of red blood cells and non-adherent organoids. After that the culture medium was changed 3 times a week. All primary cultures started at passage 1 (P1) and were split 1:3.

3.2 IF staining

Cells were grown in a culture flask until confluence. All the steps of the IF staining occurred at room temperature unless stated otherwise. During the staining procedure it is important that the sample never dries and, after incubation with antibodies, the sample is sensitive to light. Different fixations are required for different antibodies; for the p63 antibody, nuclear fixation is necessary and for F-actin staining, formaldehyde fixation is necessary.

3.2.1 Nuclear fixation

First the growth medium was aspirated and cells washed by rinsing the flasks with PBS. The samples were fixed with 3,5% formaldehyde (Sigma-Aldrich) for 5 minutes, then with a half-methanol (Sigma, St. Louis, Missouri, USA) and half-acetone (Sigma) solution for 5 minutes at -20°C Celsius. The sample was then incubated for 7 minutes in 0.1% Triton X-100 (Sigma-Aldrich) in PBS and again for 7 minutes in a fresh solution.

3.2.2 Formaldehyde fixation

The growth medium was aspirated and cells washed by rinsing the flasks with PBS. The samples were then fixed with 3,5% formaldehyde (Sigma-Aldrich) for 5 minutes, then with 7 minutes in 0,1% Triton X-100 (Sigma-Aldrich) in PBS and again for 7 minutes with a fresh solution. When changing the fixation solutions, the samples were first rinsed with PBS.

3.2.3 IF staining protocol

After fixation, non-specific binding regions were blocked with a 10% FBS (Gibco) in PBS for 5 minutes and then incubated for 30 minutes with the primary antibody in IF buffer. The fluorescent secondary antibody (Alexa Fluor®, Invitrogen, Carlsbad, California, USA) corresponding with the primary antibody was incubated for 30 minutes in a 1:1000 dilution in IF buffer followed by 15 minutes of To-Pro-3 nuclear dye (Invitrogen) in a 1:500 dilution in IF buffer. Between antibodies and the nuclear dye the samples were washed once quickly and then twice for 5 minutes. For removal of remaining salt from the PBS, samples were rinsed with water and left to dry. When dry, they were mounted with Fluoromount (Southern Biotech, Birmingham, Alabama, USA) and a deck glass. The samples were viewed in an Axioskop 2 FS confocal microscope (Zeiss, Oberkochen, Germany). A list of the primary antibodies used can be seen in table 1.

Table 1: List of antibodies used in IF staining.

Antibody	Clone	Dilution in IF staining	Species	Isotype	Producer	Number
Acetylated TUBA4A	6-11B-1	1:100	Mouse	IgG2b	Abcam (Cambridge, England)	ab11323
Cadherin, E	36	1:100	Mouse	IgG2a	BD Biosciences	BD610182
Cadherin, N	32	1:100	Mouse	IgG1	BD Biosciences	BD 610921
CK 14	RCK107	1:100	Mouse	IgG1	Abcam	ab9220
CK 5/6	D5/16B4	1:100	Mouse	IgG1	Zymed (Carlsbad, California, USA)	180267
EGFR	D38B1	1:100	Rabbit	IgG	Cell Signaling (Danvers, Massachusetts, USA)	CS4267S
EpCAM	Polyclonal	1:100	Rabbit	IgG	Abcam	ab71916
F-actin (Phalloidin)	N/A	1:40	N/A	N/A	Invitrogen	A12379
FSP	AS02	1:100	Mouse	IgG1	Calbiochem (Billerica, Massachusetts, USA)	CP28
IGR β4	3E1	1:500	Mouse	IgG1	Merk Millipore (Billerica, Massachusetts, USA)	MAB1964
IGR α6	MP4F10	1:500	Mouse	IgG2b	N/A	N/A
MUC5AC	45M1	1:100	Mouse	IgG1	Abcam	ab3649
NGFR (P75)	ME20.4	1:50	Mouse	IgG1	Abcam	ab8877
p63	7JUL	1:25	Mouse	IgG1	Novocastra (Wetzlar, Germany)	N/A
Vimentin	V9	1:100	Mouse	IgG1	Dako (Glostrup, Denmark)	M0725

3.3 Cell sorting

VA10 cells were sorted for β4 IGR and NGFR (see table 1 for information on the antibodies) with magnetic-activated cell sorting (MACS). Cells were grown in a T75 flask (BD Biosciences) until they were confluent. The cells are trypsinized, centrifuged and resuspended in 200 µl of sterile MACS buffer. They are then incubated with the antibody in the right dilution for 30 minutes at 4°C Celsius. Then the cells are washed with 3 ml of MACS buffer by centrifuging and resuspending them twice to get rid of unbound antibodies. The cells are resuspended with 160 µl of MACS buffer and 40 µl of the secondary antibodies, which are anti-mouse IgG MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany). They are incubated for 20 minutes at 4°C Celsius. The solution is filtered through a single cell filter to ensure it is a single cell suspension and run through a MACS machine (Miltenyi Biotech). After sortation, two distinct cell populations, high expressing and low expressing, are isolated and can be seeded on flasks.

3.4 RNA isolation

RNA was isolated from VA10 cells with 3 different shRNA constructs for E6/E7, both from unactivated and from TET activated cells. The cells were TET activated for 3 days prior to RNA isolation. RNA was isolated using TriReagent (Ambion, Carlsbad, California, USA). The culture medium was removed and replaced with 2.5 ml TriReagent (Ambion) per T25 flask (BD Biosciences) and incubated at room temperature for 5 minutes. The cells were then transferred to a 1.5 ml RNase free eppendorf tubes

(Axygen, Union City, California, USA) and 0.5 ml of chloroform (Sigma-Aldrich) added to the tube. The tube was vortexed and incubated for 10-15 minutes, then centrifuged at 13.000 RPM for 18 minutes at 4°C Celsius. After that the sample showed a clear division; the lower layer being pink and the upper layer clear.

The clear portion was removed with care and transferred to another RNase free tube (Axygen). 1.25 ml of isopropanol (Sigma-Aldrich) was added to the sample, cautiously mixed and left to incubate for 5-10 minutes. The sample was centrifuged at 13.000 RPM for 12 minutes at 4°-25°C Celsius. The supernatant was removed and the RNA at the bottom resuspended in 2.5 ml of 96% EtOH (Gamla apótekið, Reykjavík, Iceland) with a vortex. The sample was then centrifuged again at 8.000 RPM for 5 minutes at room temperature. Following that, the supernatant was removed and the tubes left to dry with an open cap. The RNA was then resuspended with 20 µl of RNase free water (Qiagen, Hilden, Germany). The amount of RNA is measured by a photometer and if there is a contamination of EtOH in the sample (the 260/230 ratio is under 1), the process can be repeated from the addition of isopropanol until the resuspension in RNase free water (Qiagen).

3.5 cDNA synthesis

The complementary DNA (cDNA) is made with a RevertAid First Strand cDNA Synthesis Kit (#K1621 Thermo Scientific, Waltham, Massachusetts, USA) that includes all enzymes and buffers. All reactions are carried out in RNase-free tubes (Axygen) and solutions are stored on ice. The RNA is DNase treated first to get rid of any DNA traces. 1 µg of RNA with DNase 1 and 10x reaction buffer with $MgCl_2$ in a volume of 10 µl is heated to 37°C Celsius for 30 minutes. 1 µl of 50 mM EDTA is added and the solution heated to 65°C Celsius for 10 minutes. The RNA is then ready to be used as a template for the reverse transcriptase. 1 µl of the oligo$(dT)_{18}$ primer and 1 µl of nuclease-free water are added to the solution, which is then mixed gently, centrifuged briefly and heated to 65°C Celsius for 5 minutes. Then, these components are added in the following order; 4 µl of 5x reaction buffer, 1 µl of the RiboLock RNase inhibitor, 2 µl of a 10 mM dNTP mix and 1 µl of RevertAid M-MuLV reverse transcriptase. The solution is mixed gently, centrifuged and heated to 42°C Celsius for 60 minutes. The reaction is terminated by heating it to 70°C Celsius for 5 minutes. The cDNA solution is then stored at -80°C Celsius.

3.6 qRT-PCR

cDNA of VA10 with shRNA for *E6/E7* was analyzed with qRT-PCR to verify the knockdown of *E6/E7*. The primers used were E6/E7-1-F: 5´-GAC TCT ACG CTT CGG TCG TG-3´ and E6/E7-1-R: 5´-GTG CCC ATT AAC AGG TCT TCC A-3´ (TAG Copenhagen, Copenhagen, Denmark). These were designed by the author. Maxima SYBR green/ROX qRT-PCR master mix (Thermo Scientific) was used and the reaction setup can be seen in table 1.

Table 2: Setup of qRT-PCR reaction for *E6/E7*

Maxima SYBR green/ROX qRT-PCR master mix (Thermo Scientific)	5 µl
E6/E7-1-F primer (TAG Copenhagen)	0.25 µl
E6/E7-1-R primer (TAG Copenhagen)	0.25 µl
cDNA	2 µl
Water, nuclease free (Thermo Scientific)	2.5 µl
Total volume	10 µl

The reaction was carried out in a 7500 real time PCR system (Applied biosystems, Foster City, California, USA).

3.7 3D culture in Matrigel

VA10 cells with shRNA A for *E6/E7* genes were cultured in Matrigel, (BD Biosciences); a growth factor reduced reconstituted basement membrane with HUVECs. The shRNA was activated with TET for 4 days before seeding in to Matrigel. VA10 cells and HUVECs were trypsinized and counted. 600 VA10 cells and 102.000 HUVECs were centrifuged and resuspended in 180 µl of chilled Matrigel. The Matrigel was then transferred gently to avoid bubble formation to a 48 well plate and allowed to gelatinize for 30 minutes at 37°C Celsius before the addition of 300 µl of EGM (Lonza, Basel, Switzerland) supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin (Gibco) and 5% FBS (Gibco) for the unactivated VA10 cells. The TET activation was continued by adding TET to the EGM medium (Lonza). Culture medium was changed three times a week. The cells were grown for 21 days in the Matrigel.

3.8 Staining of branching structures in Matrigel

VA10 cells with shRNA A for *E6/E7* were grown in Matrigel with HUVEC. They formed branching structures that were stained in the Matrigel to test for lumen formation. The medium was removed from the Matrigel and rinsed once with PBS. The cells were incubated in the plate with 4% formaldehyde in PBS with fluorescence labeled F-actin (Invitrogen) diluted 1:40 and To-Pro-3 nuclear stain (Invitrogen) diluted 1:500 overnight at 4°C Celsius. The Matrigel was then transferred from the plate with a Pasteur pipette to a glass. On the glass was a raised clay border surrounding the Matrigel to avoid the structures being smashed by the coverslip. The structures were viewed in an Axioskop 2 FS confocal microscope (Zeiss).

3.9 Growth curve

Cells were trypsinized and counted. 10.000 cells were seeded in triplicates into 24 well plates. Proliferation was tested for 7 days with 24 hour intervals. On each day, the corresponding plate was fixated with 4% formaldehyde for 10 minutes and washed with PBS. The cells were stained with 0.1% crystal violet for 10 minutes and washed 5 times with PBS. The plates were then allowed to dry. After the last plate had been fixed the samples were dissolved in 0.5 ml of 10% acetic acid and 100 µl were

transferred to a 96 well plate and measured in a photometer at 570 nm. The samples were diluted if their absorbance was out of range. The results were analyzed in Microsoft Excel.

3.10 Senescence staining

Primary cells were stained for senescence using the senescence β-galactosidase staining kit (#9860, Cell signaling). All solutions were mixed immediately prior to use. The cell medium was removed and the cells rinsed with PBS. The cells were fixed with a diluted 10x fixative solution provided in the kit for 15 minutes at room temperature. After rinsing two times with PBS, the cells were incubated overnight at 37°C Celsius in a β-galactosidase staining solution. The next day, the staining solution was removed and the cells viewed in an inverted brightfield microscope (Leica, Wetzlar, Germany).

3.11 Technical consideration regarding establishing bronchial primary cultures

Isolation protocols for bronchial tissue are generally quite comparable; in most cases enzymatic digestion is used for a period of time and then cells are grown in flasks with some sort of coating, most often collagen. Protease XIV has been successfully used for enzymatic digestion at this laboratory (Jónsdóttir, 2012). Fulcher and colleagues propose to use protease XIV with DNase for large tissue samples, but for smaller bronchial segments only protease XIV would be needed. The tissue is incubated at 4°C Celsius for 48 hours for large tissue samples but overnight for smaller ones. The cells are then grown on collagen I/III coated flasks, or on porous supports coated with collagen IV (Fulcher et al., 2005).

Yamaya and colleagues also used protease XIV and incubated the tissue overnight at 4°C Celsius (Yamaya et al., 1992). Tesei and colleagues used a protocol first described in use with primary epithelial cells from the mammary gland (Stingl et al., 1998) to isolate primary bronchial cells. They used a mixture of collagenase and hyaluronidase to digest the tissue after mincing. The tissue was incubated for 6-12 hours at 37°C Celsius and then cultured at low adherence to produce bronchospheres (Tesei et al., 2009). Karp and colleagues isolated primary epithelial cells using pronase and DNase and incubated them at 4°C Celsius for 24-96 hours. The cells were then grown in flasks coated with collagen type IV from human placentas (Karp et al., 2002).

Enzymatic digestion is not always needed. Epithelial cells can be harvested by placing a 2 cm segment of bronchus in culture medium, cutting the bronchial pieces into smaller segments on day 3-5 and culturing them again. After 8-12 days the epithelial cells have formed an outgrowth from the tissue bits. They are then removed and grown on Swiss 3T3 mouse feeder cells that have been growth-arrested with mitomycin (Lechner et al., 1981). Ramirez and colleagues also grow primary bronchial cells without digesting the tissue. They mince the tissue to bits around 4 mm in size, grow them in flasks coated with porcine collagen I in KSFM medium and epithelial cells start to grow out from the tissue bits (Ramirez et al., 2004).

No consensus has been reached as to what the best way to isolate primary epithelial cells from the human bronchus is.

4 Results

39 samples were obtained from lung lobectomies performed at the Landspítali University hospital in Reykjavík; thereof 20 distal alveolar samples and 19 proximal bronchial samples. The average age of the patients was just over 68 years and 15 patients were female and 5 male. 18 primary cell cultures succeeded. Ciliary function was observed in some samples.

Isolation of a VA10 cell subpopulation by markers associated with basal cells was performed. The isolation was performed 3 times by two different markers, β4 IGR and NGFR, which are known basal cell markers. The resulting sub-groups had a reduced growth rate but the marker expression profile was close to that of VA10 parental cells.

In order to get more differentiated VA10 cells a knockdown of the *E6/E7* genes in VA10 cells was pursued. The purpose was to make an improved model of branching morphogenesis in Matrigel and to test the hypothesis that a lumen in branching structures is formed by apoptosis of the cell in the middle of the structure. The knockdown in VA10 cells was successful and the cells containing the shRNA unactivated still branched in matrigel but activation of the shRNA severely reduced branching.

4.1 Primary cell culture

4.1.1 Primary cells retain ciliary function in initial culture

The protocol used to treat samples from biopsies resulted in a cell solution that contained single cells and organoids. A portion of both adhered to the collagen coating of the culture flask and survived. The organoids (see figure 3) that adhered had a dense halo of cells exhibiting ciliary function, as evidenced by live inspection in phase contrast microscope (data not shown) and expression of acetylated tubulin which is a marker for cilia (see figure 6). Cells surrounding the organoids expressed mucin and contained p63 negative cells (see figure 6 and 5 respectively). All these features correspond to more differentiated cells.

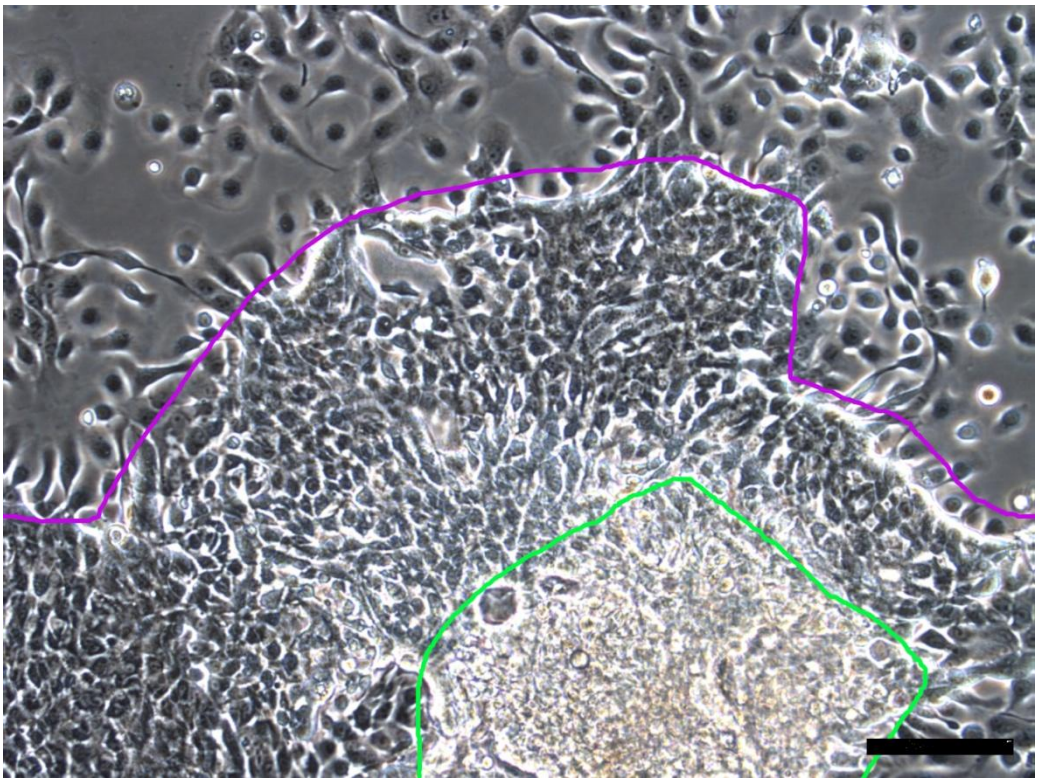

Figure 3: Epithelial cells from biopsy 8 on day 3 after initial setup.

An organoid (defined by the green border), cells with ciliary function (defined by the purple border) and larger cells that have adhered from a single cell suspension (outside the borders). Scale bar 100 µm.

Figure 3 shows a successful initial setup of bronchial epithelial organoids and single cells. Inside the green border is an organoid that has attached to the flask. Inside the purple border are cells growing out from the organoid exhibiting ciliary function as they can be seen beating in a microscope. Outside of the border are cells that have attached from a single cell suspension and do not have any cilia. The cells surrounding the organoid are quite dense and smaller than the ones that attached from a single cell suspension.

4.1.2 Primary cells from different biopsies showed a similar phenotype in culture

Primary bronchial cells from different biopsies had a similar phenotype but there are some variations. The majority of primary cells showed a curved, blunt phenotype but some cells showed a curved, elongated phenotype reminiscent of fibroblasts, with thread like protrusions. The cells seem to lie in one plane except for elongated threads that lie above the cells and seem to connect them (the black arrow in figure 4 B points to a thread). These threads are more prominent in some biopsies than others (figure 4, B). A variance was observed in the size of the cells as well. Usually the primary cells were relatively big, but from some biopsies the primary cells were quite small. The primary cells from biopsy 4 (figure 4, A) are bigger than the ones from biopsy 15 (figure 4, D).

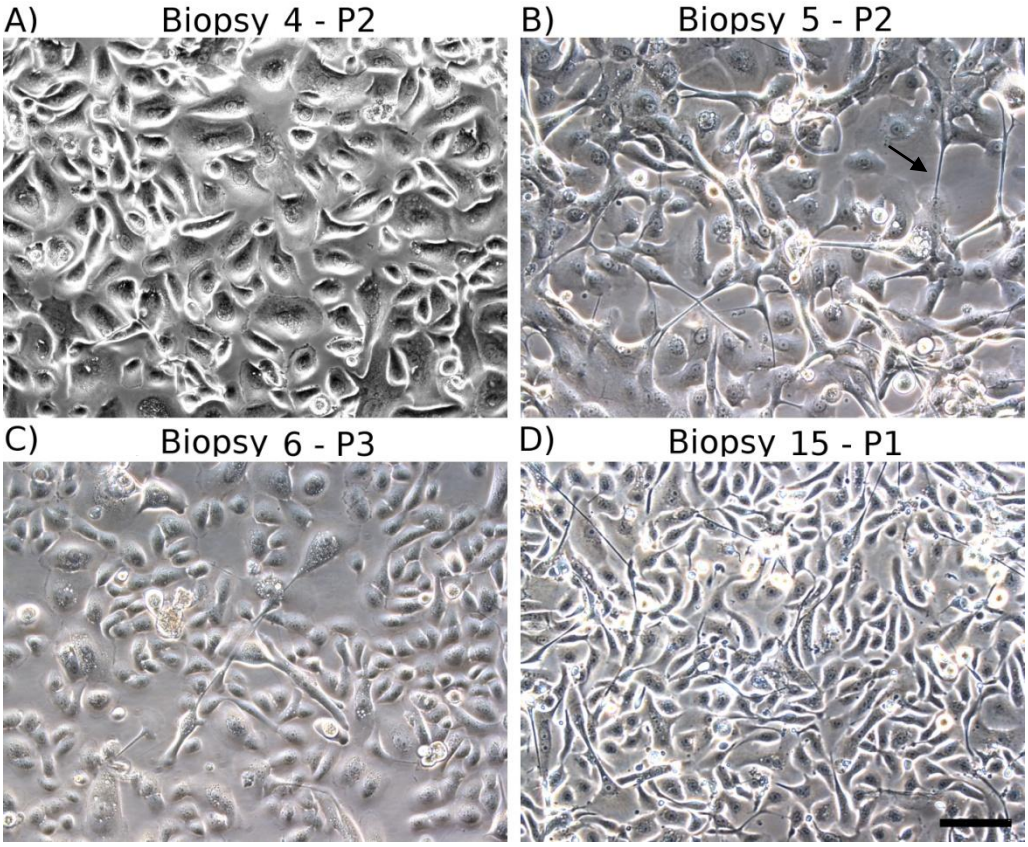

Figure 4: Comparison of primary bronchial epithelial cells from 4 different biopsies.

Different phenotypes were observed in primary epithelial cells. Some cells had an elongated phenotype with threadlike protrusions, reminiscent of fibroblasts (black arrow in B). Scale bar 100 µm.

4.1.3 Primary epithelial cells form EP bridges in cell culture

EP bridges are formed by primary bronchial epithelial cells in culture. This phenotype was observed in this project. EP bridges can be seen quite clearly in the primary cells from biopsy 5 (see figure 4, black arrow in B). There was a difference in the tendency to form EP bridges between biopsies, for example not many EP bridges were observed in biopsy 4 (see figure 4, A).

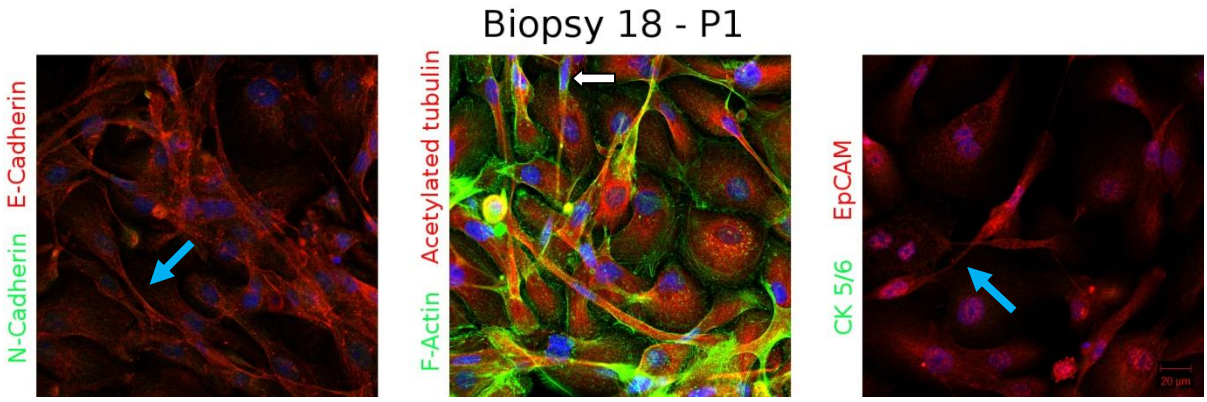

Figure 5: Three different IF stainings of primary epithelial cells from biopsy 18 showing EP bridges.

EP bridges are used for cell transport and the middle of the panel shows an epithelial cell is potentially traveling through an EP bridge (white arrow). The threadlike EP bridges stain positive for EpCAM and E-Cadherin (blue arrows). Scale bar 20 µm.

What makes EP bridges different from other cellular bridges is their composition and role: all bridges contain F-actin but EP bridges contain microtubules as well. The bridges expressed acetylated tubulin, marking them as EP bridges rather than other cellular bridges (figure 5, middle). EP bridges have been shown to transport whole cells as can be seen in figure 5 (middle, white arrow) (Zani et al., 2010).

4.1.4 Primary cells potentially change marker expression by passage number

In the first passage the primary cells exhibit ciliary function and more differentiated characters, especially around organoids that have attached. Cells in P1 do not express p63, a basal cell marker, homogenously but in P2 the expression is more homogenous (see figure 6).There is also a change in differentiation marker expression. In P1 there is an expression of mucin, a goblet cell marker, which is not to be found in P2 (see figure 7). The mucin expressing cells seem to be clustered around organoids in P1. Acetylated tubulin, which is a ciliary marker, does not seem to be affected by time.

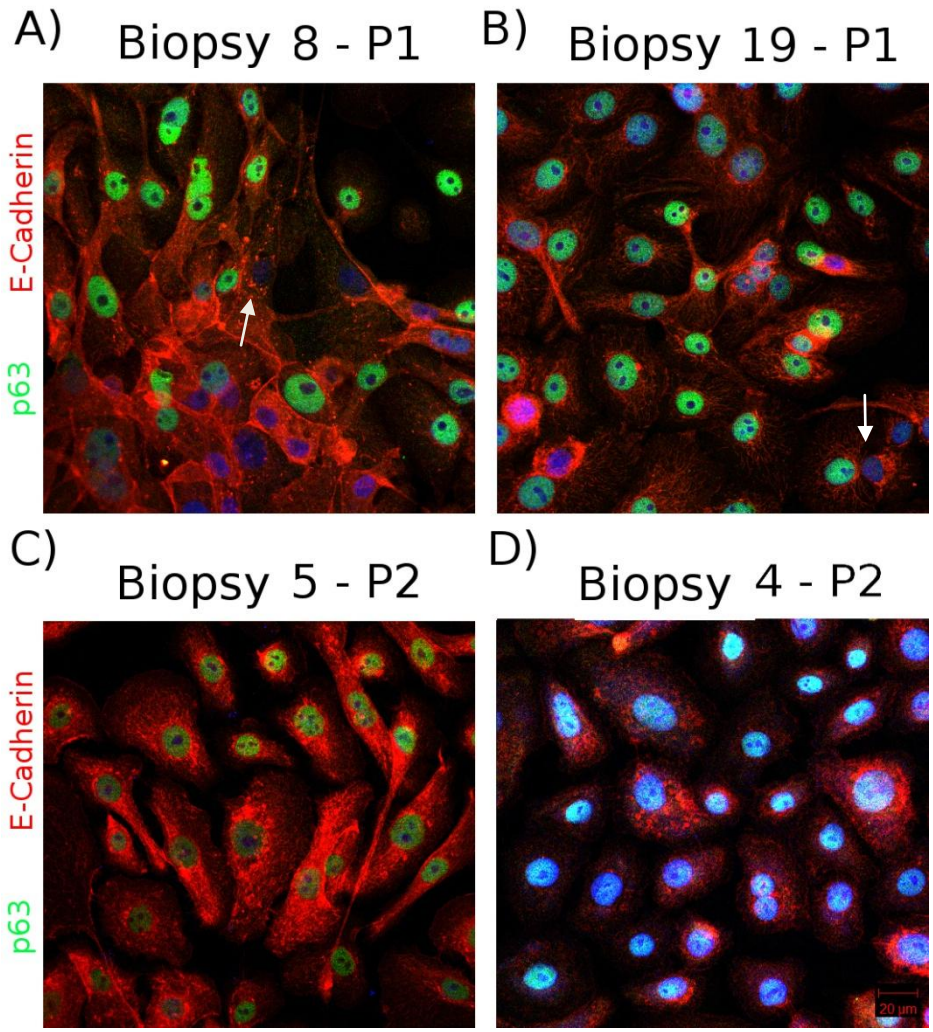

Figure 6: IF stainings of primary bronchial cells from different biopsies showing the difference in p63 expression.

Primary cells in P1 show p63 negative cells (see white arrows in A and B). Primary cells in P2 show homogeneous expression of p63. Scale bar 20 µm.

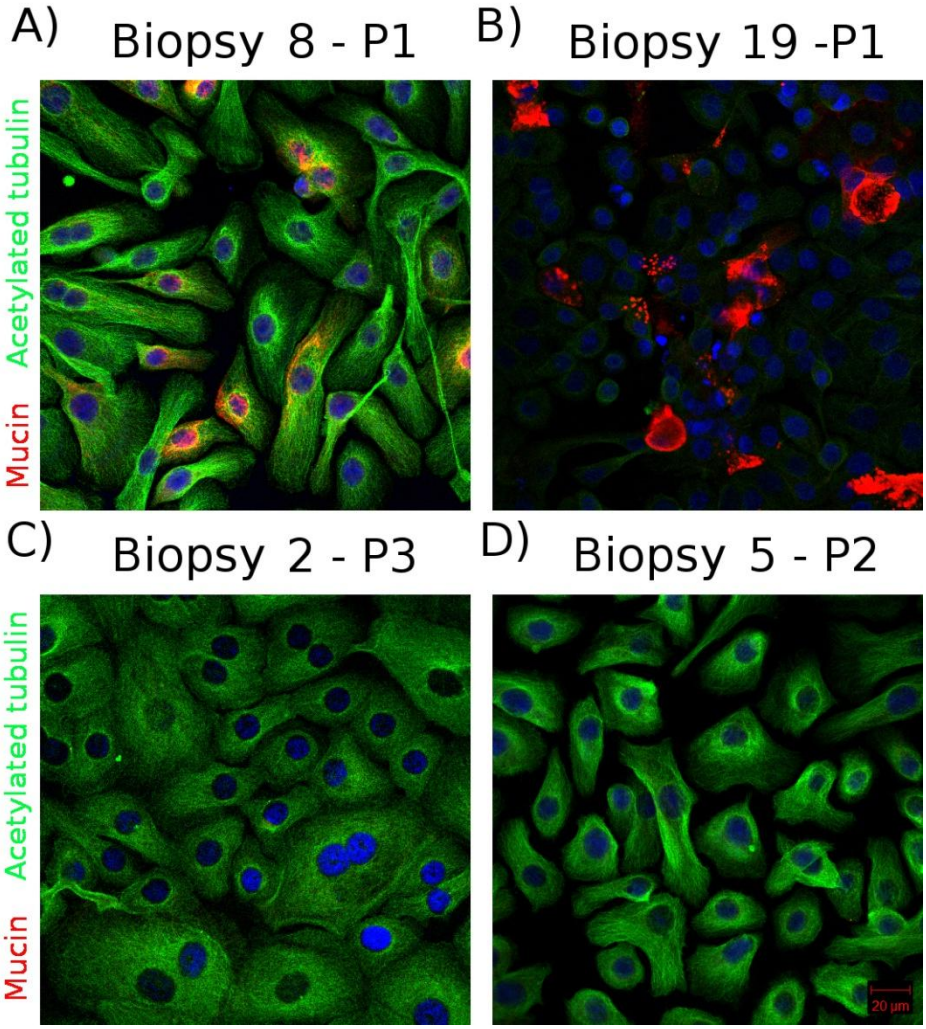

Figure 7: IF stainings of primary bronchial cells from different biopsies showing the difference in differentiation marker expression.

Acetylated tubulin is a marker of ciliary cells and mucin is a marker of goblet cells. In P1 there is a heterogeneous expression of mucin (see A and B). In P2 there is no expression of mucin. Scale bar 20 µm.

The cells surrounding organoids in passage 1 are more likely to be p63 negative than cells that have attached from a single cell suspension. Mucin positive cells are also more likely to be found surrounding organoids. To properly evaluate the changes in marker expression one should ideally perform p63 and mucin stainings on various passages of the same cell population. That has not been possible because the number of cells from each biopsy is limited. These stainings are an indication that this change in marker expression occurs. The supporting evidence is that the p63 negative cells and mucin positive cells are more likely to be found surrounding organoids, a situation which does not arise in later passages. Collectively, there is a tendency for enrichment of p63 positive cells and a reduction in mucin positive cells in P2 compared to P1.

4.1.5 Primary cells show a donor variation in various markers

Donor variance is a drawback to using primary cells, as it affects the reproducibility of the results. There are considerable donor variances but mapping the most probable ones, if that is possible, would improve the primary cell model. A variance was observed in E-Cadherin (figure 8), N-Cadherin (figure 9), epidermal growth factor receptor (EGFR), as well as vimentin (figure 10) and CK5/6 (figure 11) expression. Figure 7 also shows donor variance in acetylated tubulin expression. Cells from one biopsy (see B) seem to have a low expression of the normally abundant acetylated tubulin. These markers are not specifically chosen; all donor variation observed was reported.

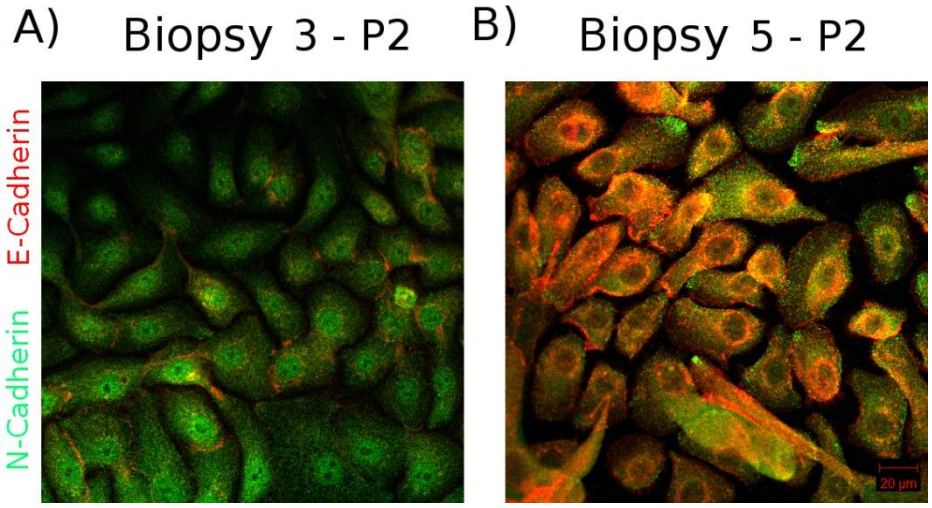

Figure 8: Donor variance in E-Cadherin expression between primary cells from 2 biopsies.

Biopsy 5 shows red stained E-Cadherin (B) and the biopsy 3 shows low expression of E-Cadherin (A). Scale bar 20 µm.

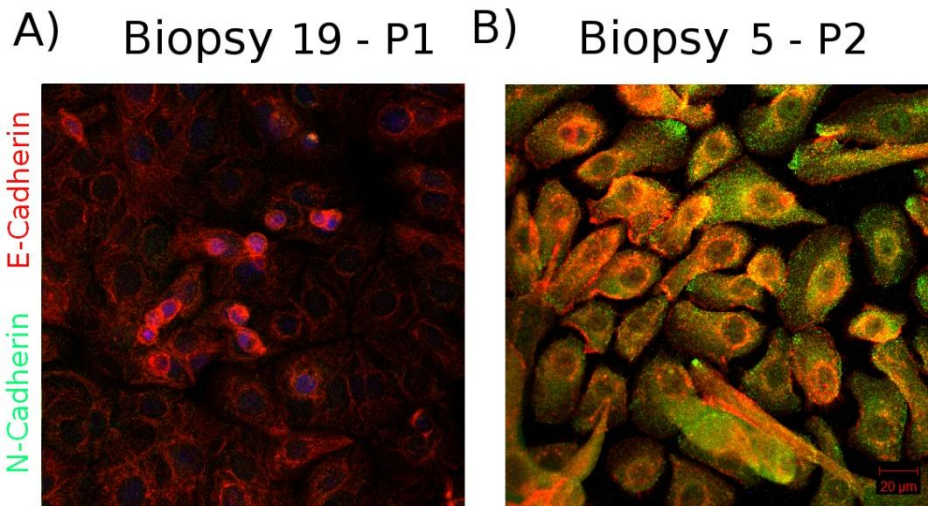

Figure 9: Donor variance in N-Cadherin expression between primary cells from 2 biopsies.

Biopsy 5 shows green stained N-Cadherin (B) and biopsy 19 shows low expression of N-Cadherin (A). Scale bar 20 µm.

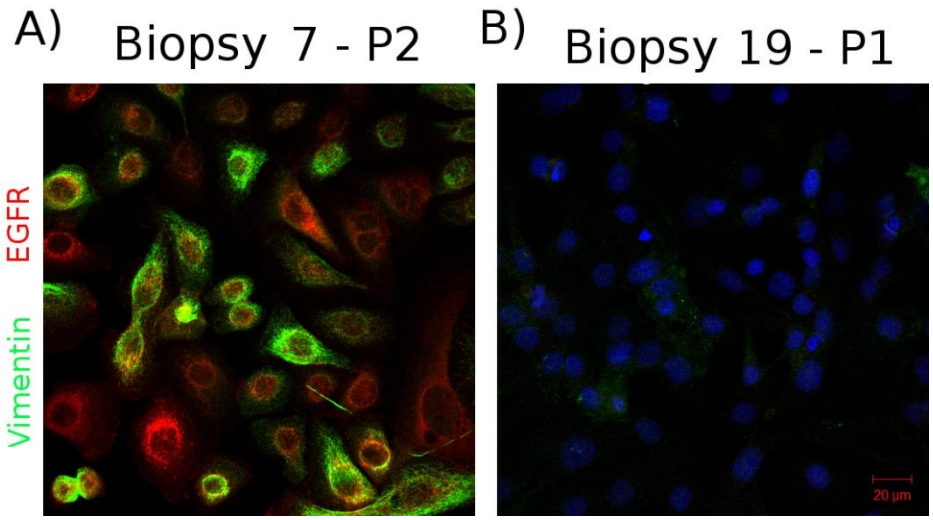

Figure 10: Donor variance in EGFR and vimentin expression between primary cells from 2 biopsies.

Biopsy 7 shows green stained vimentin and red stained EGFR (A). Biopsy 19 shows low expression of both vimentin and EGFR (B). Scale bar 20 µm.

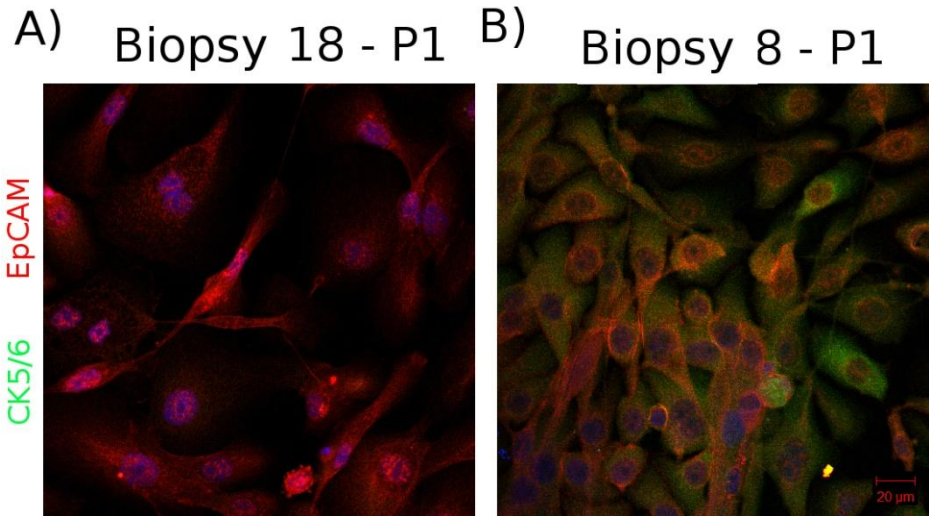

Figure 11: Donor variance in CK5/6 expression between primary cells from 2 biopsies.

Biopsy 8 shows green CK5/6 (B) and biopsy 18 shows low expression of CK5/6 (A). Scale bar 20 µm.

4.1.6 Primary cells undergo senescence after a prolonged time in culture

Primary cells have a finite lifespan and when cells have reached replicative senescence they are, although technically living, no longer feasible for research. To analyze when and in what magnitude primary cells enter senescence, primary cells were stained with β-galactosidase.

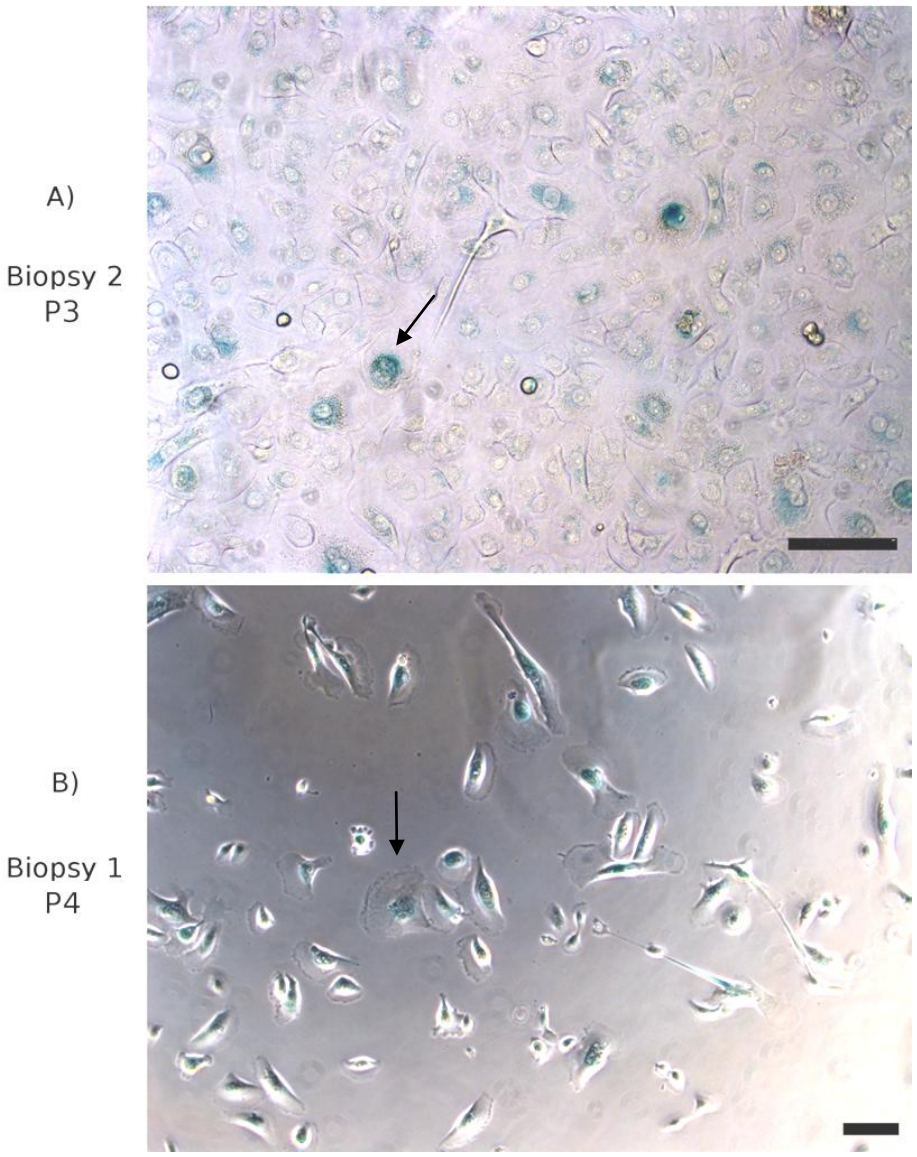

Figure 12: β-galactosidase staining of primary bronchial cells from 2 biopsies.

A) Primary cells from biopsy 2 in passage 3. Scale bar 100 µm. B) Primary cells from biopsy 1 in passage 4. Scale bar 100 µm. β-galactosidase staining marks cells that have reached replicative senescence with blue (black arrows). Note that the upper and lower parts of the figure are not taken at the same magnification.

The cells from biopsy 1 had been 6 weeks in culture (figure 12, B) and the cells from biopsy 2 had been cultured 5 weeks (figure 12, A). The cells from biopsy 1 were few and far apart because so many of them had reached replicative senescence. They show the classic senescence phenotype as well. The size of the cell increases, with the cell growing up to twice as large as it was before senescence (black arrows in figure 12). Approximately 75% of the cells have reached replicative senescence. The cells from biopsy 2 are more numerous and the percentage of cells in replicative senescence is lower. The cells that stain blue seem to have an an enlarged senescence phenotype. Approximately 20% of the cells have reached replicative senescence.

Culturing primary cells from primary bronchial tissue is a challenging task. The initial aim was to track the differentiation pattern of basal cells and isolate them for further characterization, if possible. There was no success purifying basal cells from the bulk cell population and it will be necessary to improve cell culture techniques for it to be possible to expand distinct subpopulations from the lung. In addition to this work, an aim in this research was also to analyze in better detail basal cell properties of the VA10 cell line.

4.2 VA10 cells sorted for β4 IGR and NGR show a reduction in growth

Although the VA10 cell line has basal cell properties, it is heterogeneous in terms of marker expression. In order to try to get a subpopulation of VA10 cells that had stronger basal characteristics than the parental cell line, the VA10 cells were labeled with antibodies against NGFR or β4 IGR followed by magnetic cell separation. NGFR isolation was performed twice and β4 IGR isolation once. The NGFR high and β4 IGR high portion of VA10 cells was small. The β4 IGR high portion of VA10 cell was higher than NGFR high portion but it was still a minority. That can indicate that a small segment of the VA10 cells are responsible for its basal cell abilities. In all isolations a large proportion of the cells was lost. After the sortation, few of both the high- and low-expressing cells were left to grow, so genetic diversity was lost.

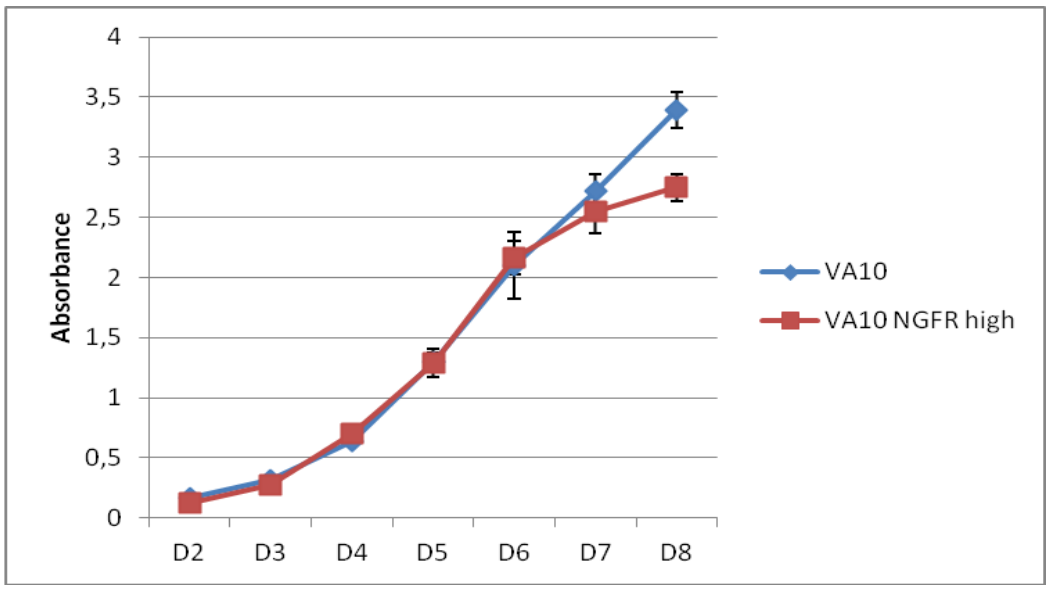

Figure 13: Growth curve comparison of unsorted VA10 cells and VA10 NGFR high.

20.000 cells were seeded and the first day of staining was 2 days after seeding. The growth curves are similar for the first 6 days (D6) but on D7 the NGFR high expressing subgroup starts showing less growth.

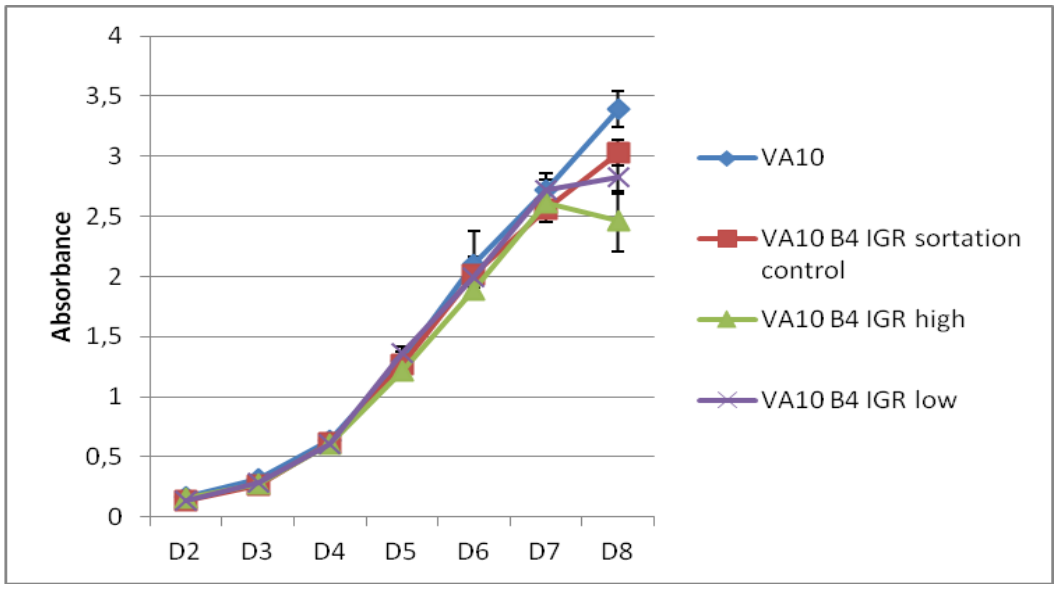

Figure 14: Growth curve comparison of unsorted VA10 cells and VA10 β4 IGR sorted cells.

20.000 cells were seeded and the first day of staining was 2 days after seeding. The sortation controls are β4 IGR high and β4 IGR low VA10 cells mixed at a 1:1 ratio. The growth curves are similar for the first 7 days (D7) but on D8 the NGFR β4 IGR isolated subgroups start showing less growth.

In figure 13 and 14 a reduction in growth is visible after magnetic cell sorting. In figure 14, combined IGR high and low are used as controls to see if the growth reduction is a result of the sortation treatment or if the β4 IGR sorted cells grow better in proximity to each other. In the sortation control, the β4 IGR high and β4 IGR low VA10 cells were mixed at a 1:1 ratio which is an arbitrary ratio and not an accurate representation of the ratio in the VA10 cells.

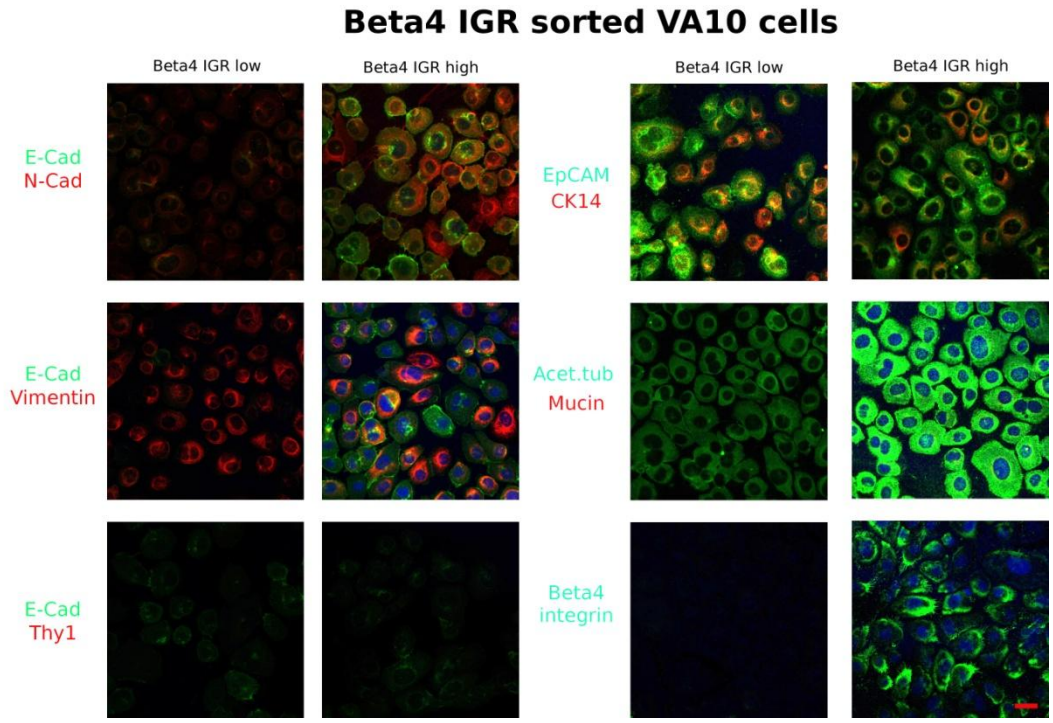

Figure 15: IF staining of β4 IGR isolated VA10 cells.

Although the two subgroups show a similar marker expression profile, the β4 IGR expression is much higher in β4 IGR high expressing cell, thus showing that the isolation was successful.. Scale bar 50 µm.

There seems to be a slightly higher expression of acetylated tubulin in β4 IGR high cells (figure 15). An increased expression of a differentiation marker does not correlate well with β4 IGR high cells being a subgroup with more stem cell abilities. Otherwise the β4 IGR high and low show a similar marker expression.

4.3 shRNA causes knockdown of *E6/E7* in VA10 cells

VA10 cells were originally generated by retroviral transfection of constructs containing the *E6* and *E7* oncogenes targeting p53 and pRb, respectively. In attempt to see if VA10 could increase the differentiation potential, lentiviral constructs containing shRNA against *E6* and *E7* were designed.

Three different shRNAs for *E6* and *E7* were tested. Two of the shRNAs, shRNA A and B, were designed and described by Rampias and colleagues (Rampias et al., 2009). The third, shRNA C, was designed by Sigríður Rut Franzdóttir. The shRNA oligonucleotides were cloned into a TET conditional (expression turned on with TET) vector with a GFP reporter gene and VA10 cells were transfected. This work was done by Sigríður Rut Franzdóttir. To evaluate the knockdown the expression of *E6/E7* was assessed with qRT-PCR (figure 16). shRNA B and C do not achieve a knockdown but shRNA A shows a seven-fold knockdown.

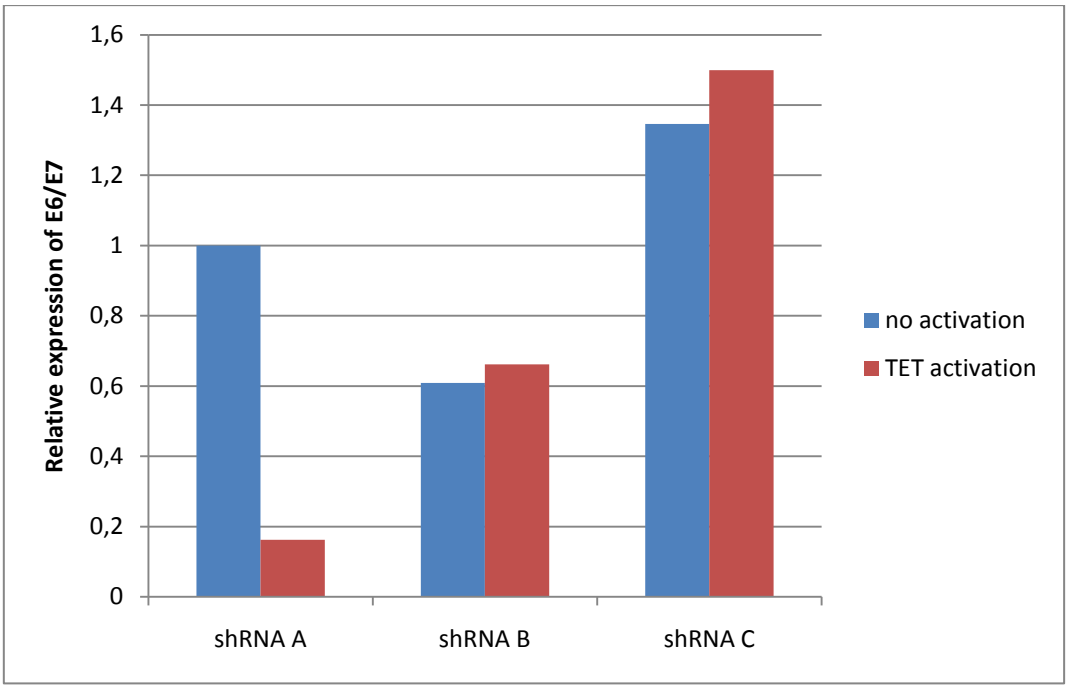

Figure 16: Knockdown of *E6/E7* in VA10 cells with 3 different shRNA constructs.

The expression of *E6/E7* was estimated with and without TET activation. β tubulin is an internal control.

To test the affects of the knockdown of *E6/E7*, VA10 cells were seeded in Matrigel with HUVECs and grown there for 21 days. The cells were TET activated for 4 days prior to seeding them in the Matrigel and for the duration of growth in Matrigel.

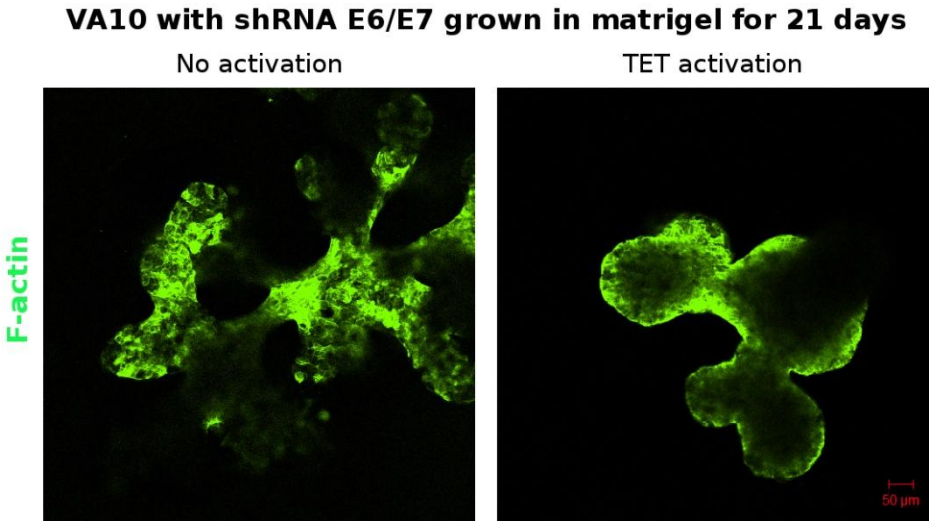

Figure 17: Examples of the branching structures formed when VA10 cells with shRNA A are grown in Matrigel with and without TET activation.

The branching structures were stained after 21 days in culture. The branching structure that was not activated with TET (left) is lumenless but the TET activated branching structure (right) shows a lumen. Scale bar 50 µm.

VA10 cells with shRNA A for *E6* and *E7* were grown in Matrigel with (see figure 17, right) and without (see figure 17, left) TET activation. A substantial difference in the number of branching colonies was observed, TET activated cells branched much less. In figure 17 an example of branching structures with and without lumen formation can be seen. The branching structure that received no activation is whole and lumenless but the TET activated structure has a lumen. Branching structures with and without lumen were observed both in TET activated and VA10 cells with no activation. The structures were stained with To-Pro 3 nuclear stain but no nuclear stain could be seen upon visualization.

5 Discussion

The research presented here further characterizes primary epithelial cells from the human bronchus, defines the donor variance observed and indicates a change in the marker expression of the cells with the passage of time. These aspects have to be taken into account when primary cells are used in research. The sortation of VA10 cells based on markers that have been associated with basal cells, i.e. β4 IGR and NGFR, resulted in VA10 cell subgroups that grew more slowly than the original cell line and had a marker expression that did not differ severely from VA10 cells. The subgroups showed changed expression of the markers used to sort the subgroups, confirming that the sortation was successful. VA10 cells were immortalized with viral *E6* and *E7* genes. A shRNA construct was designed to knock down *E6* and *E7* and it had the desired function of knocking down expression, thus making the VA10 cells mortal again. This knockdown seems to affect the branching formation of VA10 cells in Matrigel.

5.1 Primary bronchial cells

Culturing both primary cells and established cell lines has its faults. Established cell lines have been immortalized, grown for many passages in a laboratory in an artificial culture medium, all of which can influence their marker expression and behavior. Keeping primary cells in culture is not easy, as they have a very limited lifespan and go into senescence after a few passages. Isolating cells from tissue is problematic, as getting fresh tissue requires many permits and close cooperation with surgeons and/or pathologists. The advantage gained by using primary cells is that they represent the cell of origin better than established cell lines.

As for the issue of obtaining tissue, most laboratories that culture primary cells do not have the advantage of proximity to the surgery ward that this laboratory has. The surgery room is close by and I have permission to go into the surgery room and collect the sample minutes after it is removed from the body. A short amount of time passes from the tissue being removed from the body until it is processed in the laboratory. This would not possible without close cooperation with the surgeon and the surgery staff.

The patients that were sampled for this research were chosen on the basis of the quality of their lung tissue. The average age of the donors is lower than the average age of patients diagnosed with lung cancer in Iceland (70 years for males and 69 years for females) (Jónasson & Tryggvadóttir, 2012), suggesting that there is a correlation between age and lung tissue quality. The majority of the donors are females. Gender of donors can have an influence on the quality of cells. Women are, for example, less likely to have smoked in their lifetime, which is big risk factor in developing lung cancer (Jemal et al., 2011; Levitz et al., 2004). Non-smokers also develop lung cancer and these individuals are more likely to be female and have an adenocarcinoma. These cancers are linked to environmental factors such as second-hand smoke and air pollution (De Groot & Munden, 2012). Women who do smoke are also more likely to develop lung cancer, likely due to higher estrogen concentration (Gasperino, 2011).

5.1.1 Phenotype of primary cells

Primary epithelial cells show a similar phenotype between biopsies, but with certain variations. Some cells show a curved, blunt phenotype but others showed a curved, elongated phenotype reminiscent of fibroblasts with thread like protrusions called EP bridges. The prominence of the elongated phenotype was not the same in cells from different biopsies. The two different phenotypes from the primary cells can be seen in figure 4, (A for the curved blunt phenotype, B for more prominent fibroblast-like phenotype.) The majority of the cells showed a curved blunt phenotype but the elongated cells with EP bridges take up more space and are therefore more conspicuous. The EP bridges are thought to be a normal part of primary epithelial cells *in vitro* and epithelial in nature (E-Cadherin and EpCAM positive, see figure 5) (Zani et al., 2010).

5.1.2 Lifespan of primary cells

Primary cells have a limited lifespan. The exact lifespan of each primary cell culture fluctuates and depends, for example, on the size of bronchial segments, the viability of the cells and the state and condition of the donor. In the senescence staining shown in figure 12, the difference in the percentage of cells in senescence seems considerable. It must be taken into account that the cells from biopsy 2 have reached confluence and would have been subpassaged that day, bringing them to P4. Upon reseeding, the viable cells that can divide and grow may be too few, causing cellular stress that may cause more cells to go into senescence (Rodier & Campisi, 2011).

Due to the limited lifespan of primary cells and the scarcity of tissue samples it is important to have a good estimate of in which passage specific research has to be performed. For example, it is not possible to differentiate primary bronchial cells into secretory and ciliated cells after P3 (Gray et al., 1996). When the highest passage number for any particular research has been established, it is possible to maximize the cell amount acquired from each biopsy by reseeding them until that passage number.

5.1.3 Difference in marker expression

IF stainings show the variance between donors and indicate changes that occur when the cells are further cultured. p63 is uniformly expressed in P2, but in P1 there are some p63 negative cells (see figure 6). Mucin is expressed heterogeneously in primary cells in P1 (see figure 7) but in P2, mucin expression is completely gone. The p63 negative and mucin positive cells seem to cluster around organoids that have adhered. As stated before, because different biopsies are shown stained it is not possible to declare that p63 and mucin expression changes by time but the stainings suggest it.

Epithelial cells that grow from organoids are unlike the cells that adhered from a single cell suspension. The cells that form a halo surrounding organoids are smaller, denser and have beating cilia that can be seen in a microscope. In the first passage, some cells exhibit mucin expression and a few are p63 negative, which is an unusual expression pattern outside of the organoid halo. Both trypsin and protease XIV were used to digest the bronchial tissue samples. Using protease XIV seems to result in more organoids adhering and therefore more ciliary function. Using trypsin resulted in more cells that adhered from a single cell suspension.

A donor variance was observed in marker expression (see figure 8, 9, 10 and 11) and phenotype (see figure 4). That is to be expected from human samples, since the donors probably have variations in regards to smoking, prior health and lifestyle. These inconsistencies will likely affect the viability and the marker expression of the isolated cells. These donor variances must be kept in mind when using primary cells for research and using only primary cells from one donor is not a reliable method. Results must be reproduced before being accepted as valid. Donor variance has been pointed out as a drawback to primary cell culture before (Forbes, 2000; Halldórsson, et al. 2007).

5.1.4 Future aims in primary bronchial cell culture

An aim for future research would be to confirm the changes over time in p63 and mucin expression that the present stainings indicate. Then cells from one biopsy have to be cultured from start in two flasks, one would be stained in P1 and the other would be split and stained at a later passage. Care must be taken to divide the initial cell solution equally so that there is an equal number of organoids in both flasks.

Culturing primary bronchial cells on a fibroblast feeder layer is an interesting future project. A small pilot study has been performed growing VA10 cells on growth arrested 3T3 fibroblasts. This will perhaps solve the original aim of this project that was to purify basal cells from the lung epithelium. That original aim was unsuccessful mainly because of difficulties with expanding the cells in culture.

5.2 Sortation of VA10 cells

The VA10 cell line is already recognized as having basal properties that are p63 positive, generating branching bronchioalveolar-like structures and pseudostratified epithelium in 3D culture and air-liquid culture, respectively (Halldorsson et al., 2007). An attempt was made to enrich for more basal cell properties by using a cell sorting technique based on antibodies against surface proteins expressed in basal cells; NGFR and β4 IGR. The subgroups showed reduced growth speed (see figures 13 and 14) and a marker expression profile very similar to the original VA10 cells (see figure 15). The cause of that may lie in the sortation treatment. The MACS machine used is not sterile and the subgroups were quite small as a large portion of the cells was lost during the procedure.

If the high expressing cells had more stem cell abilities, a reduction in growth would not be unexpected. Following that logic, one would expect the low expressing cells to have a higher growth rate, but that is not the case. The fact that the low expressing cells show this growth reduction as well points to it being a result of the sortation treatment or clonal growth after it.

Measuring growth by crystal-violet staining is not a faultless method; rather than measuring the number of cells, it measures the area the cells occupy. This results in a false reduction in growth when cells are smaller in size. No size difference between high or low expressing cells has been noticed that could explain this growth reduction.

β4 IGR high and low expressing VA10 cells show a similar marker expression. β4 IGR high expressing cells stain positive for β4 IGR confirming that the sortation was successful. A higher expression of acetylated tubulin in β4 IGR high cells was noticed, which does not correlate well with β4 IGR high cells being a subgroup with more stem cell abilities.

In the future this isolation could be repeated using a FACS Aria rather than MACS and expression profiles then be reevaluated.

5.3 Knockdown of *E6/E7* in VA10 cells

It is important to have a cell culture model that can represent the process of branching morphogenesis as closely as possible. The model should be as accurate as possible in imitating the biological pathway that, for example, forms the lungs and other organs during embryonic development.

The viral genes *E6* and *E7* were used to immortalize the VA10 cell line. By transfecting VA10 cells with shRNA for the viral genes they are being made mortal again, reverting them to "normal" gene expression with p53 and rBp active. The knockdown of *E6/E7* was successful and with a conditional vector the knockdown can be turned on and off. VA10 cells were transfected with shRNA in an attempt to achieve branching morphogenesis with a phenotype more similar to that of *in vivo* branching. Branching structures are not complete without a lumen and if the lumen is formed by apoptosis of the cells in the middle of the structure, making the VA10 cells mortal again should improve lumen formation.

The results were that branching structures with and without a lumen were observed in both TET activated cells and cells with no activation. The TET activation seems to inhibit branching, as fewer branching structures were observed in TET activated cells compared to cells without activation. These results are preliminary and need to be reproduced.

The knockdown of *E6/E7* is not complete, so to see whole branching structures in TET activated cells does not prove the hypothesis about lumen formation with apoptosis wrong. Those branching structures could be the result of cells that have a lower or no knockdown of *E6/E7*. The decline in branching in TET activated cells is not unanticipated. The reduction in growth is in accordance with the results of Gu and colleagues (2006), which showed growth reduction as a result of a low dose shRNA lentiviral transfection. A high dose shRNA lentiviral transfection was needed to induce apoptosis and perhaps the knockdown in VA10 is not sufficient.

The reduction in branching could be a consequence of the cells being TET activated for too long (around 13 days when the cells should start branching) for them to make proper branching structures. For the 3-D culture in Matrigel seen in figure 17, the cells were TET activated 4 days before they were seeded into Matrigel. The TET activation seems to impede growth, but it is perhaps not necessary to activate the shRNA so soon before the 3-D culture starts. Further experiments should be performed to see the effects of activating the shRNA in Matrigel at various points in time; before budding or after. It would also be beneficial to determine how much time elapses from the addition of TET in the culture media until *E6/E7* gene expression is knocked down.

6 Conclusions

In summary, I have characterized the marker expression pattern of primary cells, pointing out donor variances observed in primary cell cultures and indicated changes that occur over time. I isolated a subgroup of the VA10 cell line that had enriched basal cell features, but the subgroups were reduced in growth speed and showed an expression pattern very similar to that of the parental VA10 cell line. The *E6* and *E7* immortalization genes were conditionally knocked down in VA10 cells in order to try to obtain more differentiated cells that would make branching structures with a lumen. The knockdown was successful and when cultured in 3-D Matrigel, the cells with activated knockdown branched much less that their unactivated counterparts. Further work should be done in activating the knockdown in the Matrigel at various points in time to see if that improves the branching of the cells. Another future project is growing primary bronchial cells on a growth arrested fibroblast feeder layer with the aim of getting a primary cell population that retains its stem cell abilities.

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