Characterization of human lung tissue: spatial expression of receptor tyrosine kinases and sprouty proteins *in situ* and in 3D culture

A thesis submitted for the degree of Master of Science

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Skilgreining á lungnavef manna: tjáningarmynstur týrósín kínasa viðtaka og sprouty protein *in situ* og í þrívíðu ræktunarmódeli

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Ágrip

Mannslungað myndast sem útvöxtur út frá meltingarvegi í kringum fimmtu viku þroskunar. Öndunarvegurinn þroskast með endurtekinni greinóttri formgerð þar sem þekjuvefur vex inn í stoðvefinn sem umkringir hann. Rannsóknir á lungnaþroska manna krefjast vel skilgreindra frumulína og ræktunarlíkana. Slíkt líkan hefur verið þróað á rannsóknastofunni, þar sem vel skilgreind mannafrumulína (VA10) er ræktuð í þrívíðu umhverfi og líkir þá eftir greinóttri lungnaformgerð. Rannsóknir á öndunarvegi ávaxtaflugunnar hafa sýnt að týrósín kínasa viðtakarnir (RTKs) FGFR2 og EGFR fjölskyldan ásamt stjórnpróteinum þeirra, Sprouty fjölskyldunni, séu lykilprótein í þroskun öndunarvefjar. Lítið sem ekkert er samt vitað um hlutverk þeirra í lungnaþroska manna. Uppbygging og frumusamsetning mismunandi hluta mannslungans er mjög mismunandi frá stærri öndunarvegum niður í loftskiptavef. Þessu hefur almennt lítið verið lýst og því var nauðsynlegt að skilgreina frumugerðir í mismunandi svæðum lungans til að geta betur staðsett tjáningu RTKs og Sprouty og auðvelda samanburð við ræktunarlíkanið.

Markmið þessa verkefnis var að skilgreina uppbyggingu þekju- og stoðvefjar mannslungans og einnig skilgreina tjáningarmynstur RTKs og Sprouty fjölskyldunnar í þeim. Einnig að kortleggja tjáningarmynstur þessara próteina í greinóttu formgerðinni sem myndast í þrívíða ræktunarlíkaninu.

Mótefnalitanir sýna að RTK eru tjáðir bæði í efri og neðri loftvegi en tjáningin er mismunandi eftir próteinum. Það sama á við um Sprouty fjölskylduna. Tjáningarmynstur FGFR2 og Sprouty2 í

ræktunarlíkaninu er mjög líkt því sem sýnt hefur verið fram á í þroska öndunarvegar í ávaxtaflugunni og í músum. Sum mótefni, líkt og þau gegn Sprouty fjölskyldunni, eru ekki fullreynd og því þarf að nota fleiri aðferðir til að tryggja rétta niðurstöðu. Ég setti því upp mRNA *in situ* hybridization aðferð á rannsóknastofunni.

Samantekið, þá sýna niðurstöður mínar að hægt sé að nota þrívíða ræktunarlíkanið til að rannsaka lungnaþroska manna og að hyggilegt sé að byrja á að skoða hlutverk FGFR2 og Sprouty2 í því samhengi.

Abstract

The human lung develops as an epithelial outgrowth from the fetal digestive tract around the 5th week of gestation. The complete bronchial tree is formed by repeated branching of epithelial tissue into the surrounding mesenchymal tissue (referred to as stroma in this thesis). Using the VA10 bronchial epithelial cell line, the laboratory has introduced a three dimensional cell culture assay that partially mimics human lung morphogenesis. The structure and cellular composition of the epithelial compartment from the proximal to the distal zone is very distinct, which necessitates characterization of *in situ* condition to be better able to evaluate the quality of the 3D culture model. Studies on molecular control of airway branching in *Drosophila* and mice have revealed some key players like the receptor tyrosine kinases FGFR2, the EGFR family and their intracellular pathway regulator, Sprouty2. In contrast, there is limited information on the role of RTKs and their regulators in human lung development.

The aim of this project was to characterize epithelial and stromal compartments of the adult human lung and to explore the expression profile of RTKs, particularly FGFR2, EGFR and ErbB2, in both tissue and 3D culture. My objective was also to analyze if the Sprouty expression correlated with the RTKs expression.

Immunohistochemistry shows that RTKs are expressed in bronchial epithelium and to some extent in alveoli but expression pattern is variable between them. Similar staining pattern is seen for the Sprouty family. Expression pattern of FGFR2 and Sprouty2 in branching structures from 3D cultures correlates well with pattern

shown in animal models. Some of the Sprouty antibodies were however promiscuous. Therefore I put effort into establishing mRNA *in situ* hybridization method at the laboratory, to be better able to evaluate the expression profile of Sprouty proteins in lung tissue.

In summary, my results demonstrate that the 3D culture system can be highly useful to study molecular mechanisms during human lung development and the FGFR2 and Sprouty2 are ideal candidates for initial functional studies.

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Table of contents

Figures and tables

Abbreviations

AA	Acetic anhydride
BM	Basement Membrane
BCIP	5-bromo-4-chloro-3-indol-phospate
CDS	Color Development Solution
dSprouty	*Drosophila* Sprouty
DTT	Dithiothreitol
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGM-2	Endothelial growth medium
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FBS	Foetal bovine serum
Grb2	Growth factor receptor bound protein 2
HER	Human epidermal growth factor
HUVECs	Human umbilical vascular endothelial cells
IGF	Insulin-like growth factor
MAPK	Mitogen-activated protein kinase
NBT	4-nitro blue tetrazolium chloride
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol 3-kinase
PLCγ	Phospholipase C gamma 1
PTB	Phosphotyrosine binding domain
PVDF	Polyvinylidene fluoride
rBM	Reconstituted basement membrane
RIPA buffer	Radioimmunopreciptitation assay buffer
RPM	Rotations per minute
RTKs	Receptor tyrosine kinases
SH2	Src-homology2
SHH	Sonic hedgehog
Sos	Son of sevenless
SMG	Submucosal glands
TER	Trans epithelial resistance
VEGF	Vascular endothelial growth factor

I - Introduction

1 - Histology of the human lung

The human lung is a complex organ composed of a tubular network with more than 10^5 conducting airways and 10^7 respiratory airways designed to manage gas exchange. (Bejan, 2000; Mauroy et al., 2004; West et al., 1997). The lung trachea is a continuation of the larynx and it gives rise to the two main right and left extrapulmonary bronchi. The wall of the trachea contains C-shaped hyaline cartilage rings coated with perichondrium, bound together with the trachealis smooth muscle. The wall also contains mucosa, submucosa, submucosal glands (SMG) and adipose tissue (figure 2a). The lumen of the trachea and large bronchi is lined with a pseudostratified ciliated epithelium connected to the basement membrane (BM) (figure 1 (1 and 2) and figure 2a). The BM separates the epithelium from the underlying stroma which contains extracellular matrix, fibroblasts, blood- and lymphatic vessels and immune cells (figure 1)

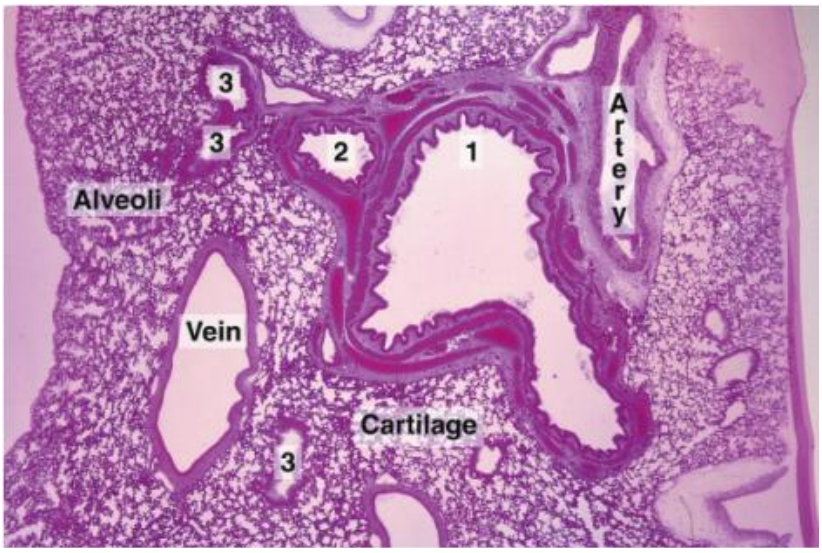

Figure 1. Overview of lung histology. Lung section showing bronchioles of different sizes (1,2,3), blood vessels and alveoli. (Junqueira & Carneiro, 2005).

(Eroschenko, 2005; Stevens & Lowe, 2005).

The two extrapulmonary bronchi enter the hilum of the lungs and give rise to the left (two) and right (three) lobar bronchi (figure 3-left). Each of them further divides into smaller intrapulmonary bronchi down to the bronchioles. As the bronchi decrease in size, the cartilage rings change from C-shaped form to being circumferential and smaller. Smooth muscle becomes more prominent and connects the cartilage plates. In the bronchioles, the cartilage is absent, and smooth muscle entirely circulates their wall.

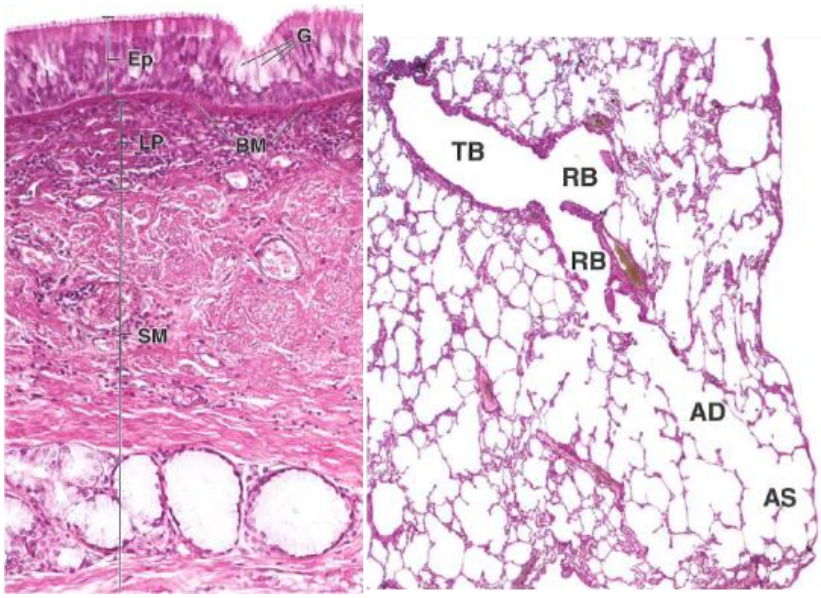

Figure 2. Histological overview of bronchial epithelium and alveoli. **A**. Bronchial epithelium (Ep) and underlying stroma. Epithelial layer contains ciliated, basal and goblet (G) cells and rests on the basement membrane (BM). The underlying lamina propria (LM, the immediate stroma beneath the BM) contains loose connective tissue. The submucosa also contains connective tissue along with blood and lymphatic vessels, nerves and mucus and serous-secreting glands. **B**. Alveolar tissue. Terminal bronchile (TB) branches into respiratory bronchioles (RB). They give rise to the alveolar ducts (AD) which end in alveolar sacs (AS). Adapted from((Ross, 2002)).

During the transition form proximal large airways to the smaller distal airways, the tall pseudostratified epithelial layer of the bronchi is reduced to a flat layer and in the terminal bronchioles, to a simple columnar or cubodial epithelium (figure 2b). No glands are located around bronchioles. Terminal bronchioles are the smallest conducting airways. They are approxamately 1mm or less in diameter and can be identified by the mucosal folds caused by contractions of the surrounding smooth muscle. The transition zone between the conducting- and the respiratory airways are the respiratory

bronchioles. It contains simple cubodial epithelium with a thin layer of smooth muscle surrounding it. Each respiratory bronchiole gives rise to an alveolar duct, a flattened epithelial lining that opens into two or three alveolar sacs (figure 2b). The main site of the gaseous exchange is in the alveolar sacs (Eroschenko, 2005; Young et al., 2006).

2 - The cellular context of human lung epithelium

The complexity of the lung epithelium from the proximal to the distal part is reflected in the number of various cell types that exist within the respiratory epithelial compartment. **Columnar ciliated cells** are the most prevalent cell type in the trachea and the upper airways**.** They are connected to the basement membrane and extend to the lumen. They are believed to arise from basal or goblet cells in the epithelium (Evans & Plopper, 1988; Kierszenbaum, 2007; Spina, 1998). **Goblet cells** produce and secrete mucus to the lumen. The mucus serves as a protective agent for the epithelium, trapping foreign objects on the luminal side. It is then mobilized up the airway by the cilia of the ciliated epithelium. Goblet cells are thought to be able to self-renew or arise from basal cells (Evans & Plopper, 1988; Kierszenbaum, 2007).

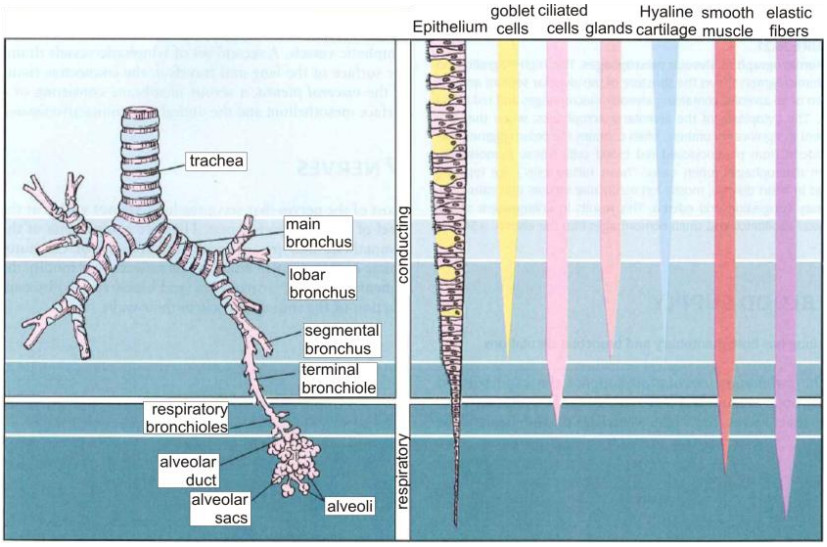

Figure 3. Division of the Bronchial tree and position of histological features. The trachea divides into the two main bronchi, which then further divide into smaller airways and end in alveolar sacs. The right panel demonstrates the position of histologic features in the bronchial tree., divided into the conducting and respiratory airways. (Ross, 2002).

Basal Cells are firmly attached to the BM and are generally considered to serve as progenitor / stem cell population in the epithelium. They do not extend to the luminal surface and play a role in connecting more apical cells to the basement membrane with hemidesmosomal complexes. The number of basal cell decreases in more distal part of the lung and they are abolished in the bronchiolar airways (Evans et al., 1990; Evans et al., 1989; Evans & Plopper, 1988; Hicks et al., 1997; Kierszenbaum, 2007). In the bronchioles, **Clara cells** are believed to be the progenitor/stem cells. They produce protective surfactant and are able to neutralize harmful inhaled substances (De Water et al., 1986; Hong et al., 2001). **Pulmonary neuroendocrine cells** are rare, atypical, innervated eptithelial cells

that play a role in the regulation of breathing. They are distributed throughout the bronchial epithelium and have been postulated to possess some somatic stem cell properties (De Proost et al., 2009). In the respiratory epithelium **Alveolar type I** cells are predominant. Altough they are fewer in number than type II cells (1:2 ratio), they cover ~95% of the surface area. The cellular organs are usually grouped around the nucleus leaving the rest of the cytoplasm relatively free of organelles. This allows the cell to strech out and become extremely thin, enabling smooth gas exchange, which is their main role (Junqueira & Carneiro, 2005; Young et al., 2006). **Alveolar type II** cells are much less conspicuous in the alveolar epithelium and cover ~5% of the surface area. They are usually found where alveolar walls unite, two or three cells together and rest on the basement membrane. They are able to give rise to the type I cells but their main function is to produce surfactant that coats the alveoli. It reduces suface tension so the alveolus does not collapse during exhalation and also decreases the force required for inhalation. The lungs are higly vascularized and the closest connection between the epithelium and the vascular endothelium is at the bronchio-alveolar junction. **Capillary endothelial** cells and alveolar type I cells share a very thin basement membrane over which the gas exchange takes place (Junqueira & Carneiro, 2005; Young et al., 2006). In general the lungs are a complex branching structure composed of various cell types.

3 - Branching morphogenesis

Branching morphogenesis is a conserved mechanism observed in the development of various tubular organs such as the vasculature, kidneys, breasts and lungs. Such a system develops from a single major conduit that gradually branches into smaller tubes until they are made up of a single cell layer. The size, shape and density of tubular networks are determined by the number of new branches and the angle between them. The lumens of all tubular systems in the body are composed of epithelial cells, except the vascular and lymphatic systems which consist of endothelial cells (reviewed in (Horowitz & Simons, 2008)).

Much research has been conducted in the field of airway branching. The two major research models are the mouse and *Drosophila*, in which many of the key factors involving branching have been identified. *Drosophila´*s simple structure and accessible genetics has given much of the basic mechanisms of airway branching (Affolter & Caussinus, 2008; Glazer & Shilo, 1991; Klambt et al., 1992; Samakovlis et al., 1996). Understanding the molecular mechanisms governing the pattern of branching and how normal cellular turnover in adult tissues is maintained is a major goal in developmental biology. The process of lung development is highly complex and driven by a strict molecular control. Various lung diseases including pulmonary fibrosis and lung cancer can occur as a result of deregulation of these fine tuned cellular processes. Therefore it is important to understand the mechanisms behind these developmental processes. Although many players have been

identified as important in this regard, receptor tyrosine kinases (RTKs) are believed to play a central role.

4 - Receptor tyrosine kinases (RTKs)

Growth factor signaling through RTKs controls numerous cellular activities including proliferation, differentiation, migration and metabolism. RTKs play an important role in processes like embryonic development, organogenesis, tissue maintenance including stem cell biology and oncogenesis. RTKs are a large family of transmembrane receptors that include the fibroblast growth factor receptor (FGFR) family and epidermal growth factor receptor (EGFR) family. These receptors are activated through binding to their respective extracellular ligands such as FGFs and EGF. Members of these RTKs like FGFR2, EGFR1 and ErbB2 have been thoroughly studied with regard to their role in development and oncogenesis (Blume-Jensen & Hunter, 2001; Schlessinger, 2000). Most studies, however, have been conducted in animal models and less is known about their expression and functional role in human lung development.

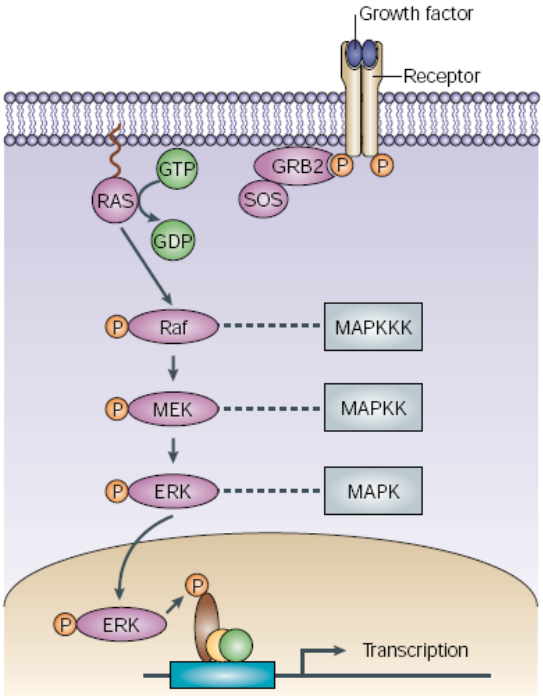

Figure 4. RTK signaling to ERK intracellular pathway. At the plasma membrane, activated RTKs recruit Grb2/Sos complexes and activate RAS. It then results in phosphorylation of Raf, MEK and ERK (MAPK kinase kinase, MAPK kinase and MAPK, respectively) Phosphorylated ERK translocates to the nucleus and phosphorylates and activates numerous transcription factors. (Mason et al., 2006)

RTK signaling can be highly complex and activate an intricate signaling network e.g. during organogenesis. Extracellular ligands bind to their cognate receptors and trigger intracellular pathways. This leads to transcription of many genes, including genes that serve as regulators of the pathway in action which may act as a negative feedback loop within the signaling network. Two of the main intracellular signaling cascades related to RTKs and development are the mitogen-activated protein kinase (MAPK) pathway (figure 4) and

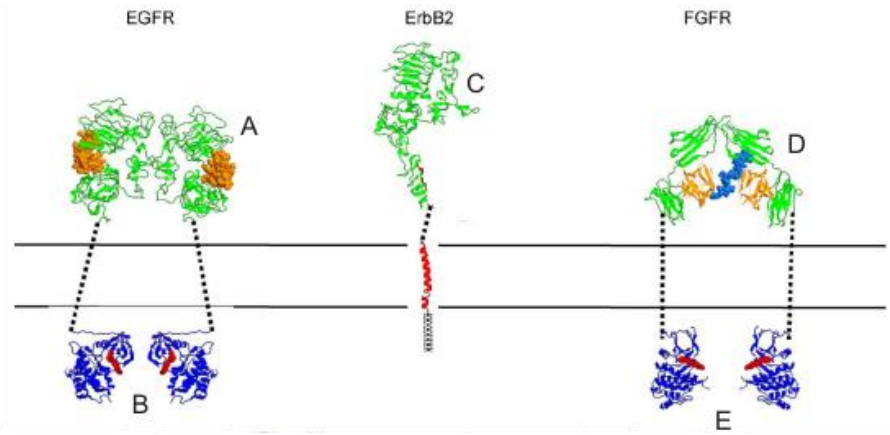

Figure 5. Structural overview of selected RTKs. A,C and **D** show extracellular domain of EGFR, ErbB2 and FGFR2, respectively. **B** and **E**, intracellular domains of EGFR and FGFR2, respectively. Structures originally extracted from the Protein Data Bank (PDB, http://www.pdb.org). Adapted from ((Bennasroune et al., 2004))

phosphatidylinositol 3-kinase (PI-3 kinase) pathways (Chalhoub & Baker, 2009; Morrison & Davis, 2003).

RTKs contain glycosylated, extracellular ligand-binding domain, a single transmembrane region and a cytoplasmic portion with a conserved protein tyrosine kinase domain (figure 5). When inactive, most RTKs are found as monomers but upon binding with their ligands, they usually form dimers. When activated by dimerization, the tyrosine residues on the intracellular domain binding sites phosphorylate (Bennasroune et al., 2004; Schlessinger, 2000). These binding sites are specific for proteins that contain Src-homolgy 2 (SH2) or phosphotyrosine binding (PTB) domains. This activation then leads to intracellular signaling cascades such as the MAPK pathway (reviewed in (McKay & Morrison, 2007)).

4.1 - FGFR2

Fibroblast Growth factor Receptor 2 (FGFR2) is by far the most studied RTK to date with regard to airway branching morphogenesis (Warburton & Bellusci, 2004).

The approximately 10.000 tubes used for gaseous transportation in the tracheal system of *Drosophila* originate from 10 separate clusters of epithelial cells (Manning & Krasnow, 1993). The clusters form epithelial sacs by invaginating to the ectoderm simultaneously and seal off from it. This process is highly genetically stereotyped and it has been shown that expression of different groups of markers is linked to different stages of this branching formation. This includes both markers necessary for each state of branching and also markers that promote cellular events on the next state of branching. These data suggest that there is a hierarchy in gene expression during branching (Samakovlis et al., 1996).

One of the first gene to play a critical role in branching of trachea in *Drosophila* is the pan-epithelial gene *trachealess*, a homolog for murine NPAS3 (Zhou et al., 2009). In *trachealess* mutants, tracheal placodes fail to invaginate, and a number of tracheal genes are not expressed (Isaac & Andrew, 1996; Wilk et al., 1996). One of those genes is *breathless*, the homolog of human FGFR2. Normally it turns on just before primary branching in tracheal cells, driven by *trachealess* (Glazer & Shilo, 1991). Simultaneously in the ectoderm, small clusters of epidermal and mesodermal cells around the tracheal sac, start to express *branchless*, the ligand for *breathless* and homolog of human FGF. This causes the tracheal epithelial cells

to migrate toward the *branchless* (FGF) source and thus creating a branch (figure 6a). FGF acts as a chemoattractant and drives primary sprouting and controls the sites of each primary branch (Klambt et al., 1992; Sutherland et al., 1996). FGF signaling is a dynamic process. When the tracheal cells reach FGF secreting cell clusters, the expression of the receptor stops and migration ceases. Upon activation of *breathless* (FGFR2) in tracheal cells, the Ras/MAPK pathway is activated and also a number of genes necessary for secondary branching (Gabay et al., 1997; Sutherland et al., 1996).

FGFR2 is highly expressed along the proximal-distal axis of the mouse respiratory epithelium at early stages of development (Cardoso et al., 1997; K. G. Peters et al., 1992). Mouse models have shown that FGF ligands, expressed in stromal tissue, can act as chemoattractors for FGFR2 expressing epithelial cells. In mouse lung development, one exracellular ligand for FGFR2, FGF-10, has been shown play a similar major role as *branchless* in *Drosophila.* (figure 6b). Furthermore, mesenchymal-free cultures with FGF-10 have been shown to induce epithelial budding (Bellusci et al., 1997). FGFR2 null mice have severe lung aplasia and FGF-10 null mice do not develop lungs (Min et al., 1998; K. Peters et al., 1994; Sekine et al., 1999).

FGF expression also activates expression of a large number of genes, some of which act as negative feedback regulators limiting further branching. One such such regulator is the Sprouty family of genes (see below).

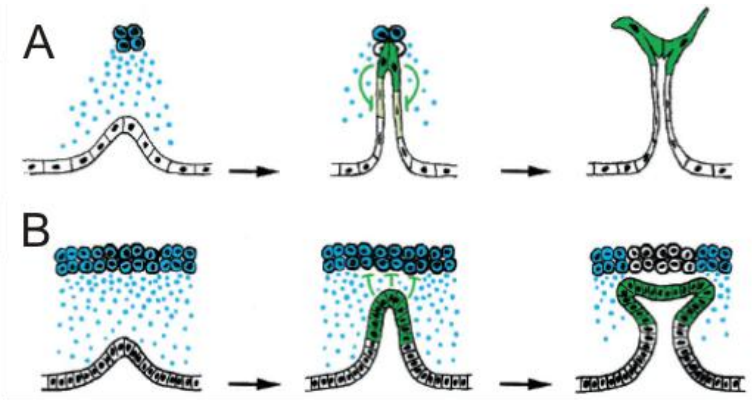

Figure 6. Airway branching of *Drosophila* and mouse. **A**. Model of *Drosophila* branching. Cells in the ectoderm (top) express branchless (FGF,blue) and guide the migration and proliferation of tracheal cells as they form branches toward the branchless origin. Sprouty (green) limits the range of FGF signaling and restricts secondary branch to cells closest to FGF center. **B**. Model of mouse branching. FGF-10 (blue) is secreted by mesenchymal cells and drives airway epithelial cells to proliferate and migrate to the FGF-10 source. Sonic hedgehog (green) in considered to be secreted by epithelial cells and downregulate FGF-10 expression in the mesenchyme. Adapted from ((Metzger & Krasnow, 1999))

4.2 - The EGFR family

The Epidermal Growth factor Receptor (EGFR) family is well studied, both with regard to development and cancer. This family contains four receptors, EGFR1, ErbB2, ErbB3 and ErbB4. The latter two have been less studied and characterized.

Signals through EGFR1 have been shown to participate in modulating mouse lung branching morphogenesis. Lung epithelial and mesenchymal cell proliferation along with markers of cytodifferentiation has been shown to be stimulated by EGFR signaling (Schuger et al., 1996; Warburton et al., 1992). Loss-of-function and knockout experiments result in decreased branching morphogenesis in culture and mouse embryonic lungs and is lethal in

null mutants (Miettinen et al., 1995; Raaberg, Nexo, Jorgensen, et al., 1995; Raaberg, Nexo, Poulsen, et al., 1995; Seth et al., 1993; Warburton et al., 1992). EGFR has also been shown to be expressed in rat alveolar epithelial cells (Raaberg et al., 1992). Signal amplifying mutations in EGFR are well known in lung cancer and are currently a therapeutic target (Gazdar, 2009).

Epidermal Growth factor Receptor 2 (ErbB2, HER2) can form dimers with other RTKs. It has been widely studied regarding cancer and is upregulated or constitutively active in many cancer types. ErbB2 null mice are lethal at day E.10.5 due to heart developmental abnormalities (Lee et al., 1995), explaining why little has been documented about ErbB2 and lung development. Therefore, development of *in vitro* culture models that mimic lung development and branching morphogenesis is an important task in developmental biology. This is especially urgent with regard to human developmental biology, since models that mimic human lung development are lacking. In summary, although much is known about RTKs in mouse and *Drosophila* airway development, their spatial location and functional role in normal lung morphogenesis is not well characterized.

5 - The Sprouty protein family

Like many branching regulatory genes, Sprouty was first discovered in *Drosophila* (dSprouty) (Hacohen et al., 1998). It has been shown to be a widespread feedback regulator of RTK mediated Ras signaling during organogenesis. Sprouty-null flies have phenotypes indicating

uncontrolled EGF- and FGF signaling (Minowada et al., 1999; Tefft et al., 1999).

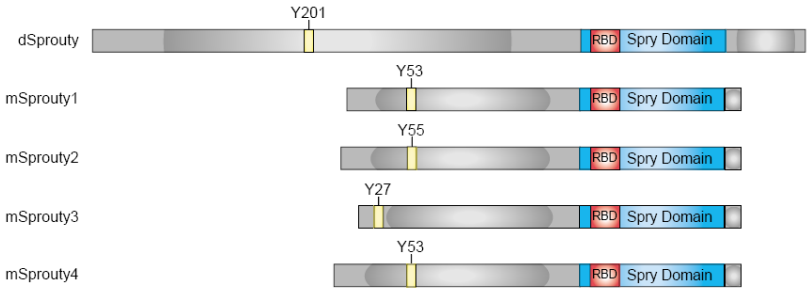

Figure 7. Structure of *Drosophila* and mouse Sprouty proteins. Mouse Sproutys are shorter than dSprouty. The highest similarity of the proteins is in the cysteine rich region (blue). Within that region is the highly conserved Raf1 binding motif (red). Altough the N-terminal is more divergent (left side), there is an invariable tyrosine residue (yellow) which is responsible for many of the inhibitory functions of Sprouty. Adapted from ((Mason et al., 2006)).

Four mammalian Sprouty genes have been identified, based on sequence similarity with dSprouty (figure 7). The human homolog proteins are shorter than dSprouty but share high similarity in the cysteine-rich C terminal. The N terminal is more diverse (Mason et al., 2006). The exact function of Sprouty inhibition is still under investigation, but to date, multiple mechanisms of function have been shown. Upon RTK activation, Sprouty proteins are phosphorylated and translocate to the cell membrane (Hanafusa et al., 2002). Sprouty proteins have been shown to inhibit signaling downstream of RTKs at the level of both Ras and Raf (Figure 8a) (Gross et al., 2001; Reich et al., 1999; Yusoff et al., 2002) and by competing with the Shp2 (Src-homology-2) tyrosine phosphatase and the FRS2 (FGFR substrate-2) adaptor protein for binding to the Grb2-SOS complex (figure 8b)

(Hanafusa et al., 2002). Sprouty has also been shown to facilitate EGF-mediated signaling by binding to the ubiquitin ligase c-CBL. The c-CBL is then unable to degrade activated EGFR and terminate signaling (Waterman et al., 2002). Expression of Sprouty proteins is induced by RTK mediated signaling (Hacohen et al., 1998; Sasaki et al., 2001; Yigzaw et al., 2003). In mammals, the Sprouty expression has been linked to sites of FGF activity and to a lesser degree to EGF signaling (Chambers & Mason, 2000; de Maximy et al., 1999; Minowada et al., 1999).

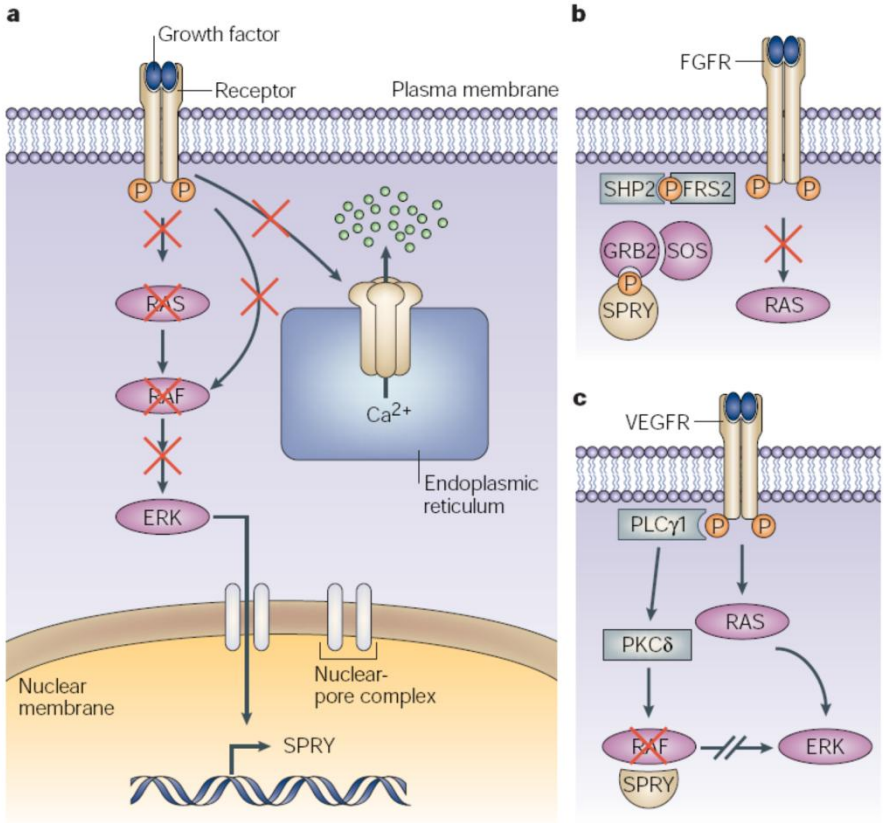

Figure 8. Inhibitory locations of Sprouty. A. After activation of RTK, Sprouty can inhibit ERK/MAPK signaling at various locations (red crosses). It also can inhibit calcium mobilization after RTK activation. **B** Activation of FGFR can result in phosphorylation of FRS2 (FGFR substrate2) or SHP2 (Src-homolgy-2-domain-containing protein tyrosine phosphatase-2). The Grb2-Sos (growth-factor-receptor bound-protein-2 – Son of sevenless) complex is then recruited to the phosphorylated tyrosine residue of FRS2 or SHP2 resulting in activation of RAS. Sprouty phosphorylates during FGF downstream signaling and competes for the Grb2-Sos complex. **C**. Signaling through Vascular endothelial growth factor receptor (VEGFR) can result in activation of protein kinase C-δ (PKCδ) by phospholipase C-γ1 (PLCγ1) and thus activating RAF. Sprouty binds to RAF and inhibits activation of ERK. Adapted from ((H. J. Kim & Bar-Sagi, 2004)).

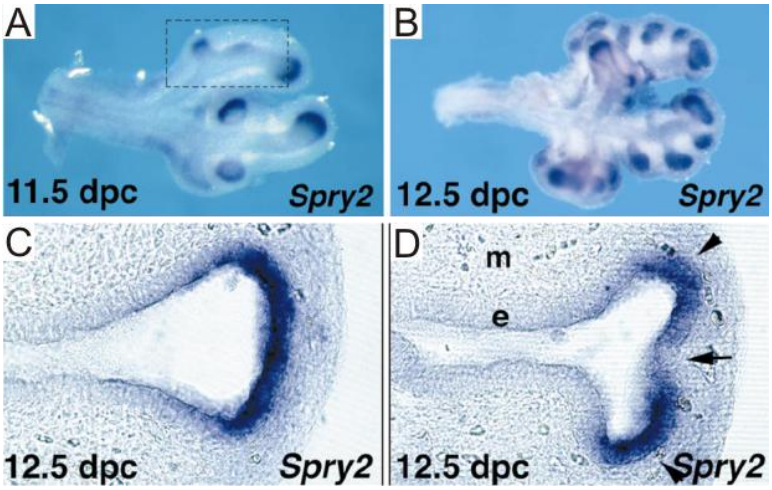

Figure 9. Sprouty2 expression during early mouse lung development shown with *in situ* hybridization. A,B. Sprouty2 has highest expression distally in growing mouse airway buds. **C,D**. Sections of growing bud. Sprouty2 expression is restricted to the tip of the bud (C), but is downregulated at the cleft when it bifurcates. Adapted from ((Mailleux et al., 2001)).

During early mouse lung development, Sprouty2 mRNA is expressed in the epithelial layer, with highest expression in the distal branching buds (figure 9a and 9b). It is also detected in the surrounding mesenchyme. When a branching bud bifurcates, Sprouty2 is downregulated at the central cleft (Figure 9c and 9d) (Mailleux et al., 2001).

The common denominator of functional studies on Sprouty proteins is that overexpressed Sprouty reduces cell proliferation and branching, but loss of function results in excessive proliferation and branching.

Drosophila knockout for Sprouty shows disrupted and ectopic tracheal branching at the secondary branching stage (Hacohen et al., 1998). In mice, Sprouty2 knockdown studies show increased tracheal

branching and epithelial cell proliferation, especially at early stages of development (Metzger et al., 2008; Tefft et al., 1999). Overexpression of Sprouty2 results in reduced branching of mouse lung (Mailleux et al., 2001). Continuous expression of Sprouty4 in mouse lung results in severe defects in lobulation and lung hypoplasia (Perl et al., 2003). Taken together, there is much literature regarding the expression pattern of RTKs and Sprouty proteins in *Drosophila* and mouse models but data from humans are very limited.

6 - Three dimensional cell cultures

For developmental studies, it is important to apply a research model that mimics as well as possible the true *in vivo* environment. Monolayer cell cultures have proven to be an important tool to answer basic biological questions. However, monolayer cultured cells have shown tendencies to lose many functional and structural properties (Weaver et al., 1996). Furthermore, extracellular matrix (ECM) is necessary for adhesion, growth and differentiation of cells and the cellular interaction with the ECM is atypical in monolayer cultures (Gudjonsson et al., 2003). Animal models, such as *Drosophila* and mouse have also proven to be highly useful tools in developmental research (see above). *In vivo* experiments however have their limitations; e.g. manipulation of markers like extracellular growth factors is difficult. Therefore three dimensional cultures utilizing diverse extracellular matrix such as hydrated collagen 1 gels (Elsdale & Bard, 1972) or reconstituted basement membrane (rBM) have been used to capture *in vivo*-like environment. An example of this

experimental setup is the possibility of managing key constituents of epithelial-stromal interactions. For studying lung development, fetal mouse lung mesenchyme and epithelium have been co-cultured in rBM (Hyatt et al., 2004). However, significant differences exist between mouse and human lung tissue. And since human fetal research is highly limited, three dimensional cell culture utilizing cells with stem-cell like phenotype is a useful tool to study lung development. Several research groups like the Keith Mostov group, Mina Bissell group and Joan Brugge group have shown the value of three dimensional cell cultures in various cell types. Mostov and colleagues have shown extensive work in developmental biology by culturing epithelial cells in 3D culture and forming lumens and other different epithelial structures (O'Brien et al., 2002). The Bissell group has done important work both regarding breast development and cancer (Bissell & Radisky, 2001; Schmeichel & Bissell, 2003) and the Brugge group has made considerable contributions to cancer biology (Debnath & Brugge, 2005).

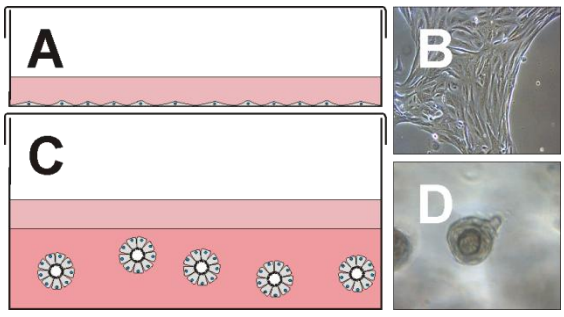

Figure 10. Schematic outlining of monolayer versus 3D cultures. A,B. Monolayer culture. Cells are cultured at the bottom of culture flask with culture medium on top. **C,D**. In a 3D culture, cells can adapt a different phenotype, sometimes more resembling their original form. (Ingthorsson, 2008)

Another cell culture model, air liquid interface (ALI) has been used with success to mimic the histology of the upper airways (Verkman et al., 2003). In this setup, cells are cultured on a transwell filter with the culture medium beneath the cell layer with the apical side exposed to air. Some lung cell lines are able to form a pseudostratified epithelial layer in this assay (Forbes et al., 2003; Grainger et al., 2006; S. Halldorsson et al., 2007). This model enables studies on lung defense, cancer and drug transport.

7 - VA10, a human bronchial epithelial cell line

To be able to study human lung morphogenesis and molecular signaling in the developing human lung, it is necessary to have access to representative cell culture models and well defined cell lines that capture specific phenotypic traits. There has been limited access to well characterized cell lines that have shown specific phenotypic traits of different lung cell populations. Recently, a human bronchial cell line with somatic stem cell properties was established at the laboratory, referred to as VA10 (S. Halldorsson et al., 2007). The VA10 was immortalized with retroviral transfection of the E6 and E7 oncogenes. VA10 has been thoroughly characterized, both in terms of functional abilities and phenotypic characteristics. It expresses a panel of cytokeratins including basal keratins 5/6, 14 and 17 and the luminal keratin 18. It also expresses the basal cell transcription factor p63 that is associated with stem cell phenotype in a number of organs (Crum

& McKeon, 2010). When cultured in air-liquid culture it forms pseudostratified epithelium with beating cilia (S Halldorsson, 2010), expressing p63 basally and forms functional tight junction proteins. It also forms polarized bronchio-alveolar like structures with branching morphogenesis, when co-cultured with endothelial cells in 3D cell culture model (manuscript in preparation). Co-culturing endothelial cells and epithelial cells is a relatively novel approach to study cell-cell interactions (Gudjonsson et al., 2003) and has been gaining more attention recently.

II - Aim of Study.

The general aim of this project was to explore the expressional pattern of RTKs and their regulators, the Sprouty protein family, in human lung epithelium *in situ* and in culture. RTKs are a large family of receptors and in this study I focused on FGFR2, EGFR and ErbB2 due to their importance in mouse lung biology and development. A 3D culture model mimicking human lung morphogenesis has been established at the laboratory and my objective was to compare the RTK and Sprouty expression between the *in situ* and 3D conditions. Given the complexity of the lung, I wanted however to begin my experimental part by conducting a general characterization of the lung tissue. This would help me to localize the RTK and Sprouty expression to a particular cell type *in situ*. Expression profiling to RTKs and Sprouty in human lung and 3D culture generates basis for further functional studies on branching morphogenesis in human lung. Understanding how RTKs signaling is regulated in lung morphogenesis also is valuable in order to comprehend its role in lung cancer.

Specific tasks:

1) Characterization of epithelial and stromal compartments along with expression of RTKs and Sprouty proteins in fresh lung tissue.
2) Establishment of an *in situ* hybridization method to support protein expression studies.

3) Expression profiling of the specific RTKs (FGFR2, EGFR and ErbB2) in 3D culture of lung epithelial cells and compare this to the normal lung tissue.
4) Analyze expression of Sprouty proteins as in task 3) and compare to the expression in normal lung tissue.

III - Materials and methods

1 - Cell culture

1.1 - The VA10 cell line

For maintenance, VA10 was cultured on T25 culture flasks (Becton Dickinson Labware, Bedford, MA) in commercially bought lung epithelial cell medum, Bronchial Epithelial Growth Medium (BEGM) (Lonza Group Ltd.), supplemented with Bovine Pituitary Extract (BPE), hydrocortisone, human epidermal growth factor (hEGF), epinephrine, transferrin, insulin, retinoic acid, triiodothyronine and 50 UI/ml penicillin and 50 µg/ml streptomycin (Gibco, Burlington, Canada). Culture medium was changed three times a week and the cells subcultured at about 80% confluence.

1.2 - HUVECs

Primary human umbilical vain endothelial cells (HUVECs) were kindly supplied by Haraldur Halldórsson and Brynhildur Thors at the Department of Pharmacology and Toxicology at the University of Iceland. Since the cells were not immortalized they were only cultured up to 8 passages. Cells were cultured on T75 tissue culture flasks (Becton Dickinson) on commercially bought endothelial medium EBM-2 that is supplemented with human recombinant Epidermal Growth Factor (hEGF) ,human Fibroblast Growth Factor-Basic (hFGF-B), Vascular Endothelial Growth Factor (VEGF), human recombinant Insulin-like Growth Factor (R_3-IGF-1),

Hydrocortisone, Heparin, Ascorbic Acid, Gentamycin, Ampothericin-B, 50 UI/ml penicillin and 50 µg/ml streptomycin (Gibco). Culture media was supplemented with 30% Fetal bovine serum (FBS) (Gibco) when fresh cells were received to induce proliferation but reduced to 10% FBS after first passage.

1.3 – 3D culture models

For 3 dimensional cell culture, reconstituted basement membrane (rBM, Growth factor reduced Matrigel matrix, BD-Biosciences) was used. At 0-4°C the Matrigel is liquid and cells can be seeded into it. At higher temperature the Matrigel gelatinizes and cells stay embedded.

To induce VA10 cells to undergo branching morphogenesis, 1000 VA10 cells were co-cultured with 250.000 HUVECs in 300µl rBM. Cells were centrifuged and re-suspended in rBM incubated at 37°C for 30 minutes for the gel to gelatinize. The culture was then supplemented with EGM with 5% FBS. The medium was changed 3 times a week.

To make round colonies of VA10 in the same setup, 10.000 VA10 cells were seeded instead of 1000. When cultured at this density, VA10 cells do not branch.

2 - Immunohistochemistry

Monolayer VA10 cells or cryosectioned lung tissue slides were fixed with methanol at -20°C for 10 minutes or at room temperature with 3.5% Formaldehyde/PBS for 5 minutes followed by 0.1% Triton X-100 for 2x7 minutes. Fixation method was based on the manufacturers' antibody datasheets.

Paraffin embedded lung tissue slides were deparaffinized for 2x5 minutes in Xylene. Slides were rehydrated with 99% → 70% EtOH. Antigen retrieval was carried out be boiling slides in mild TE buffer (10mM Tris, 1mM EDTA) for 20 minutes, then cooled and placed in PBS.

Staining s amples were treated with PBS/10% FBS blocking solution for 5 minutes. All washing steps involved 2x5 minutes in PBS/10%FBS. Primary antibodies were pre-mixed in PBS/10% FBS and incubated for 30 minutes. Samples were washed and incubated with Rabbit anti-mouse immunoglobulins (DAKO, Z0259, 1:25 dilution) for 30 minutes. For Rabbit primary antibodies, incubation with Mouse anti-rabbit immunoglobulin (DAKO, M0737 1:50 dilution) was required before Z0259 incubation. After Z0259, cells were washed and then incubated with PAP, mouse, monoclonal (DAKO, P0850). For visualization of markers, 3.3-diaminobenzidine (DAB, DAKO S3000) was activated with 0.5 mg/ml 30%H_2O_2 and incubated on cells for 10 minutes. Nuclear staining was performed using Harris´ Haematoxylin. Images were acquired using a Leica DFC320 digital camera.

Branching bronchio-alveolar like structures from 3D cultures were isolated from the rBM. rBM was dissolved by Matrigel lysis buffer (500µl 0.5M EDTA, 250µl 0.2M $NaCI_4$, 250µl 0.25M NaF in 500ml 1x PBS) on ice at mild rotation for 60 minutes. Branching structures were then spun down in pre-cooled rotator at 1000 rpm for 5 minutes at 2°C. They were then re-suspended in 30 µl PBS and left to dry on glass slides.

Microscope slides were then incubated with primary antibodies mixed in PBS containing 10% FBS for 30 minutes. The slides were then washed and incubated with isotype specific fluorescent antibodies (Alexa fluor, 488 (green), 546 (red) Invitrogen) mixed in PBS containing 10% FBS for 30 minutes in the dark. The specimens were then washed and incubated with fluorescent nuclear counterstain (TOPRO-3, Invitrogen) for 10 minutes in the dark. Samples were again rinsed and then mounted with coverslips using Fluoromount-G (Southern Biotech) for preservation of the fluorescent signal. Immunofluorescence was visualized using a Zeiss LSM 5 Pascal laser scanning confocal microscope.

Table 1. List of antibodies used in the study.

Antibody	Clone	Species	Isotype	Dilution	Fixation	Company - Order number
Sprouty2	Poly	Rabbit	IgG	1:50	Methanol	Upstate – 07-524
Sprouty3	Poly	Rabbit	IgG	1:50	Methanol	Upstate – 07-530
Sprouty4	Poly	Rabbit	IgG	1:25	Formaldehyde	Zymed – 40-9300
FGFR2	Poly	Rabbit	IgG	1:50	Formaldehyde	Abcam – ab10607
EGFR	Poly	Rabbit	IgG	1:50	Formaldehyde	Abcam – ab2430
EGFR2	Poly	Rabbit	IgG	1:50	Formaldehyde	Abcam – ab2428
β-4 integrin	3E1	Mouse	IgG1	1:250	Methanol	Millipore – MAB1964
CD31	JC/70A	Mouse	IgG1	1:50	Methanol	DAKO – M0823
ck18	CD10	Mouse	IgG1	1:50	Methanol	DAKO – M7010
P63	7JUL	Mouse	IgG1	1:25	Formaldehyde	NovoCastra - NCL-P63
Thy-1	ASO02	Mouse	IgG1	1:100	Methanol	Dianova – Dia 100
ck5/6	D5/16B4	Mouse	IgG1	1:100	Methanol	Invitrogen - 18-0267

3 - RNA isolation

Monolayer cells were dissolved using 1 ml TriReagent (Ambion) for 5 minutes. The solution was then transferred to eppendorf tube and mixed with 0.2 ml chloroform and then vortexed for 15 seconds and left standing for 10 minutes at RT. The solution was centrifuged at 13000 rpm at 4°C for 18 minutes. The upper aqueous phase containing the total RNA was then transferred to a new eppendorf tube. RNA was then precipitated with 0.5 ml isopropanol. Tube was left standing at RT for 10 minutes and then centrifuged at 13000 rpm for 12 minutes at 4°C. The RNA pellet was washed with 1 ml 96% EtOH, vortexed and spun down at 8000rpm at 4°C. Lastly the RNA was redissolved with 20μl of RNase free water.

4 - Protein isolation

4.1 - Protein isolation from monolayer culture

Monolayer cultures were washed with cold PBS. PBS was then removed thoroughly and replaced with 170 μl of RIPA lysis buffer (20 nM Tris (pH 7,4), 1% Triton-X, 1% sodium deoxycholate, 10% glycerol, 150 mM NaCl, 2,5 mM EDTA 1% aprotinin, 1mM PMSF, 100mM Na_3Vo_4 in PBS). The flask was left on ice for 10 minutes and cells then scraped from the bottom with cell scraper (BD Biosciences). Long DNA chains were broken by pumping the solution through a needle with syringe. The solution was placed in an eppendorf tube and left on ice for 10 minutes and subsequently centrifuged for 20 minutes at 4°C at 13000 rpm. The supernatant was then transferred to a clean eppendorf tube and protein concentration measured.

4.2 - Protein isolation from 3 dimensional cell cultures

Branching bronchio-alveolar like structures from 3D cultures were isolated from the rBM as previously described. The branching structures were redissolved in 50 μl RIPA lysis buffer and kept on ice for 10 minutes. The solution was then sonicated for 3 minutes. Solution was centrifuged at 13000 rpm for 20 minutes at 4°C. Supernatant was then transferred to a clean eppendorf tube and protein concentration measured.

5 - Western blot

5 µg of protein were mixed with 2.5µl sample buffer (ref) and dH_2O to a total volume of 10 µl. Samples were reduced with 1 µl Mercaptoethanol (Sigma) at 70°C for 10 minutes. Samples were loaded on 10% SDS Bis-Tris gels (Invitrogen) and run on 200V for 35 minutes in 1x MES NuPage running buffer (Invitrogen). PVDF membrane (Invitrogen) was activated in methanol for 30 seconds and then paired with the Bis-Tris gel. Proteins were transferred at a 30V constant for 1 hour in 1x NuPage transfer buffer containing 10% methanol and 0.1% antioxidant (Invitrogen).

After transfer, membrane was washed in PBS at RT for 5 minutes and then blocked with 5% milk in 0.25% Tween/PBS for 1 hour. Then afain washed with PBS for 5 minutes. Incubation with primary antibody was performed overnight at RT with 5%milk/PBS.
The day after, the membrane was washed for 5 minutes in 0.25% Tween/PBS and 2x5 minutes in PBS. Secondary antibody incubation was performed with 1:2000 dilution in 0.25% Tween/PBS for 1 hour at RT. Membrane was washed 1x5 minutes with 0.25% Tween/PBS, 2x5 minutes with PBS and 1x5 minutes with dH_2O.

Signal detection was performed with ECL solution (GE Healthcare). 1ml of prepared solution was dripped on the membrane and then the membrane was wrapped in vita-wrap and placed in an x-ray box in the dark room. Membrane was exposed using Hyperfilm (GE healthcare) for 30 seconds up to 7 minutes depending on signal strength. Film was then developed in a film developer (Dürr Medicine 260).

6 - PCR of Sprouty2-4 segments

Primers for Sprouty2-4 were generated with Primer 3 (Rozen & Skaletsky, 2000).

Table 2. List of primers used for Sprouty PCR

mRNA	Forward primer	Reverse primer	Segment length
Sprouty2	ttgcacatcgcagaaagaag	gacatgtacctgctgggtga	232
Sprouty3	tctgcttttctcagcatgga	gagtagagcgcagctgttca	223
Sprouty4	agcctgtattgagcggtttg	ggtcaatgggtaggatggtg	220

Previously generated complementary DNA (cDNA) from the MDA-MB-231 breast cancer cell line provided by Valgarður Sigurðsson, a PhD student at the lab, was used as template for PCR of Sprouty 2 and 4. For Sprouty3, cDNA from VA10 cells was used. 1μg of template DNA was mixed with 5μl of 10X PCR buffer (÷Mg), 1μl 10mM dNTP mixture, 1μl of primers (10μM each), 6,5μl 50mM $CgCl_2$, 1 unit of Taq polymerase and filled up to 50μl with dH_2O. The reaction was carried out as follows;

1. 94°C for 1 minute,
2. 20x (30 seconds at 94°C, 65°C for 30 seconds and lowers by 0.5°C every lap ending in 55°C, 72°C for 30 seconds),
3. 20x(94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds),
4. 72°C for 5 minutes and then lowered to 4°C until stopped.

7 - TOPO TA cloning

The Sprouty2-4 PCR products were cloned into a plasmid pCR®2.1-TOPO® (Invitrogen). TOPO® Cloning reaction was as follows: 2 μl of fresh PCR products, 1 μl of salt solution, 2 μl of sterile H2O and 1 μl of TOPO® plasmid are mixed gently together and incubated from 5 minutes at RT. The reaction was then transformed into One Shot® Chemically competent *E.coli* using protocol from the manufacturer (Invitrogen).

8 - Isolation of plasmid DNA

Plasmid DNA was isolated using Qia-quick® plasmid isolation kit from Qiagen. Plasmid DNA was resuspended in 50ml sterile DNase-free H_2O.

9 - Restriction digestion

DNA was digested with EcoRI restriction endonuclease (Fermentas), both sides, to be sure that the construct had inserted into the pCR®2.1-TOPO® plasmid. 10 ml of plasmid DNA, 7 ml of H2O, 2 ml EcoRI buffer and 1 ml EcoRI restriction endonuclease was mixed together and incubated in 37°C incubator for 1 hour. Then the plasmid DNA was inserted to 1% agarose gel and electrophorated to see if the length of the insert was correct.

10 - q-RT-PCR

10.1 - cDNA synthesis

Reverse transctiption was carried out with the RevertAid First Strand cDNA Synthesis Kit (Fermentas). For growth-arrest RNA, VA10 cells were cultured on growth-factor reduced medium for 24 hours and RNA isolated. For proliferation RNA, RNA was isolated from 60% confluent VA10 cells, 2 hours after change to fresh medium.

First, 1 µl RNA, 1 µl 10x reaction buffer, 7 µl water and 1 µl DNAse was mixed together in an eppendorf tube. Then the solution was incubated at 37°C for 10 minutes and 1 µl of 25 µM EDTA was added to the mix. After incubation for 10 minutes at 65°C the solution was ready for cDNA synthesis. The RNA solution was mixed with 1 µl of water and 1 µl oligo(dt) (0,5 µg/µl). The solution was incubated at 70°C for 15 minutes and cooled on ice. Then 4 µl of 5x reaction buffer, 1 µl of ribonuclease inhibitor (20U/µl) and 2 µl 10mM dNTP mix were added to the mixture and incubated at 37°C for 5 minutes. Finally 1 µl of reverse transcriptase was added to mixture and incubated at 42°C for 60 minutes and then stopped by inactivation of the transcriptase at 70°C for 10 minutes.

10.2 – RT PCR

TaqMan primers for Sprouty2-4, EGFR, ErbB2, FGFR2 and GAPDH were purchased from Applied Biosystems (Table 3).

For real time PCR; 10 µl master mix (Applied Biosystems), 1 µl primers, 9 µl cDNA sample were mixed in a 96 well PCR tray. The reaction was run and analyzed by quantification method in 7500 Real time PCR system from Applied Biosystems. The reaction cycle was as follows;

1. 50°C for 2 minutes (1cycle),
2. 95°C for 10 minutes (1 cycle),
3. 95°C for 15 seconds
4. 60°C for 1 minute (40 cycles)
5. 72°C for 10 minutes (1 cycle).

Quantitative analysis of results was calculated with the $2^{\wedge-\Delta\Delta CT}$ method, as carried out by the manufacturer (Applied-Biosystems, 1997).

Table 3. List of Taq-man real time primers.

Primers for	Order number
Sprouty2	Hs00183386_m1
Sprouty3	Hs00538856_m1
Sprouty4	Hs00540086_m1
EGFR	Hs01076092_m1
ErbB2	Hs01001599_m1
FGFR2	01552920_m1
GAPDH	4326317E

11 - q-RT-PCR standard curve

To further expand the usage of q-RT-PCR, a copy number standard curve was made for Sprouty mRNAs. This connects a CT value to number of copies of mRNAs and enables comparison of mRNA expression between experiments. This gives an alternative to the regular protocol where expression is relative to expression of a housekeeping gene.

Solutions containing pCR®2.1-TOPO® vectors with Sprouty2-4 segments from chapter 3.6 were measured with Nano-Drop (Thermo Scientific). Number of copies for each plasmid was calculated with online copy number calculator (Staroscik, 2004) as follows:

For Sprouty2

Vector length + PCR segment length = plasmid length
3931bp + 232bp = 4163bp
Nano-Drop mesasured 104,7ng/µl
→ 2,33*10^10 copies/µl

For Sprouty3

Vector length + PCR segment length = plasmid length
3931bp + 223bp = 4154bp
Nano-Drop mesasured 174,07ng/µl
→ 3,88*10^10 copies/µl

For Sprouty4

Vector length + PCR segment length = plasmid length
3931bp + 220bp = 4151bp
Nano-Drop mesasured 121,1ng/µl
→ 2,705*10^10 copies/µl

Series of 10x dilutions for each plasmid were made and real time PCR with primers for Sprouty2-4 was run on all dilutions in the 7500 Real time PCR system from Applied Biosystems.

Linear equation for Sproutys reveals the formulas for calculating copy numbers:

Sprouty2:

CT value(y) = -3,2831(copy number(x)) + 37,99. →

Copy number = (CT value – 37,99)/3,2837 →

And since the X-axis is on $\log_{10}$ scale →

Copy number = $10^{(\text{CT value} - 37{,}99)/3{,}2837}$

The same formula applies for equations for Sprouty3 and Sprouty4 (figure 11).

Furthermore, the equations show that expression of Sprouty2 mRNA is zero at CT value of 37,999, Sprouty3 at CT level of 37,125 and Sprouty4 at CT level 52,831. This indicates a different efficiency of the primers for different Sprouty mRNAs.

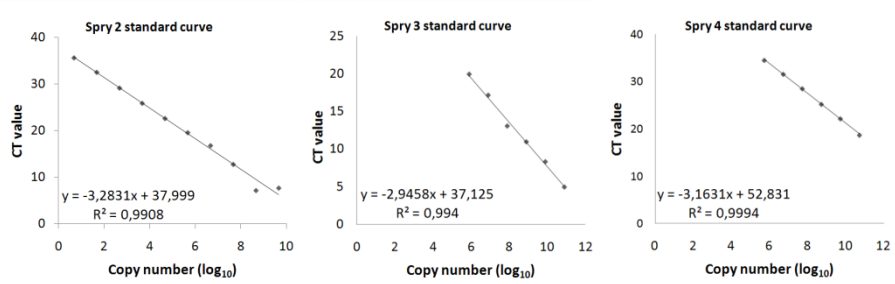

Figure 11. Standard curve for Sprouty primers. Quantitative real-time PCR of 10x dilutions of Sprouty plasmids with known concentrations. Plotting the results reveals a linear equation that can be used to calculate copy number of mRNAs from a CT value. X-axis in on $\log_{10}$ scale for simplification of the graphs.

IV - Technical approach to *In situ* Hybridization

To our best knowledge, mRNA *in situ* hybridization is not conducted systematically by any research group in Iceland. This method is very helpful to back up protein expression studies, in particular when working with poorly defined antibodies or markers that do not stain with commercial antibodies. The human Sprouty antibodies available are polyclonal and their use in the literature is limited. To support the expressional data, I set up *in situ* hybridization method. This method, however, requires many optimization steps and delicate handling of materials. Given the effort to establish the *in situ* method in our laboratory I will discuss the setup and pitfalls of this methodology. Figure 12 outlines the working process of the *in situ* hybridization.

1 - Probe synthesis

For probe synthesis, a DNA plasmid containing segment of mRNA of interest is necessary. The segments should be between 250 to 1500 nt in length and approximately 800 nt long segments exhibit the highest probe sensitivity according to Ambion, a manufacturer of probe labeling kits (Ambion, 2010b).

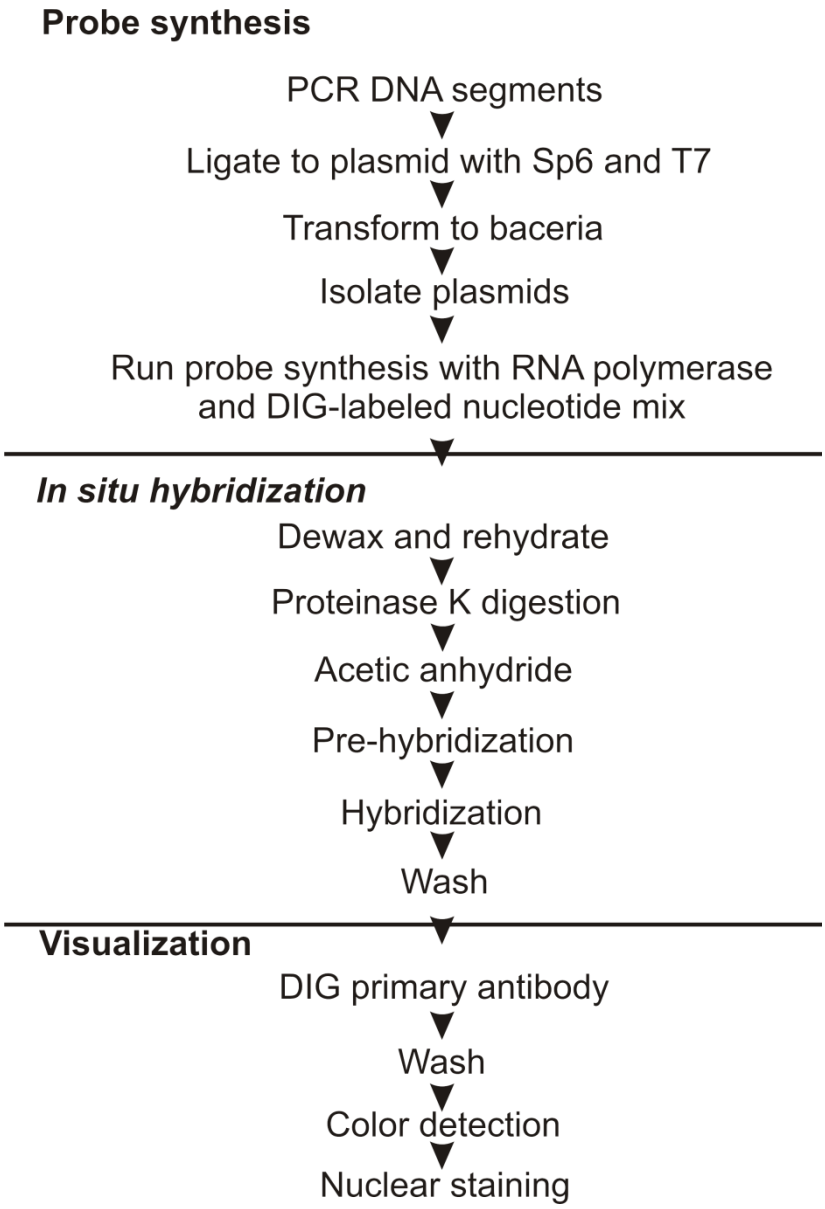

Figure 12. Flow diagram of *in situ* hybridization. First, probes are synthesized. Tissue slides are then prepared and hybridized with the probes. The last step is the visualization of hybridized probes.

I had previously prepared plasmids with Sprouty 2-4 segments. However, they contained under 250 nt long segments. In the laboratory, two plasmids were previously made by a former master student, Silja Dögg Andradóttir (Andradottir, 2006). One contained a 981nt segment for Sprouty2 and the other 917 nt segment for Sprouty3. Since the size of the segments was suitable, it was decided to use them. They were transformed into competent *E.coli*, and isolated (see Materials and Methods). In optimal probe synthesis, a vector with two, different, opposable RNA polymerase promoters is used (e.g. T7 and Sp6 promoters). This is because the optimal negative control for RNA probe (antisense strand) is a probe with the "sense" strand (GeneDetect.com, 2010). However, the plasmid with Sprouty segments available was pCR®2.1-TOPO® plasmid. It only has one promoter, the T7 promoter. Thus, the Sprouty inserts were restriction digested with EcoRI and religated back into the plasmid leading to random orientation. Then the clones were identified with both sense and antisense direction with automated DNA sequencing. Sense strand preparation for Sprouty2 was successful but not the Sprouty3 construct.

To make a Dioxygenin-labeled probe, the commercially available DIG RNA labeling mix was used (Roche), containing 10mM ATP, CTP, GTP, 6.5 mM UTP plus 3.5 mM DIG-11-UTP. In this method DIG-11-UTP is incorporated every ~20 nucleotides on the probe. Protocol for probe synthesis is listed in the Appendix.

2 - *In situ* hybridization (ISH)

2.1 - Dewax and rehydrate tissue slides

Paraffin embedded lung tissue sections were generously provided by Dr. Jóhannes Björnsson. Cryostat sections of frozen tissue can also be used for ISH, and were the first choice for this experimental setup. Fresh lung tissue was provided by Dr. Tómas Guðbjartsson from lobectomies and frozen in liquid nitrogen at the lab. As the lung is a sporous tissue, especially the alveolar gaseous exchange region. It collapses easily when frozen in liquid nitrogen, and the frozen lung tissue is difficult to cut in cryostat to sections thinner than 10 μm. For these reasons it was decided to use paraffin embedded sections. In general, paraffin embedded tissues show better morphology than frozen tissue. However, more care is required for paraffin embedding since tissue processing is increased and risk of RNA damage is higher (Tournier et al., 1987). For paraffin embedded tissue, the sections were first dewaxed and rehydrated.

2.2 Proteinase k digestion

Next the tissue was digested with Proteinase K for 10 minutes at RT. This step of permeabilization of tissues is necessary to facilitate probe diffusion to cellular mRNA and it also reduces nonspecific probe binding to tissue macromolecules (Tesch et al.). Excessive deproteination can result in both loss of signal and morphological deterioration. This has to be tested for each set of tissue samples. 10 minutes of digestion was determined sufficient for

the lung tissue slides used in this experimental setup. Another variable is the fixation of tissue. Whether the tissue is fixed with precipitating fixatives (e.g. ethanol/acetic acid or Carnoy´s Solution) or by cross-linking fixatives (gluteraldehyde, formalin or paraformaldehyde), can affect the period of proteinase digestion or even affect if another method should be used (Ambion, 2010a). Another permeabilization method, is usage of diluted Hydrochloric acid (HCL). In our experiments, that was better suited for permeabilizing VA10 cell cultures than tissue slides.

2.3 - Acetic anhydride treatment

Next, background signal was reduced with acetic anhydride solution treatment. Background signal is mostly caused by probes retained nonspecifically in the tissues. This happens primarily when probes make electrostatic interactions with tissue macromolecules, especially amine and carboxylate groups of proteins in the tissue (Ambion, 2010a). Acetic anhydride (AA) is mixed with the amine free buffer triethanolamine (0.1M) (1:200, respectively) and kept on tissue for 10 minutes. The AA acetylates free amines on proteins and therefore neutralizes positive charges to reduce electrostatic non specific binding of probes (Joanna Yeh, 2010).

2.4 - Pre-hybridization & hybridization

The use of pre-hybridization buffer is widely acknowledged and was also applied in this setup. The pre-hybridization buffer contains formamide, sodium chloride/sodium phosphate/EDTA

(SSPE) buffer and Denhardt´s solution (containing Bovine serum albumin (BSA), Ficoll and polyvinylpyrrolidone). The components of Denhardt´s solution saturate the binding sites on proteins and therefore reduce unspecific binding of probes (Ambion, 2010a). The pre hybridization buffer is also used as hybridization buffer with addition of 100ng of DIG-labeled probe and 400ng tRNA as blocking agent. The formamide is used to increase stability of mRNA and probe-mRNA binding specificity (GeneDetect.com, 2010). Time of incubation can vary but is usually from 12 – 36 hours. The temperature is highly important; probe-mRNA binding is more specific with higher temperature. The normal starting temperature is 50°C. Troubleshooting these factors lead to the result that hybridization was specific and relatively background-free at 55°C for 16 hours in humidified hybridization chamber.

2.5 - DIG primay antibody & visualization

The next day, the slides were blocked and incubated with blocking buffer containing Alkaline phosphatase-conjugated Fab fragments from anti-DIG antibody, diluted 1:500. Less dilution of antibody resulted in non specific staining.

To develop color from Fab fragments conjugated with alkaline phosphatase, a buffer containing 5-bromo-4-chloro-3-indol-phospate (BCIP), 4-nitro blue tetrazolium chloride (NBT) and levamisole was used (Color development solution (CDS)). When BCIP and NBT react with alkaline phosphatase they yield insoluble black-purple precipitate *in situ*. It is a steady rate reaction and thus the strength of

the signal is easily controllable according to the manufacturer (Thermo-Scientific, 2010). Levamisole inhibits endogenous alkaline phosphatase activity in tissues and increases signal specificity. Depending on mRNA abundance, CDS reaction time can vary from 30 minutes to 24 hours. In this experimental setup, 4-5 hours seemed to develop a clear signal. Lastly, nuclei was stained with nuclear fast red (0.1 mg/ml), coverslipped with permount and photographed.

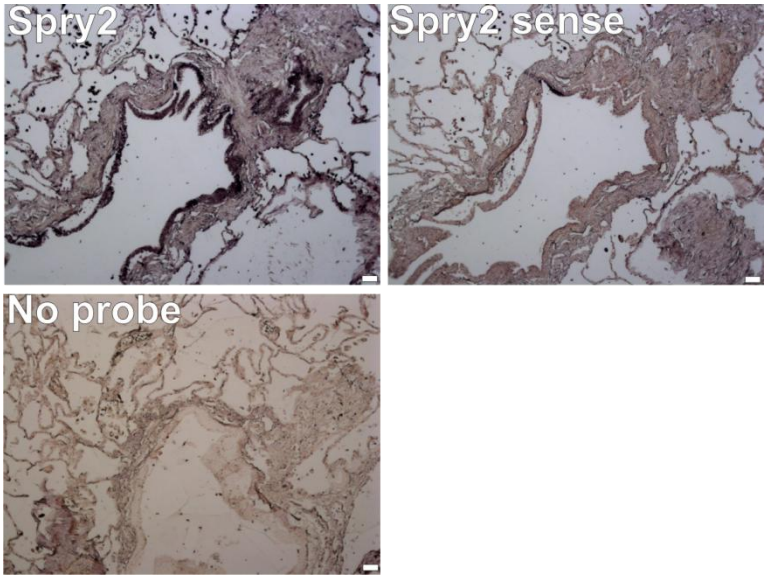

Figure 13. Successful *in situ* hybridization. Sprouty2 probes reveal mRNA expression in bronchial epithelium, alveoli and vague expression in stroma compared to the control sense probe. Compared to no probe, the sense probe shows some non specific background staining. Scale bar 100μm.

3 - Revision and additional troubleshooting

Here I discuss some aspects of the method that could have been done otherwise and review additional purposes for the method at our laboratory.

1. Addition of Dextran Sulfate to the hybridization buffer could be useful. It can increase probe concentration in hybridizing solution and therefore increasing hybridization rates. This can occur because it becomes strongly hydrated and reduces the amount of hydrating water for dissolving the nucleotides in use (Hrabovszky & Petersen, 2002).

2. Once the RNA probe binds to mRNA target, the hybrid becomes much more resistant to RNAses. Therefore, a digestion with RNase after hybridization could be useful to remove non-hybridized RNA probe and reduce background staining. This could be suitable in my approach, since the sense control shows some non specific background.

3. For ISH analysis of bronchio-alveolar like structures from 3D culture, it will be necessary to make cross-sections of the culture gel on glass slides. This can be done by either freezing the whole gel or to embed it to paraffin wax. Freezing the gel directly in liquid nitrogen could be too harsh, since the gel might lose its structure in the "boiling" nitrogen. Freezing with dry ice and -70°C n-Hexan might be more applicable since that kind of freezing is much gentler. Since the facility to embed in paraffin is not available at the moment at the

laboratory, this would probably be the best option. However, to be able to comprehend some 3 D aspects of the slides, much thicker slides (at least 20μm) must be used than the 3μm paraffin lung tissue slides. This could lead to more background staining during ISH and signal could be misinterpreted.

V - Results

1 - Epithelial and stromal tissues in the human lung are characterized by a panel of distinct markers.

As mentioned above, there are limited articles on phenotypic characterization of human lungs in terms of marker profile for different cell types and compartments. Most of the information available is from text books in histology with limited expression profile of individual cell types within the human lung. To gain better overview over marker profile of different cell types in epithelial and stromal compartments, normal lung tissue sectiones were stained with antibodies against number of markers including, epithelial cytokeratins, stromal- and endothelial markers. Cytokeratin (CK) 18 is expressed in most epithelial tissue (Ueno et al., 2005). In normal lung CK18 is expressed in surface (ciliated) epithelium of larger bronchi and bronchioles (figure 14a and c) and also in the alveolar cells of the distal lungs (figure 14b and d). In contrast, CK5/6 is expressed in larger airways in cells located near the basement membrane, most likely basal cells. (figure 14e and g).

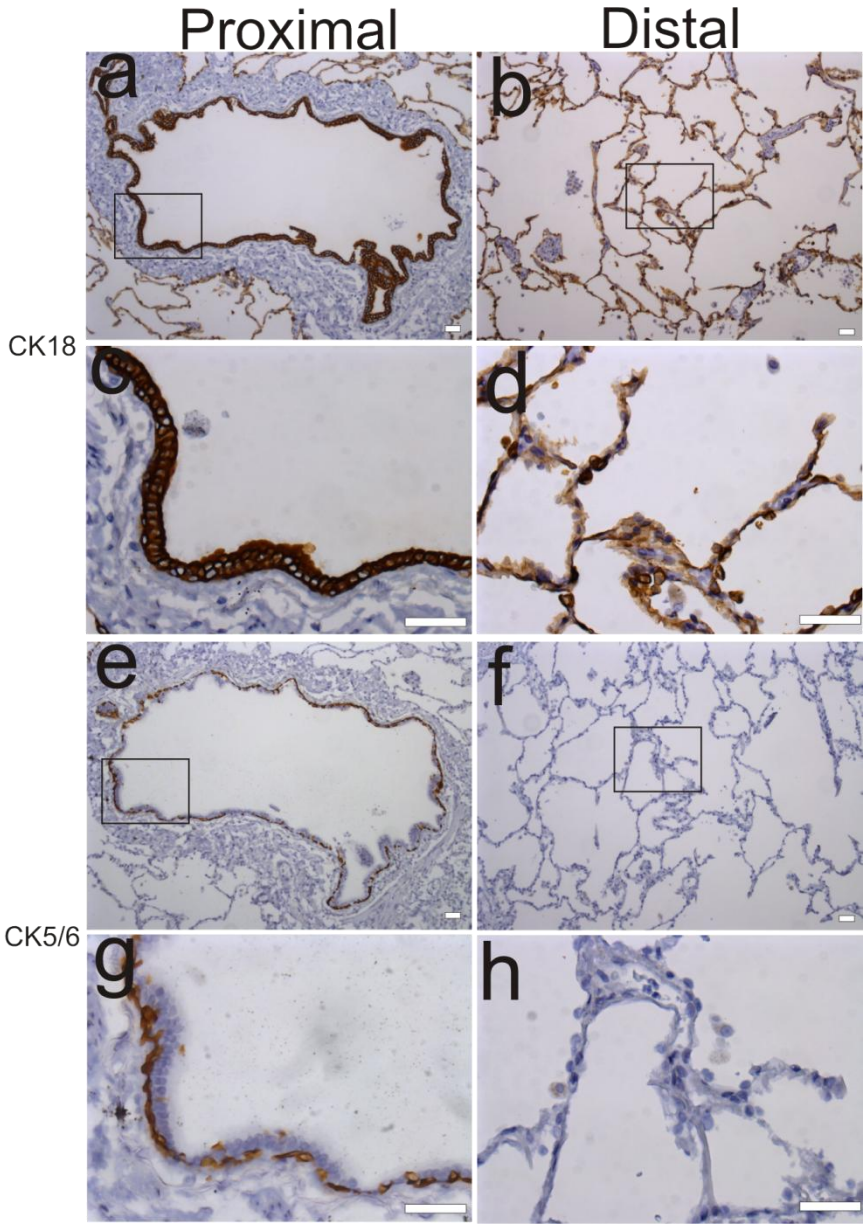

Figure 14. Lung epithelium is defined by expression of cytokeratins. Cytokeratin 18 is expressed in surface epithelium of larger bronci, bronchioles (a,c) and in alveolar cells of the distal lungs (b,d). Cytokeratin 5/6 is expressed in more basal layer of the larger airways (e,g), however it is not expressed in distal lung alveolar region (f,h).Figures c,d,g,h represent magnified areas of a,b,e,f, respectively. Paraffin embedded human lung tissue slides. Nuclear staining haematoxylin blue. Scale bar 100μm.

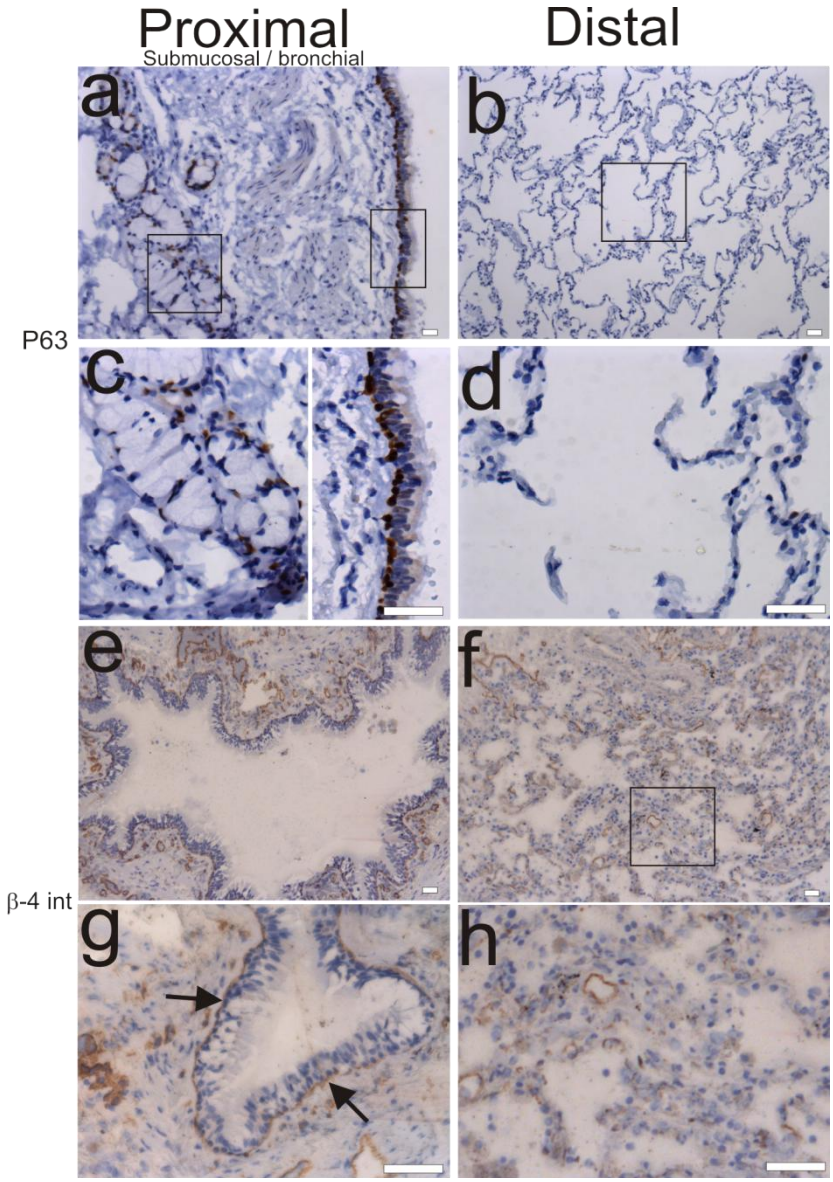

Figure 15. Basal cells are in close contact to the basement membrane. P63 is a restricted basal cell marker found in basal cells of the bronchial epithelium (a and c-right) and cells in the submucosal glands of proximal lung tissue (a and c-left). Basal cells are absent in distal alveolar lung tissue (b,d). β-4 integrin connects bronchial epithelium to the basal lamina (e,g). β-4 integrin has limited expression in distal alveolar region, it is possibly expressed in alveolar ducts (f,h). A-d, paraffin embedded lung tissue slides, e-h, frozen sections of lung tissue Figures c,d,h represent magnified areas of a,b,f, respectively. Figure g represents magnified area of same slide as figure e, but not present on it. Nuclear staining haematoxylin blue. Scale bar 100μm.

In the distal alveolar epithelium basal cells are absent (figure 14f and h).

P63 expression is restricted to the basal layer of the bronchial epithelium and to the cells in submucosal glands (figures 15a and c). In submucosal glands the P63 positive cells are most likely myoepithelial cells (figure 15c) as has been documented in other organs such as salivary glands and breast (Barbareschi et al., 2001; Yang & McKeon, 2000). Basal cells defined by p63 expression are absent in alveolar lung tissue (figures 15b and d). β-4 integrin connects the bronchial epithelium to the basement membrane, which separates the epithelium from the surrounding stromal tissue, as shown in figures 15e and 15g. In the distal lung alveolar region, β-4 integrin is mostly expressed in smallest airway tubes (figures 15f and h) whereas expression in the alveoli is more promiscuous.

Expression of the endothelial marker CD31 demonstrates the vasculature in the lung tissue (figures 16a-d). Figures 16b and 16d show the highly vascularized alveoli. Comparing epithelial (figure 14b) and endothelial staining of the alveoli (figure 16d) gives view of the close connection between the airway and vasculature in the gaseous

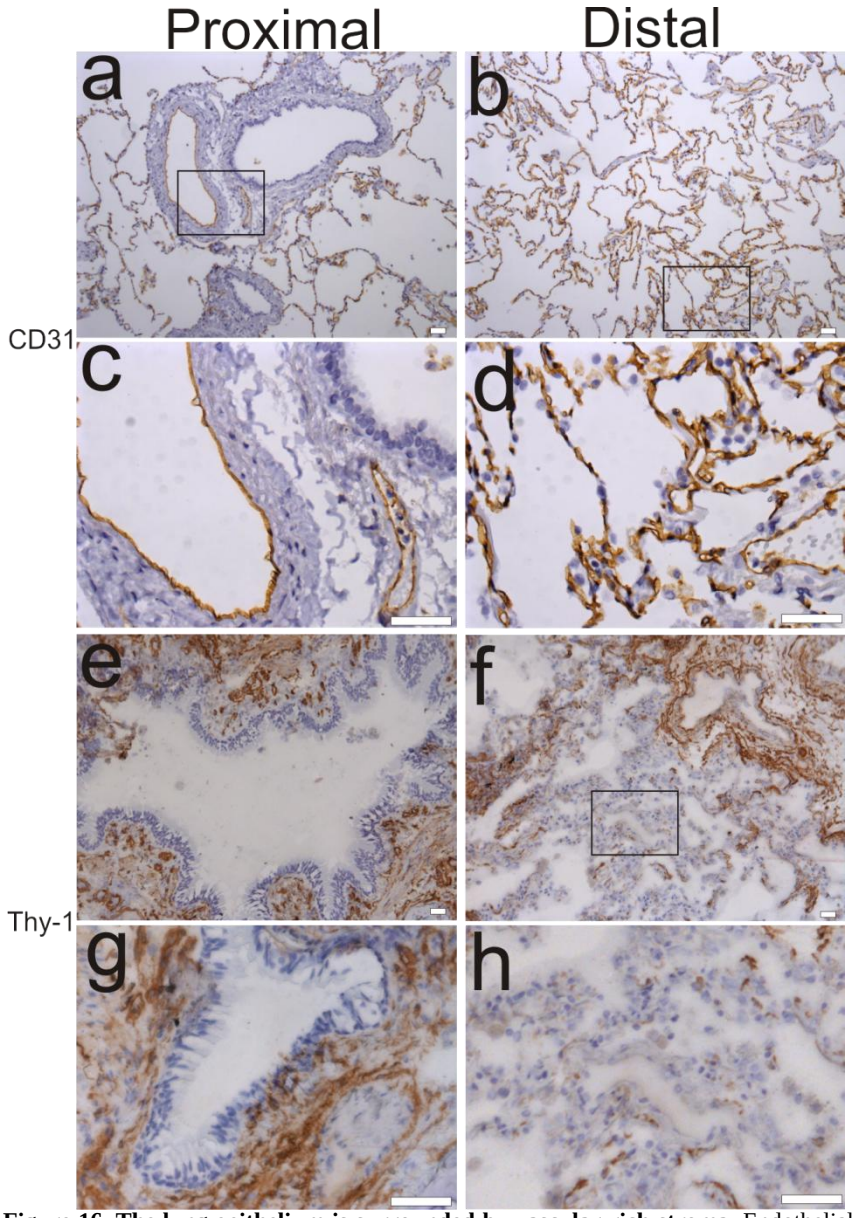

Figure 16. The lung epithelium is surrounded by vascular rich stroma. Endothelial cells in lung tissue are revealed with CD31 staining. Large and small blood vessels (a,c). The distal alveolar region is highly vascularized (b,d). Thy-1 reveals stromal tissue surrounding large airway (e,g). Thy-1 is not much expressed in alveoli.Positive staining is most likely alveolar macrophagues. . Paraffin embedded lung tissue (a-d) and frosen sections of lung tissue (e-h). Figures c,d,h represent magnified areas of a,b,f, respectively. Figure g represents magnified area of same slide as figure e, but not present on it. Nuclear staining haematoxylin blue. Scale bar 100μm.

exchange areas. Staining with stromal marker Thy-1 indicates the fibroblast rich stromal tissue surrounding both airways and vasculature (figures 16e and 16f). Thy-1 staining is shown in alveolar lung tissue, possibly revealing macrophages (figures 16f and 16h).

2 - Spatial location of RTKs and Sprouty proteins in lung tissue.

2.1 Expression pattern of RTKs in lung tissue

The receptor tyrosine kinases FGFR2, EGFR and ErbB2 have received much attention in lung biology both with regard to cancer biology (Bennasroune et al., 2004) and development (Warburton & Bellusci, 2004; Warburton et al., 1992). Most of these studies have been conducted in animal models. In contrast, there are very limited data on their expression pattern in normal adult human lung. Based on the initial phenotypic characterization above, the expression of these receptors was studied with focus on the precise location within the epithelial compartment of both epithelium in proximal airways and in the distal alveolar region of the lung. Furthermore, Sprouty expression was examined to see if it followed the expression pattern of the RTKs.

To examine the expression of RTKs and the Sprouty protein family in normal adult lung, tissue slides were stained with respective antibodies. FGFR2, EGFR and ErbB2 are all expressed in bronchial epithelium. Expression pattern of FGFR2 is mainly in basal cells, it is

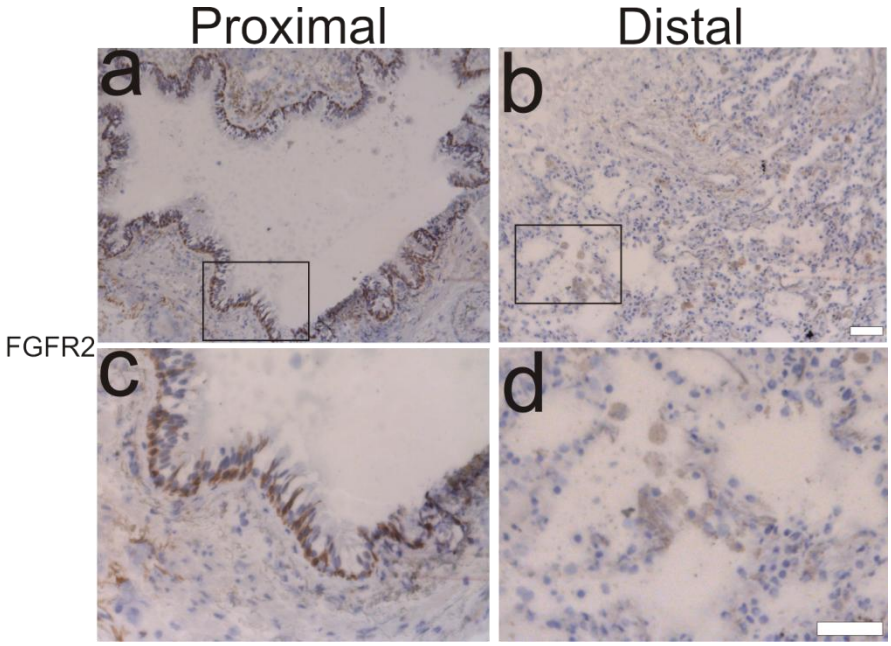

Figure 17. FGFR2 expression is restricted to the basal layer of the proximal airways and not in distal lung tissue. In bronchial epithelium, FGFR2 is expressed near basal lamina but not in apical region (a and c). FGFR2 is sparsely expressed in distal lung alveolar region. Frozen sections of lung tissue. Figures c and d represent magnified areas of a and b respectively. Nuclear staining haematoxylin blue. Scale bar 100μm.

expressed near the basement membrane but also extending between cells towards the lumen (17a and c). No evident expression is on the apical side. In the alveolar region, FGFR2 is vaguely expressed (figures 17b and d).

EGFR is expressed in bronchial epithelium but the expression is strongest at the luminal side (18a and c-right). EGFR is also expressed in the submucosal glands of the upper airways. It seems to be only expressed in the granular serous cells of the glands but not the white mucous cells (18a and c-left). EGFR is expressed in alveolar epithelium but the expression level is lower (figures 18b and d).

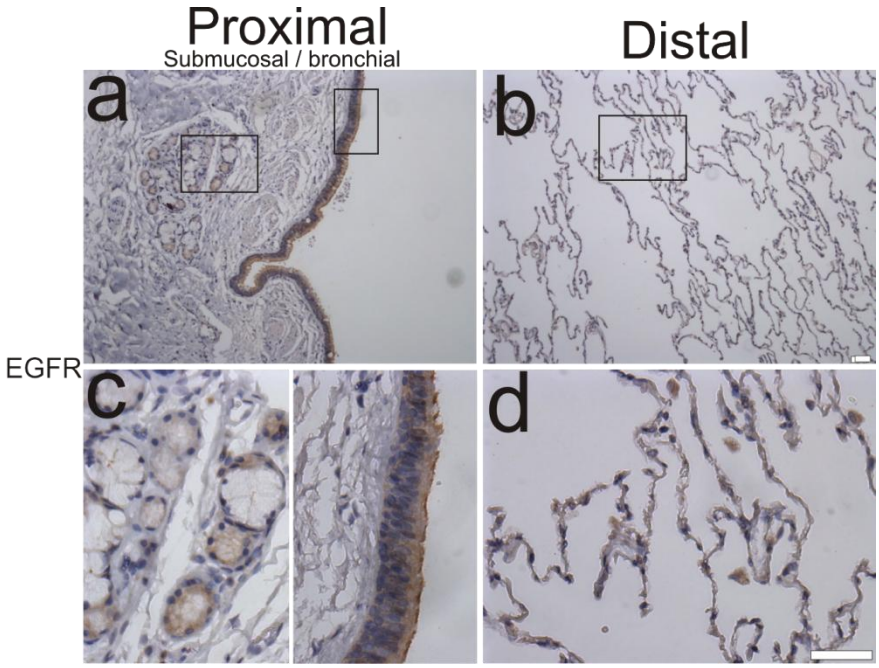

Figure 18. EGFR is more expressed in apical region of proximal bronchial airways. Apical expression of EGFR in bronchial epithelium (a and c-right). EGFR is expressed in serous cells of submucosal glands (a and c-left). In alveolar tissue, EGFR expression level is somewhat lower than in proximal tissue. Paraffin embedded human lung tissue slides. Figures c and d represent magnified areas of a and b respectively. Nuclear staining haematoxylin blue. Scale bar 100μm.

ErbB2 has a similar expression pattern to FGFR2. It mostly has a basal expression in bronchial epithelium (figures 19a and c) but much lower expression at the alveolar region (figures 19b and d). ErbB2 is expressed in the submucosal glands of the upper airways (figure 19c). In general, all three RTKs are less expressed in the distal alveolar region than in the proximal epithelium.

2.2 Expression pattern of Sproutys in lung tissue

Expression of Sprouty2 has been documented in the developing mouse lung and in developing *Drosophila* tracheal system

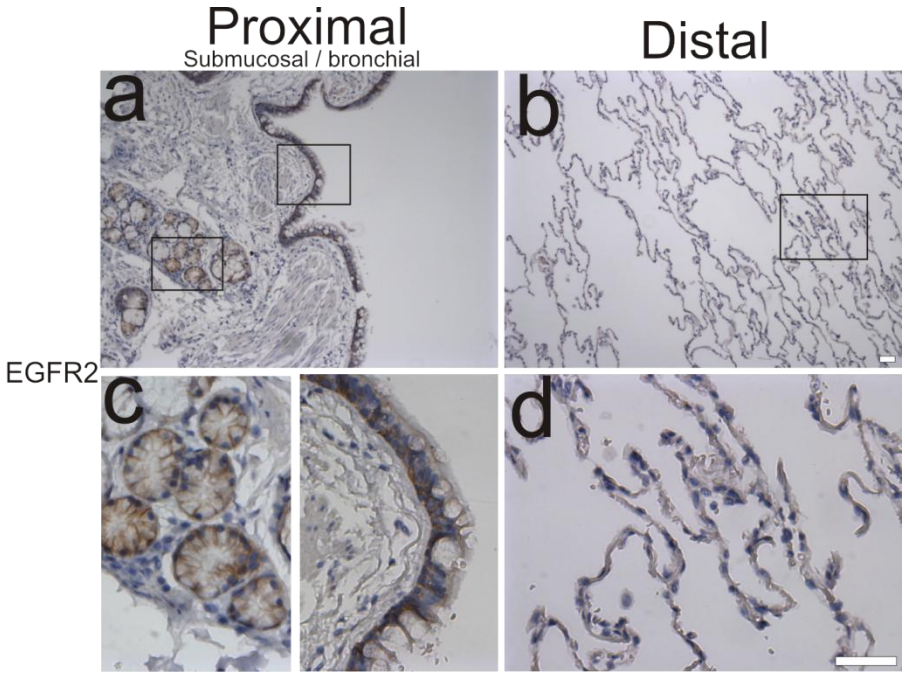

Figure 19. EGFR2 is expressed near basal lamina and in submucosal glands. EGFR2 is expressed on the basolateral side of bronchial epithelium (a and c-right). It is also expressed in submucosal glands (a and c-left). Expression levels are lower in distal alveolar lung. Paraffin embedded human lung tissue slides. Figures c and d represent magnified areas of a and b respectively. Nuclear staining haematoxylin blue. Scale bar 100μm.

(Hacohen et al., 1998; Mailleux et al., 2001). However, less is known about the expression in the human adult lung. Figures 20a and 20c show that Sprouty2 is expressed throughout the bronchial epithelium and to some extent in the surrounding stroma, as previously shown in mouse studies (Mailleux et al., 2001). Sprouty2 also has expression in alveolar tissue (20b and d). It is clear that Sprouty2 in more prominent in epithelium than endothelium and stromal cells in human lungs. There is currently limited data from different organs on Sprouty3 expression and its role in development.

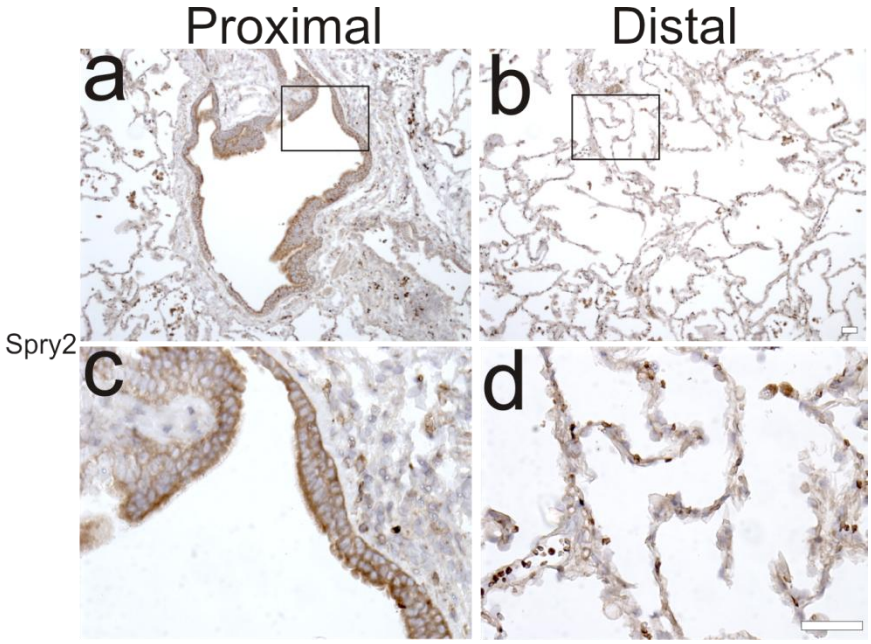

Figure 20. Sprouty2 is expressed in both proximal and distal lung. Sprouty 2 is highly expressed throughout bronchial epithelium (a,c). Expression in alveolar tissue is also high. Paraffin embedded human lung tissue slides. Figures c and d represent magnified areas of a and b respectively. Nuclear staining haematoxylin blue. Scale bar 100μm.

Figure 21 shows expression of Sprouty3 in human adult lung. In bronchial epithelium, it is expressed basolaterally. The expression is strongest near the basement membrane but stretches up between cells (21a and c). Expression levels in distal alveolar region are significantly lower (21b and d).

Sprouty4 has been reported to be expressed in the developing mouse lung and overexpression of it blocks branching morphogenesis in the

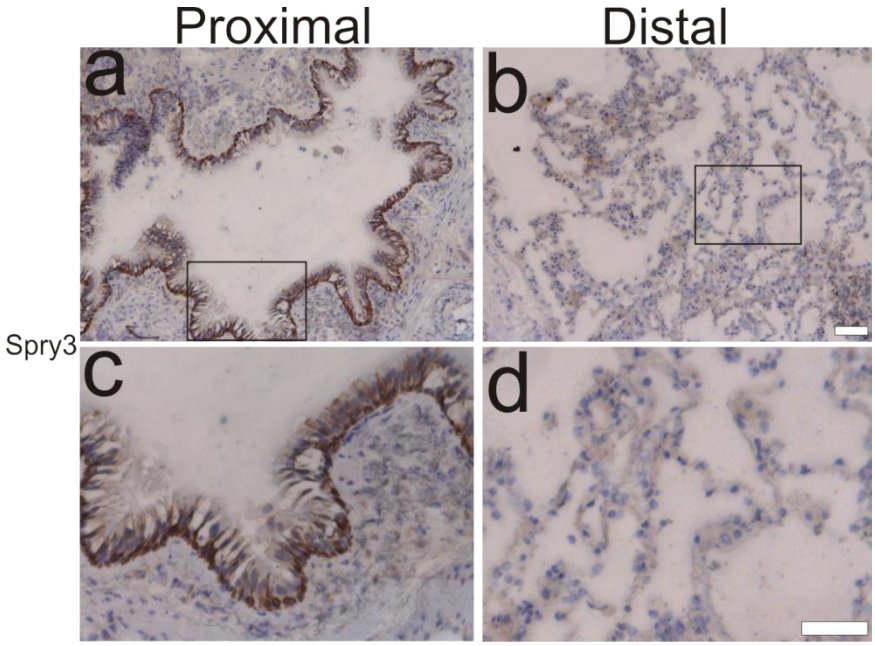

Figure 21. Spry 3 is expressed in basolateral side of conducting airways but expression in distal lung is much lower. Sprouty 3 has a basolateral expression pattern in proximal conducting airways (a,c). Expression levels in alveolar tissue are lower (b,d). Frozen sections of lung tissue. Figures c and d represent magnified areas of a and b respectively. Nuclear staining haematoxylin blue. Scale bar 100μm.

lung (Perl et al., 2003; Zhang et al., 2001). Other reports claim that Sprouty4 deficiency does not enhance lung branching, possibly because of compensation of other Sproutys (Taniguchi et al., 2007). In normal human lung, Sprouty4 is expressed to some extent in bronchial epithelium. However, the most obvious expression is in the submucosal glands of the upper airways (figures 22a and c). Sprouty4 is completely absent in distal alveolar tissue (22b and d).

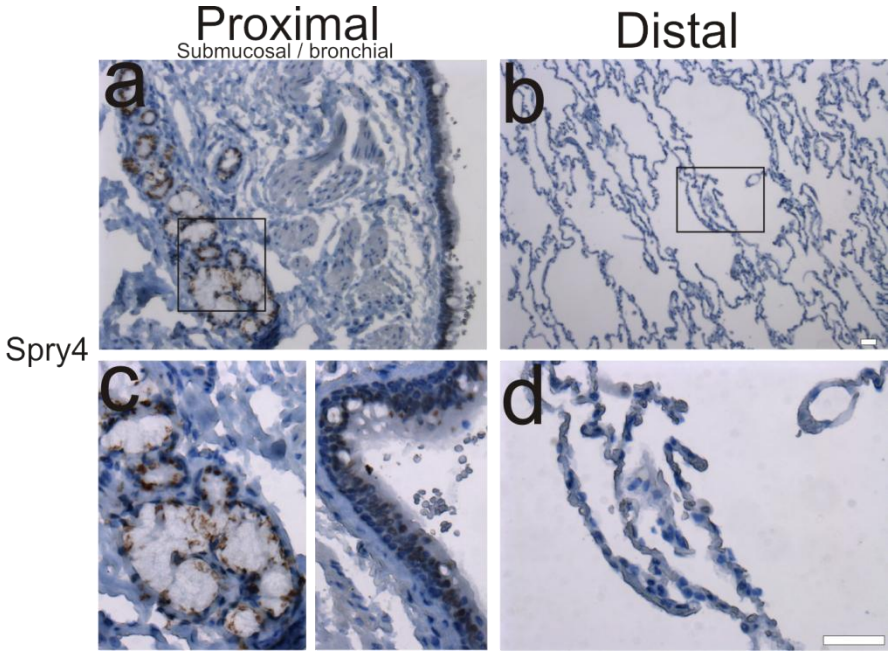

Figure 22. Spry 4 is expressed in submucosal glands and bronchial epithelium. Sprouty 4 has low expression in bronchial epithelium though expressed in some cells (a and c-right). It is expressed in submucosal glands, both in serous and mucus cells (a and c-left). Sprouty 4 is not expressed in distal alveolar tissue. Paraffin embedded human lung tissue slides. Figures c and d represent magnified areas of a and b respectively. Nuclear staining haematoxylin blue. Scale bar 100μm.

3 - *In situ* hybridization

To verify results from immunohisthochemistry, lung tissue slides were labeled with *in situ* hybridization. Probes against Sprouty2 mRNA and Sprouty3 mRNA were used. Sprouty2 hybridization shows that mRNA is expressed throughout the bronchial epithelium and to some extent in alveoli (figure 22a and b). This correlates well with the immunohistochemisty results. Furthermore, it shows that Sprouty2 is expressed in submucosal glands of the proximal airways, which had not been captured on the immunohistochemical slides (figure 23b). Sprouty3 hybridization is more inconclusive. It is shown to be expressed throughout the bronchial epithelium in some experiments (figure 23c), but less in others (figure 23d). It is also expressed to some extent in alveoli. Whether this is due to poor hybridization remains to be explored. This does not confirm expressional pattern shown by antibody staining and this data therefore needs to be taken provisionally.

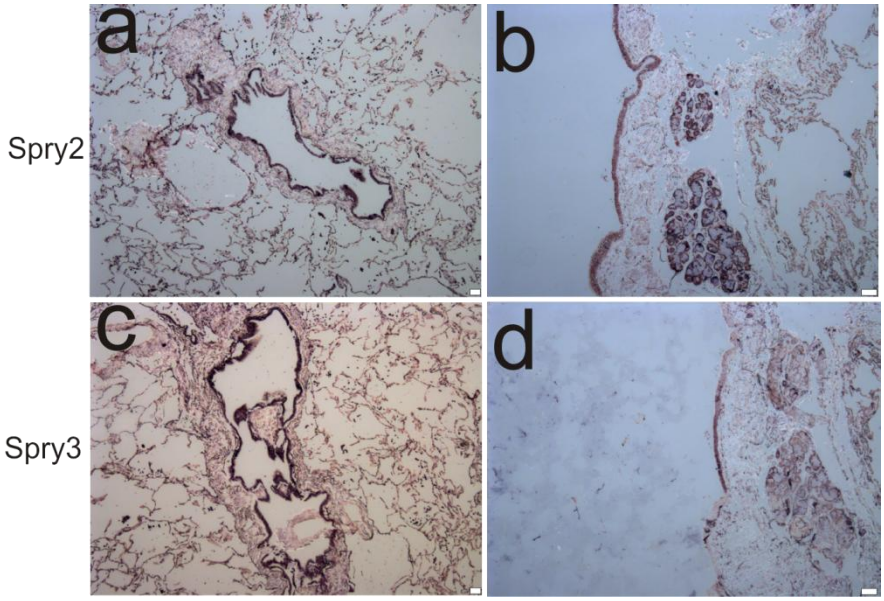

Figure 23. Sprouty2 and 3 expression in lung tissue with *in situ* hybridization. Sprouty2 mRNA is expressed throughout the bronchial epithelium and to some extent in alveoli (a,b). Sprouty3 hybridization shows differences in expression levels between experiments and needs further studies (c,d). Nuclear staining nuclear fast red. Scale bar 100μm

4 - Expression pattern of RTKs and Sprouty proteins in cultured lung epithelial cells

Before culturing VA10 in 3D culture model to establish bronchio-alveolar structures I analyzed the baseline expression of markers of interest in the VA10 cell line in conventional 2D culture. This was done to verify that no major expressional disturbances were evident in VA10, and to explore potential differences in expressional pattern between 2D and 3D cultures. FGFR2 and EGFR are expressed in all VA10 cells. ErbB2 also has a uniform expression but however has a more modest expression than the other RTKs (figure 24). VA10 has high expression of Sprouty2 and Sprouty3. Sprouty4 staining is very weak (figure 25).

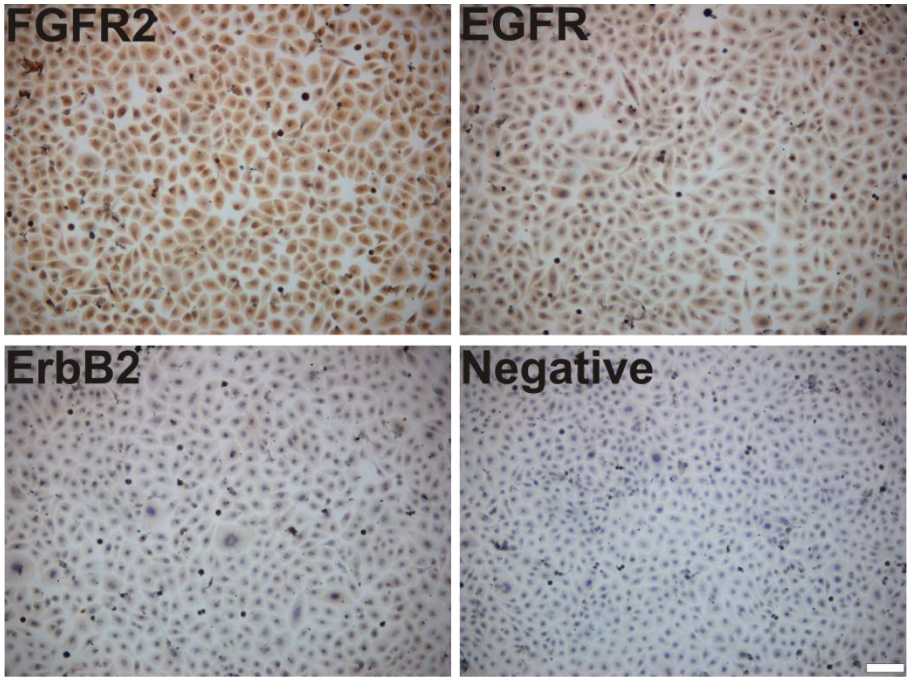

Figure 24. RTK expression in monolayer VA10 cells. Confluent monolayer culture stained with antibodies against RTKs. FGFR2 and EGFR are expressed in all VA10 cells but ErbB2 staining is less expressed. Nuclear staining haematoxylin blue. Scale bar 100μm.

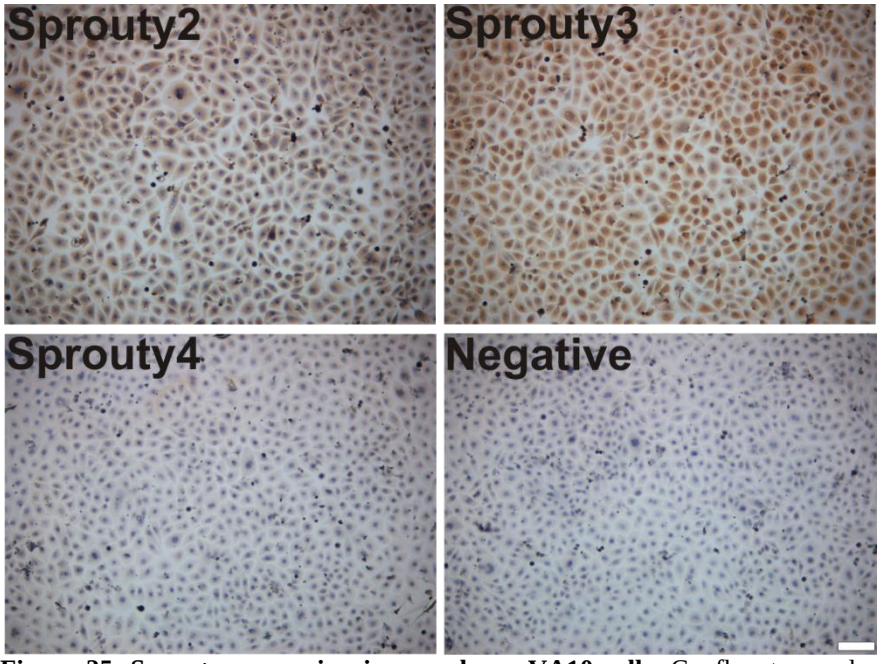

Figure 25. Sprouty expression in monolayer VA10 cells. Confluent monolayer culture stained with antibodies against Sproutys. Sprouty2 and 3 are expressed in all VA10 cells but Sprouty4 staining is very weak. Nuclear staining haematoxylin blue. Scale bar 100μm.

5 - Q-RT-PCR analysis on RTK and Sprouty expression

Quantitative Real time PCR shows that mRNA expression of Sprouty2, 3 and 4 in 2D culture is upregulated during growth of VA10 cells, compared to a growth-arrest phase used as control (given the value 1) (figure 26). This is especially noticeable for Sprouty2 and 4. However the expression of the RTKs FGFR2, EGFR and ErbB2 is more stable during growth.

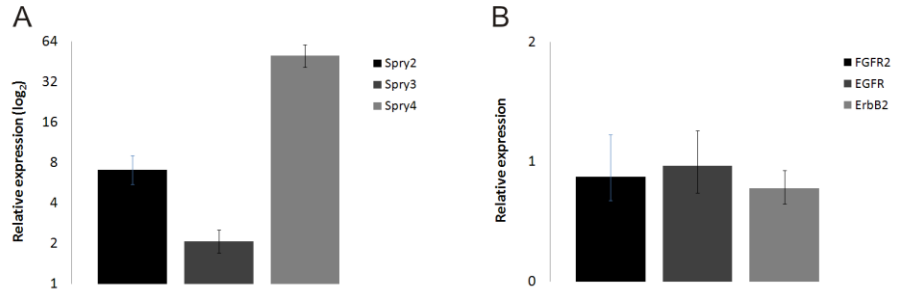

Figure 26. Quantitative real-time PCR of Sprouty and RTK mRNA. Graph shows fold induction in mRNA expression compared to control mRNA extracted from VA10 cells starved for 24 hours (not shown, given value of 1 for each marker). **A**. All Sprouty mRNA levels are increased during proliferation of VA10 (Spry2 ~7fold, Spry3 ~2fold, Spry4 ~50fold). **B**. RTK mRNA values show no significant change compared to control.

6 - Expression of RTKs and Sprouty proteins in bronchio-alveolar structures in 3D culture

In order to study the expression of these critical regulators of branching morphogenesis we set out to map the expression pattern in a novel 3D model of branching morphogenesis developed in the laboratory. Recent studies from our laboratory have shown that co-culture of endothelial cells with epithelial cells stimulate growth and branching morphogenesis of the epithelial cells (Axelsson, 2010; Ingthorsson, 2008) The stimulatory effects on branching morphogenesis of endothelial co-culture with VA10 can be seen in figure 27. When cultured without endothelial cells the epithelial cells form round colonies. Co-culture leads to extensive branching terminating in alveolar like structures. These structures express the clara cell and type II cell specific marker thyroid transcription factor-1, suggesting alveolar differentiation. (Axelsson, 2010) (figure 27).

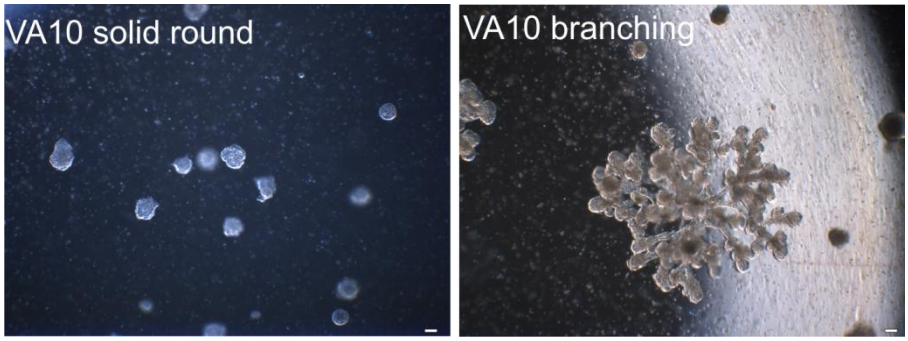

Figure 27. Endothelial cells stimulate branching of VA10 cells. VA10 cultured in 3D culture. When cultured alone or with endothelial cells up to 7 days, the VA10 forms solid round colonies. When cultured over 7 days in co-culture with endothelial cells, the VA10 solid round colonies start to branch and form bronchio-alveolar like structures.

FGFR2 is highly expressed at the tips of branching buds of bronchio-alveolar like structures (figure 28). This correlates well with studies on airway branching of *Drosophila* where it is expressed at the tips of migrating and proliferating airways towards an FGF signaling center (Ohshiro et al., 2002). EGFR is expressed in single cells in branching structures, more around branching areas than at the center of the structures (figure 29). ErbB2 has much more uniform expression than FGFR2 and EGFR. It is expressed all over the structure (figure 30). Sprouty2 is expressed at the tips of branching buds and less between the tips. Western blot shows that expression is downregulated when structures start to branch from solid round colonies (figure 31). Sprouty3 has more uniform expression at the periphery of the structures than Sprouty2. It is expressed near the epithelial-basement membrane barrier, as seen on the lung tissue slides (figure 32). Unlike Sprouty2 and 3, Sprouty 4 is not specifically expressed at the

periphery of the structures and not obviously bound to specific area, although expression pattern indicates more expression in branching areas (figure 33). All structures were stained with β-4 integrin (green). It is expressed at the periphery of the structures, since they are polarized against the extracellular matrix. Its expression was used to outline the structures and to find proper focus point inside the structures during confocal microscopy.

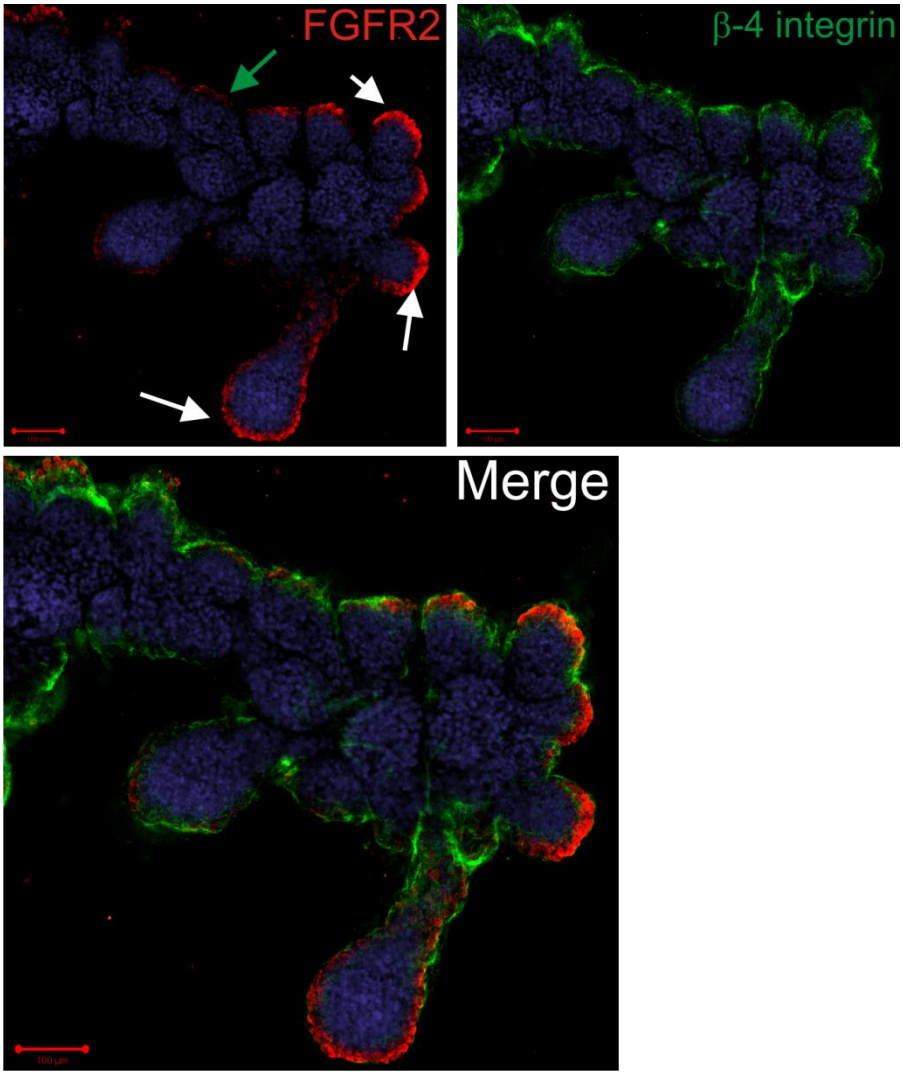

Figure 28. FGFR2 is expressed at the tip of branching buds of VA10 cells in 3D culture. FGFR2 (red) is highly expressed at the tips of branching buds (white arrows) and not between them and in areas not expanding into the rBM (green arrow). β-4 integrin (green). Nuclear TOPRO-3 staining blue. Scale bar 100μm

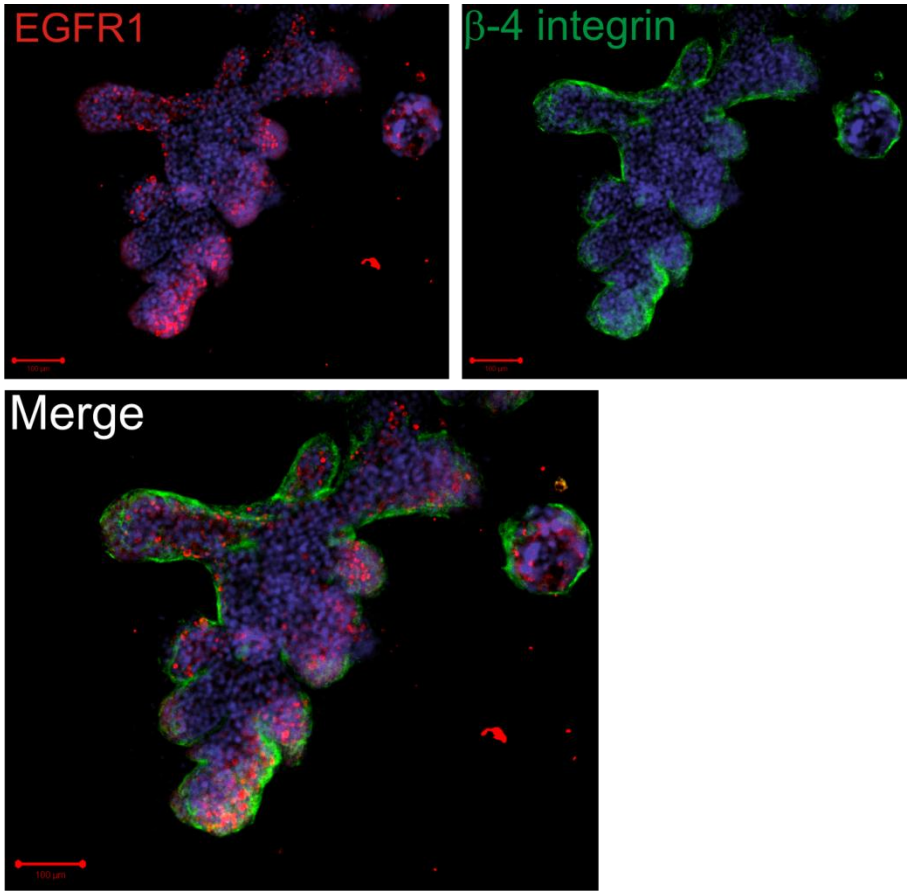

Figure 29. EGFR is expressed in single cells in branching structures. EGFR (red) is expressed in single cells and not spread around areas of the structures. Expression pattern is mostly near or inside branching buds. β-4 integrin (green). Nuclear TOPRO-3 staining blue. Scale bar 100μm

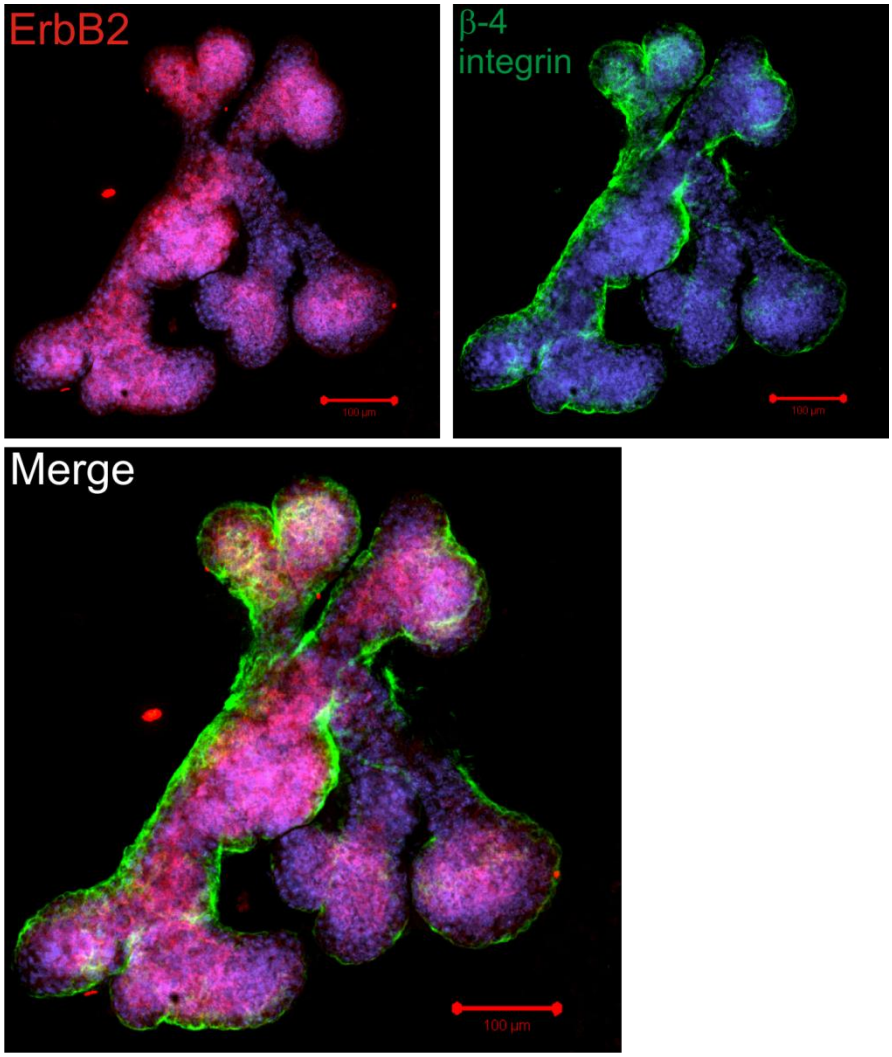

Figure 30. ErbB2 has a uniform expression in branching structures. ErbB2 (red) is widely expressed in branching structures. It has no specific expression area at the structures. β-4 integrin (green. Nuclear TOPRO-3 staining blue. Scale bar 100µm.

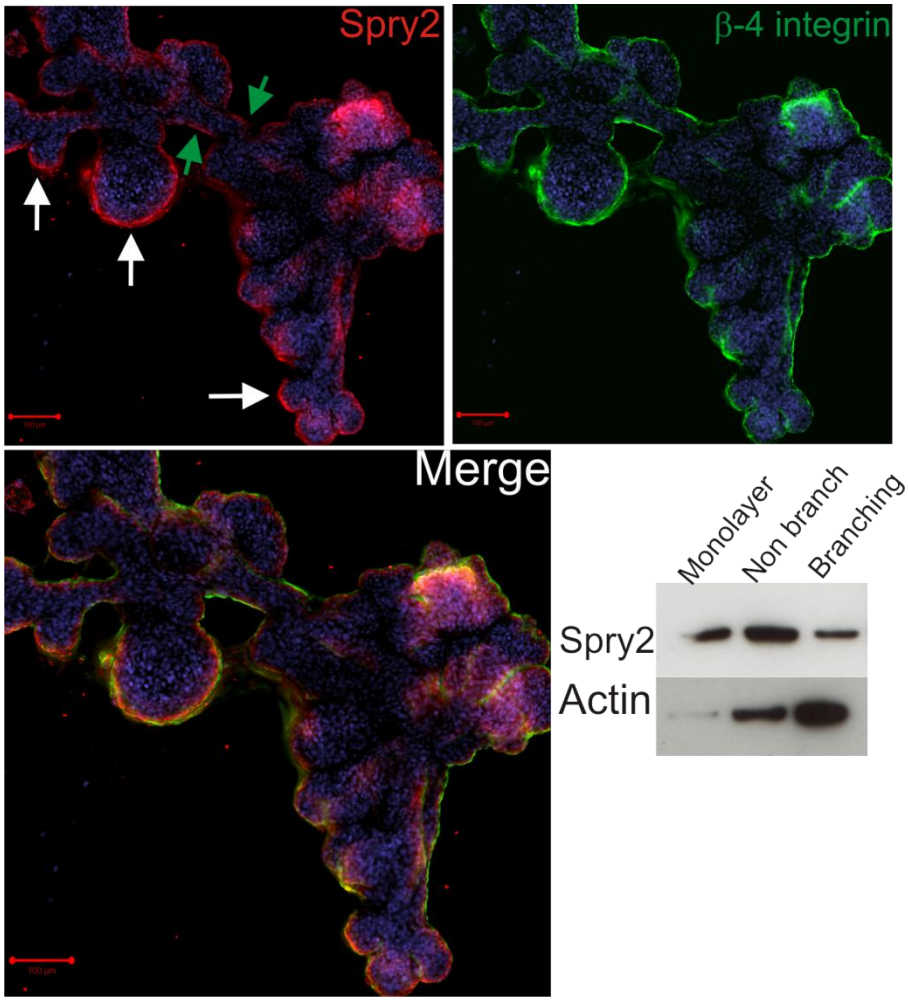

Figure 31. Sprouty2 is expressed at tips of branching buds in structures. Sprouty2 (red) is more expressed at tips of branching buds (white arrows) and less between them (green arrows). Western blot shows that Sprouty2 expression is downregulated in branching structures compared to non-branching structures. β-4 integrin (green. Nuclear TOPRO-3 staining blue. Scale bar 100μm.

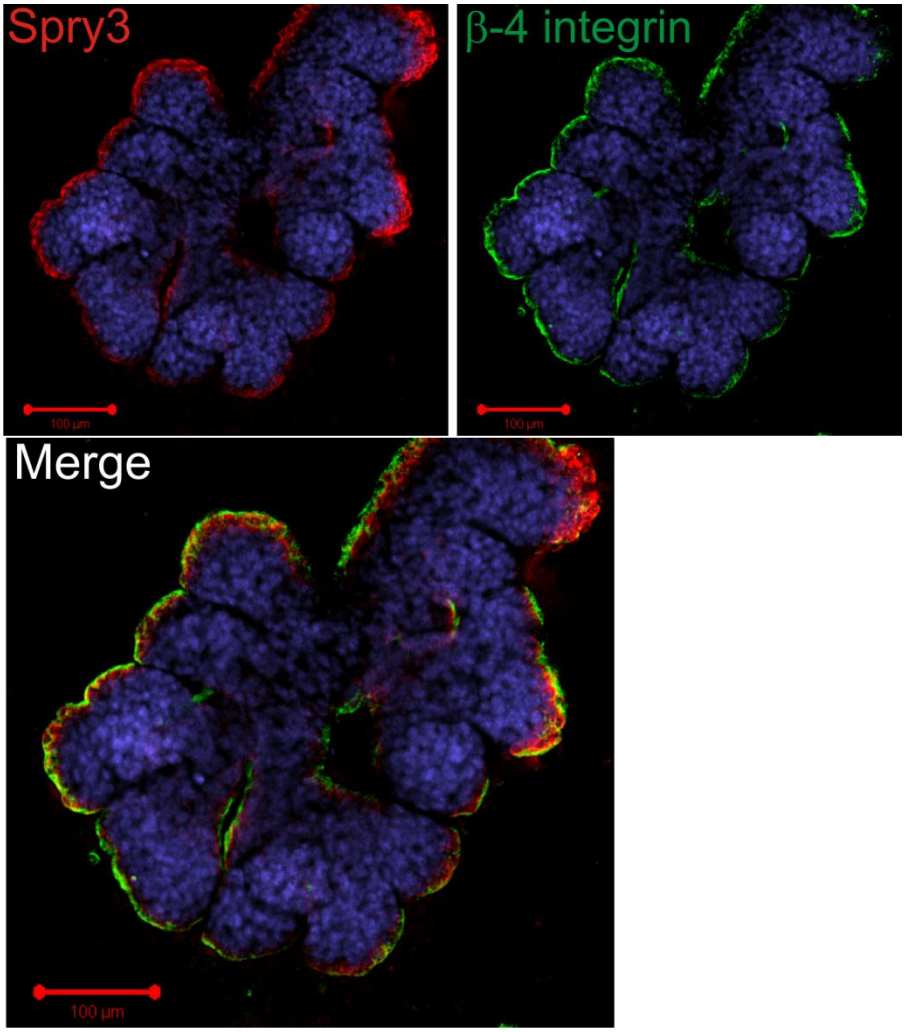

Figure 32. Sprouty3 has a peripheral expression pattern in branching structures. Sprouty 3 (red) is expressed at the epithelial-matrix interface. β-4 integrin (green). Nuclear TOPRO-3 staining blue. Scale bar 100µm.

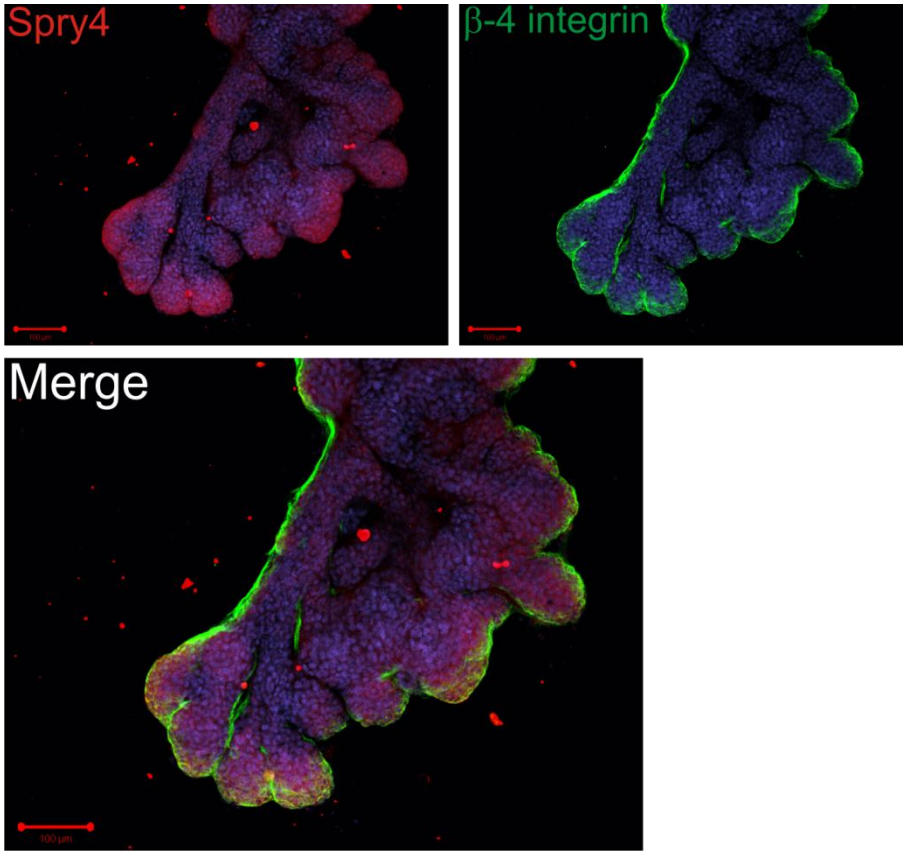

Figure 33. Sprouty4 has low and uniform expression in branching structures. Sprouty 4 expression is not bound to a specific area of the structures. β-4 integrin (green. Nuclear TOPRO-3 staining blue. Scale bar 100μm.

VI – Discussion

1- Summary

In this research project I have focused on the phenotypic characterization of epithelial and stromal compartments of the human lung. Specifically, I have explored the expression pattern of RTKs and their well-known regulators, Sproutys, in human lung tissue and in a well characterized human lung cell line during 3D culture, allowing the modelling of lung branching morphogenesis. This project is the first part of a long term research effort aimed at exploring the functional role of RTKs and Sprouty proteins during human lung morphogenesis. This expressional study, analyzing the spatial location of the RTKs and Sproutys in the 3D culture system, lays the ground for further research studying the functional role of these genes in branching morphogenesis.

2 - RTKs and Sprouty expression in lung tissue and branching structures

Receptor tyrosine kinases are well studied proteins, especially in regard to their role in development and cancer. However, the role of RTKs in human lung development has been less studied. This is partially due to lack of representative culture models and well defined cell lines. Here, a 3D cell culture model utilizing a well characterized airway epithelial cell line (VA10), is used to explore expression of three important RTKs in human lung branching morphogenesis and

their regulators, the Sprouty family. In the 3D co-culture model, the most studied RTK in airway development, FGFR2 has a very similar expression pattern at that seen to *Drosophila* airway development, especially with regard to branching morphogenesis and the proliferative end-buds showing high expression of Sprouty2 (Ohshiro et al., 2002). In the *Drosophila*, the expression pattern has been shown to be due to the presence of the stromal derived ligand *branchless* (Sutherland et al., 1996). The similar expression pattern in our model suggests that endothelial cells might be a source of an FGFR-ligand, e.g. FGF-10, suggested to be critical in stiumulating branching in the mouse model (Bellusci et al., 1997).

The culture medium contains basic FGF (bFGF/FGF2) which has been shown to stimulate branching (Nogawa & Ito, 1995). However, branching of lung structures occurs very rarely when cultured without endothelial cells. This might suggest that a certain amount of bFGF is needed to drive branching of lung structures and the endothelium supplies the additional ligand or another ligand acting in concert with bFGF. Since it has been well documented in mouse models (Bellusci et al., 1997), an interisting FGF candidate apart from bFGF would be the FGF-10. It would be interesting to culture VA10 cells without endothelium in 3D culture with beads soaked in FGF-10 to explore if it would drive branching. Work along this line with increasing bFGF in culture medium is currently being performed at the laboratory.

FGFR2 is expressed basally in adult human lung bronchial tissue, near the basement membrane. This could indicate

communication with FGF ligands from the surrounding stromal tissue, including messages from endothelial cells to bronchial basal cells. Less is expressed in alveolar tissue, where basal cells are absent. This could represent the low replicative activity in the alveolar tissue compared to the developing lung or the in vitro 3D model.

The EGF receptors do not appear to have as obvious expression profile in the 3D cultured structures. Although EGFR is in proximity to expanding branching regions, it is present in single cells and not spread throughout the adjacent area. *In situ,* EGFR is expressed in the bronchial eptihelium, is mostly at the luminal side. This correlates well with studies showing that EGFR stimulates pulmonary mucin expression (S. Kim et al., 2005). EGFR is also expressed in serous cells of submucosal glands and in the alveoli.

ErbB2 has a universal expression in 3D cultures. It is expressed basolaterally in the bronchial epithelium and in submucosal glands. In an elegant study, Vermeer et al. showed that ErbB2 is expressed basally and its regulator, heregulin-α, is expressed apically in polarized, cultured lung epithelium. They proposed that when epithelial integrity is compromised, the ligand flows through the epithelium and activates the receptor, resulting in cell proliferation and injury repair (Vermeer et al., 2003). This correlates well with the basolateral expression profile shown in adult human lung. Furthermore, this could suggest that non-polarized cells during development express this ligand for their own use to drive proliferation. Again, this hypothesis correlates well with the uniform expression profile shown in the 3D cultured structures.

Sprouty 2 has been shown to be a widespread regulator of RTK signaling, and it´s structure is highly conserved between species. During mouse lung branching, Sprouty2 is expressed at the tip of branching airways, and is downregulated in the cleft between branches. In VA10 branching structures, Sprouty2 has a similar expression pattern; it is expressed at the tips of branching buds and is less expressed between them. It would be very interesting to look at the expression pattern in an early branching bud during the direct process of bifurcating but that is very difficult to capture The fact that Sprouty is a negative regulator of RTK signaling and the fact that it is downregulated at the cleft, where proliferation ceases, is contradictory and quite interesting. This phenomenom could be explained by other restricting mechanisms at work, negatively regulating RTK signaling upstream of Sprouty and thus inhibiting further Sprouty expression. Sonic hedgehog (Shh) affects the FGF-10 expression in the mesenchyme in front of a branching bud (Warburton et al., 2005). Bone morphogenetic protein 4 (Bmp4) expression has been shown to be induced by FGF-10 signaling in the mouse lung, and to be able to limit branch growth (Bellusci et al., 1996). Altough interesting, it is beyond the scope of this thesis to explore this connection. Sprouty2 is highly expressed in the bronchial epithelium of the adult human lung. It is also expressed to some extent in the surrounding stromal tissue. Expression in alveoli is observed, although it is uncertain in which cells. It is clear that Sprouty2 is associated with human lung cell function and is highly interisting in this regard.

Sprouty3 has a more uniform expresssion at the periphery of the branching structures. Since very little is known about the role of Sprouty3, it will be interesting to further study its role in this model. In the adult human lung, Sprouty3 has similar expression pattern as FGFR2. Therfore it would be interesting to explore if there is a connection between FGFR2 downstream signaling and Sprouty3 in lung development. *In situ* hybridization results demonstrate that further work needs to be done to fully confirm expression pattern of Sprouty3, and later on, the function of it. The laboratory is currently working on a lentiviral approach to silence Sprouty genes with shRNA constructs.

Sprouty4 has a less distinct epithelial expression, especially in the *in vitro* model. In the normal lung, the expression was prominent in the submucosal glands and our model system does not represent the cells present there.

The fact that critical markers like FGFR2 and Sprouty2 have been shown to have similar expression profiles in the 3D culture model as in well studied model organisms supports the relevance of the model system to study branching mechanism further with functional studies.

The air-liquid interface setup gives furthermore a great oppurtunity to explore the expression and possibly function of these markers in VA10, during the generation of the pseudostratified epithelial layer. Cross-section confocal microscopy could give good expression pattern, and this could be compared to the normal *in situ* pseduostratified epithelium.

3 – Expression in monolayer

The expression of RTKs and Sprouty2 and 3 is uniform in monolayer culture. This has possibly to do with the culture medium. In monolayer the cells are grown on BEGM which might be more of a maintenance medium. Skarphéðinn Halldórsson showed in his PhD thesis that VA10 cells cultured at the air-liquid interface with DMEM supplemented with Ultroser G can differentiate to other bronchial cell types such as ciliated cells (S Halldorsson, 2010). Therfore, one could imagine that a more heterogenous expression pattern of these markers in monlayer VA10 could be seen when cultured in DMEM and Ultroser G or other medium that stimulates cell differention.

4 - Revision on antibody stainings

The accuracy of the 3D structure antibody staining is under constant consideration in the laboratory. The fact that some critical proteins in lung development are not expressed in cells deep inside the structures raises questions. It is possible that antibodies have less access to cells deep in the structures. The TOPRO-3 nuclear stain is a smaller molecule and could therefore have easier access to these deeper lying cells. There is a difference between antibodies, some show clear expression in the center of the structures, like the ErbB2 (and other antibodies used at the lab but not in this paper). This would support that the antibodies have access deep into the structures but the possibility that it might differ between antibodies cannot be excluded. To explore this, it is possible to either freeze the whole gel, or isolate structures and freeze them in freezing compound. Then the structures

could be cryosectioned and stained. Some three dimensional aspects would be lost by this approach but the staining pattern could be confirmed.

5 – *In situ* hybridization

Due to the fact that some anibodies can have non-specific binding, especially those not widely used e.g. the Sprouty antibodies, it was decided to support the data with mRNA *in situ* hybridization. As the lab had never conducted *in situ* hybridization before, I had to establish this method at the laboratory. This work was both time-consuming and required extensive troubleshooting. A successful method was established and it is now available as a routine method in the laboratory. In situ hybridization confirmed Sprouty2 expression. Sprouty3 however, has a slight different mRNA expression than protein expression possibly suggesting that the antibody might be non-specific. Furthermore, the individual Sprouty3 results within the hybridization method varied. Further troubleshooting is therfore required.

6 - Further utilization of the culture model

In a very important paper, Metzger and colleagues described the complete pattern of mouse lung branching and the lineage of the bronchial tree. They also introduced three geometrically distinct modes of branching coupled in three different sequences (Metzger et al., 2008).

Domain branching occurs when daughter branches form in rows at different positions around the circumference of the parent branch in a 90° rotation. This mode of branching is mostly used first and it generates the central scaffold of each lobe (figure 34).

Planar bifurcation is when the tip of a tube bifurcates and expands in the same 3D plane. This kind of branching is mostly observed in the thin edges of lobes.

Orthogonal bifurcation is mostly found distally in the lungs. The tip of a parent branch bifurcates like in planar bifurcation but between each round of branching there is a 90° rotation in the bifurcation plane. This rotation continues and generates a net-like branching system that creates lobe surfaces and fills the interior.

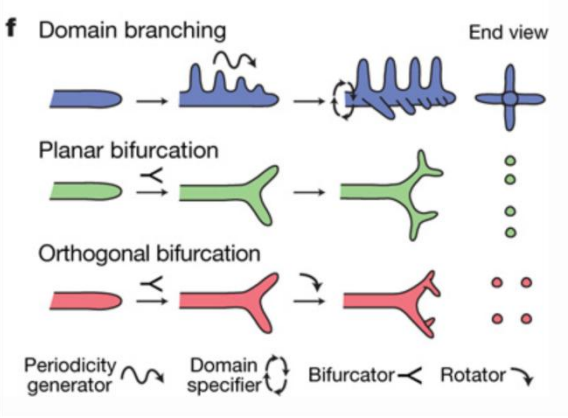

Figure 34. Three modes of branching in mouse lung. In domain branching, daughter branches form in rows around the circumference of the parent branch. Planar bifurcation is the bifurcation of a parent branch in two daughter branches in a 3D plane. Orthogonal bifurcation forms daughter branches in 90° rotation.

Studies on *Drosophila* have shown that different branching-related gene expression is evident at the primary, secondary and tertiary branching of airways.

It is an interesting approach to study the 3D cell culture branching system with regard to these three modes of branching. If one mode is dominant or all evident and equally distributed, it would be interesting to explore the expression and function of critical regulators in relationship to these different modes of branching.

This culture system however, does not fully replicate the *in vivo* situation and therefore it is possible that these branching events as clear as in the developmental in vivo setting. Although it is clear that endothelial cell signaling stimulates branching in the culture model, many other cell types lie in the stromal tissue surrounding the human epithelium. It cannot be excluded that other cell types contribute to epithelial branching *in vivo*, and therefore this branching model might lack some critical aspects of the three modes of branching introduced by Metzger and colleagues.

VII - Conclusions

In summary, I have explored the expression pattern of the receptor tyrosine kinases FGFR2, EGFR and ErbB2 in human adult lung tissue and in branching morphogenesis model in 3D culture. I have furthermore explored the pattern of Sprouty2, 3 and 4 expression and tried to correlate the expression pattern of Sprouty proteins to RTK expression. In order to confirm some of the findings I have also established *in situ* hybridization method at the laboratory. Expression pattern of FGFR2 and Sprouty2 correlates well to developmental pattern shown in animal models. My findings demonstrate that the 3D culture model is usable to study human lung mophogenesis and the findings lay ground for interesting functional studies on RTKs and Sproutys during lung branching morphogenesis in near future.

VIII - Appendix

RNA probe synthesis

Mix in the following order at RT.

a. Nuclease free-H_2O (to final volume of 20μl)
b. 5× transcription buffer (4μl)
c. 0.1M DTT (2μl)
d. Nucleotide mix (2μl)
e. Linearized plasmid (1 μg/μl) (1-3μl)
f. RNase inhibitor (0.5μl)
g.T7 RNA polymerase (20 units)

1. Add half of the polymerase first, incubate at 37°C for 1 h, then add the rest of the polymerase and incubate for another hour at 37°C.
2. Analyse a 1μl aliquot of the reaction on 1% agarose gel. Run the gel for 15 min at 50-100V. RNA band ~10-fold more intense than the plasmid should be seen.
3. Add 1μl of RNase free-DNase and incubate at 37°C for 15 min.
4. Add 100μl H_2O, 10μl 4 M LiCl and 300μl ethanol. Keep at -70°C for ~30 min.
5. Centrifuge at 4°C for 10 min, wash the pellet once with 75% EtOH, once with 96% EtOH and air-dry.
6. Redissolve the pellet in 50% formamide at about 0.1 μg/μl (100 μl) and store at -20°C.

Protocol for *In situ* hybridization.

1. Dewax and Rehydrate
    a. 2x5 minutes Xylene
    b. 2x5 min 100% EtOH
    c. 2x2 min 2X SSC buffer
    d. Pre warm Pre hybridization buffer to 50°C

2. Proteinase K
    a. Proteinase K for 10 minutes
    b. Wash 2x5 minutes 2X SSB buffer

3. Acetic anhydride
    a. Mix just before treating 50 ml 0.1M triethanolamine and 0.25ml acetic anhydride
    b. Add to slides for 10 minutes
    c. Wash 2x5 minutes in 2X SSC buffer

4. Prehybridization
    a. Use 100µl Pre hybridization buffer
    b. Incubate at 50°C in hybridization chamber in sealed box moistened with formamide/5X SSPE buffer (1/1) for 2 hours

5. Hybridization
    a. Remove pre hybridization buffer with vacuum
    b. Add to 100µl of pre hyb buffer 100ng probe and 400ng tRNA
    c. Incubate at 55°C for 12-16 hours in sealed box moistened with formamide/5X SSPE buffer (1/1)
    d. Warning: Slides tend to dry, completely seal box and add enough moist

6. Wash
    a. Wash 4x10 minutes with 4X SSC buffer

7. Dig Primary antibody
    a. Rinse in Buffer 1 for 5 minutes
    b. Add blocking buffer for 1 hour at RT
    c. Add 100µl blocking buffer with antibody (1:500) for 4 hours, light sealed at RT

8. Wash
    a. Wash 3x10 minutes in Buffer 1
    b. Wash for 5 minutes in Buffer 2.

9. Color detection
    a. Add 450µl Color development solution and incubate for 4 hours in sealed container in dark at RT.
    b. Stop reaction with mild Tris-EDTA buffer.

10. Nuclear staining
    a. Rinse thoroughly with dH_2O.
    b. Stain in nuclear fast red for 2 minutes.
    c. Add permount and coverslip

XI - References

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