Dissertation
submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

presented by:

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Oral Examination: 28.02.2019

Reflection confocal microscopy for the study of airway surface liquid dysregulation in cystic fibrosis

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Abbreviations

AEC	airway epithelial cell
ASL	airway surface liquid
bp	base pairs
CaCC	Ca^{2+} activated Cl^- channel
cAMP	3'-5' cyclic adenosine monophosphate
CCSP	club cell secretory protein
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
COPD	chronic obstructive pulmonary disease
ENaC	epithelial Na^+ channel
βENaC	β subunit of ENaC
FRT	Fischer rat thyroid
IBMX	isobuthylmethylxanthine
MCC	mucociliary clearance
n	number
pAEC	primary airway epithelial cell
PBS	phosphate buffered saline
PCL	periciliary liquid layer
PCR	polymerase chain reaction
RT	room temperature
Tg	transgenic
UTP	uridine triphosphate
UC	Ussing chamber
WT	wild-type
ZO-1	Zonula occludens protein 1

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Summary

Measurements of airway surface liquid (ASL) on primary airway epithelial cultures (AECs) grown at air-liquid interface (ALI) identified ASL depletion as a characteristic abnormality in cystic fibrosis (CF) and may be used as an endpoint for preclinical testing of strategies to improve airway surface hydration in patients with CF. Traditionally, ASL height has been determined by confocal fluorescence microscopy after addition of fluorescently labelled liquid to the apical side of the epithelium, however, several hours are required to restore steady state ASL depth after this volume challenge. Moreover, the current protocol requires fluorescently labelled liquid addition to the apical side of the epithelium resulting in non-physiological conditions.

The aim of this project is to determine the suitability of confocal reflection microscopy as a novel approach to study ASL regulation without requirement of fluorescent labelling/volume. Reflection microscopy detects the inherent differences in refractive indices between the permeable membrane, cytosol of cells and liquid layer. Primary airway epithelial cells were prepared from wild-type mice and βENaC-overexpressing mice as a model of CF lung disease, and also from non-CF and CF patients, and were cultured under physiological conditions at air-liquid interface. ASL homeostasis was investigated by confocal fluorescence and reflection microscopy.

Using the fluorescence-based volume challenge protocol, directly after the addition of the fluorescent dye in a volume of 20 µl, ASL height on AECs from wild-type mice determined by confocal reflection microscopy did not differ from values obtained by fluorescence microscopy. Two hours after volume challenge, ASL height was reduced and at 24 hour time point ASL height recovered to normal levels that did not differ between the two techniques. In AECs from βENaC-overexpressing mice, ASL height was similar at early time points, but remained reduced at 24 hour time point. Similar to studies in wild-type AECs, ASL height data did not differ between reflection and fluorescence based

measurements. Moreover, continuous measurements of unperturbed steady state measurements using reflection confocal microscopy revealed a stable ASL height measured in both wild-type and βENaC-overexpressing AECs for 5 hours. Similar to comparison studies wild-type mice derived AECs showed higher ASL height than βENaC-overexpressing cells. In addition to murine primary AECs, measurements were performed also with human primary AECs from non-CF and CF patients. Steady state measurements of non-CF patient derived AECs showed a higher ASL height than CF patient derived AECs.With the basolateral addition of benzamil, due to blocked Na^+ and liquid absorption ASL height of non-CF AECs was increased, showing that reflection confocal microscopy is capable of measuring even the small changes in ASL height. However, due to lack of functional CFTR channels, ASL height did not change in CF AECs.

In conclusion, these results support that reflection confocal microscopy can be used for accurate measurements of ASL height (dys)regulation without the need of addition of fluorescently labelled dye. This approach may facilitate preclinical evaluation of novel drugs designed to improve airway surface hydration in patients with CF under more physiological steady state conditions.

Zusammenfassung

Messungen der Atemwegsoberflächenflüssigkeit (ASL) an primären Atemwegsepithelkulturen (AECs), die an der Luft-Flüssigkeitsschnittstelle (ALI) angebaut wurden, identifizierten die ASL-Depletion als eine charakteristische Anomalie bei Mukoviszidose (CF) und können als Endpunkt für präklinische Tests von Strategien zur Verbesserung der Hydratation der Atemwegsoberfläche bei Patienten mit CF verwendet werden. Traditionell wurde die ASL-Höhe durch konfokale Fluoreszenzmikroskopie nach Zugabe von fluoreszenzmarkierter Flüssigkeit zur apikalen Seite des Epithels bestimmt, jedoch sind mehrere Stunden erforderlich, um die stationäre ASL-Tiefe nach dieser Volumenaufgabe wiederherzustellen. Außerdem erfordert das aktuelle Protokoll die fluoreszierend markierte Flüssigkeitszugabe zur apikalen Seite des Epithels, was zu unphysiologischen Bedingungen führt.

Ziel dieses Projekts ist es, die Eignung der konfokalen Reflexionsmikroskopie als neuartigen Ansatz zur Untersuchung der ASL-Regulierung ohne Anforderung von Fluoreszenzmarkierungen/ Volumen zu bestimmen. Die Reflexionsmikroskopie erkennt die inhärenten Unterschiede in den Brechungsindizes zwischen der permeablen Membran, dem Zytosol der Zellen und der Flüssigkeitsschicht. Primäre Epithelzellen der Atemwege wurden aus Wildtyp-Mäusen und βENaC überexprimierenden Mäusen als Modell der CF-Lungenerkrankung sowie von Nicht-CF- und CF-Patienten hergestellt und unter physiologischen Bedingungen an der Luft-Flüssigkeitsgrenze kultiviert. Die ASL-Homöostase wurde mittels konfokaler Fluoreszenz- und Reflexionsmikroskopie untersucht.

Unter Verwendung des fluoreszenzbasierten Volumenherausforderungsprotokolls unterschied sich die ASL-Höhe an AECs von Wildtyp-Mäusen, die durch konfokale Reflexionsmikroskopie bestimmt wurden, direkt nach der Zugabe des Fluoreszenzfarbstoffs in einem Volumen von 20 µl nicht von den Werten der Fluoreszenzmikroskopie. Zwei Stunden nach

der Volumenprobe wurde die ASL-Höhe reduziert und nach 24 Stunden wieder auf ein normales Niveau gebracht, das sich zwischen den beiden Techniken nicht unterschied. In AECs von βENaC überexprimierenden Mäusen war die ASL-Höhe zu frühen Zeitpunkten ähnlich, blieb aber zum 24-Stunden-Zeitpunkt reduziert. Ähnlich wie bei Studien mit Wildtyp-AECs unterschieden sich die ASL-Höhendaten nicht zwischen reflexions- und fluoreszenzbasierten Messungen. Darüber hinaus ergaben kontinuierliche Messungen von ungestörten stationären Messungen mit Hilfe der konfokalen Reflexionsmikroskopie eine stabile ASL-Höhe, die sowohl bei Wildtypen als auch bei βENaC-überexprimierenden AECs für 5 Stunden gemessen wurde. Ähnlich wie bei Vergleichsstudien zeigen Wildtyp-Mäuse, die von AECs abgeleitet wurden, eine höhere ASL-Höhe als βENaC-überexprimierende Zellen. Zusätzlich zu den murinen primären AECs werden auch Messungen mit humanen primären AECs von Nicht-CF- und CF-Patienten durchgeführt. Mit der basolateralen Zugabe von Benzamil, aufgrund der blockierten Na+- und Flüssigkeitsabsorption, wurde die ASL-Höhe von Nicht-CF-AECs erhöht, was zeigt, dass die konfokale Reflexionsmikroskopie in der Lage ist, selbst die kleinen Veränderungen der ASL-Höhe zu messen. Aufgrund fehlender funktionaler CFTR-Kanäle änderte sich die Höhe der CFTR-Kanäle in den CF-AECs jedoch nicht.

Zusammenfassend lässt sich sagen, dass diese Ergebnisse bestätigen, dass die konfokale Reflexionsmikroskopie für genaue Messungen der ASL-Höhen-(Dys)-Regulierung verwendet werden kann, ohne dass ein fluoreszierend markierter Farbstoff hinzugefügt werden muss. Dieser Ansatz kann die präklinische Bewertung neuartiger Medikamente zur Verbesserung der Hydratation der Atemwegsoberfläche bei Patienten mit CF unter physiologischeren stationären Bedingungen erleichtern.

1. Introduction

1.1 Cystic Fibrosis (CF) and CFTR

Cystic fibrosis (CF) remains one of the most common hereditary fatal diseases in Caucasian populations and is an autosomal recessive genetic disorder caused by mutations in cystic fibrosis transmembrane regulator (*CFTR*) gene (Kerem et al., 1989). CFTR is a cAMP dependent anion channel which transports Cl^- and HCO_3^- through epithelia (Anderson et al., 1991). In addition to Cl^- secretion, CFTR is also important for inhibition of Na^+ absorption through epithelial sodium channel ENaC (Stutts et al., 1995).

Since CFTR is expressed in many different epithelial tissues, CF affects multiple organs including the intestines, liver, pancreas, and vas deferens, however in most of the patients morbidity and mortality is caused by the respiratory disease (Fraser-Pitt and O'Neil, 2015). In airways of CF patients, the absence of functional CFTR causes basically two main defects; decreased Cl^- secretion and Na^+ hyperabsorption, which in turn leads to dehydration of the ASL surface liquid (ASL). This dehydration of the airway surfaces is hypothesized to impair ciliary beating and mucociliary clearance and constitutes the basic disease initiating defect in CF airways (Knowles and Boucher, 2002). Further progression of the CF disease causes mucus obstruction, chronic airway inflammation, bacterial infection, progressive lung damage and ultimately respiratory failure (Mall et al., 2004).

CFTR mutations are classified into six groups from Class I to Class VI. (Figure 1) Class I mutations are the most severe ones. Nonsense mutations such as G542X and deletion or insertion mutations like ΔT1078 in CFTR gene result in defective protein production. Class II mutations cause defective protein processing and misfolded protein. The vast majority of the patients carry at least one allele of ΔF508 mutation, which causes a complex class II, class III and class VI defect. Class III mutations generate defective protein regulation, also called as gating mutation including G551D mutation. Class IV mutations lead to defective

conductance of CFTR. Class V mutations produce less amount of protein and finally Class VI mutations results in protein instability on the membrane. (Amaral and Farinha, 2013; Virant-Young et al., 2015)

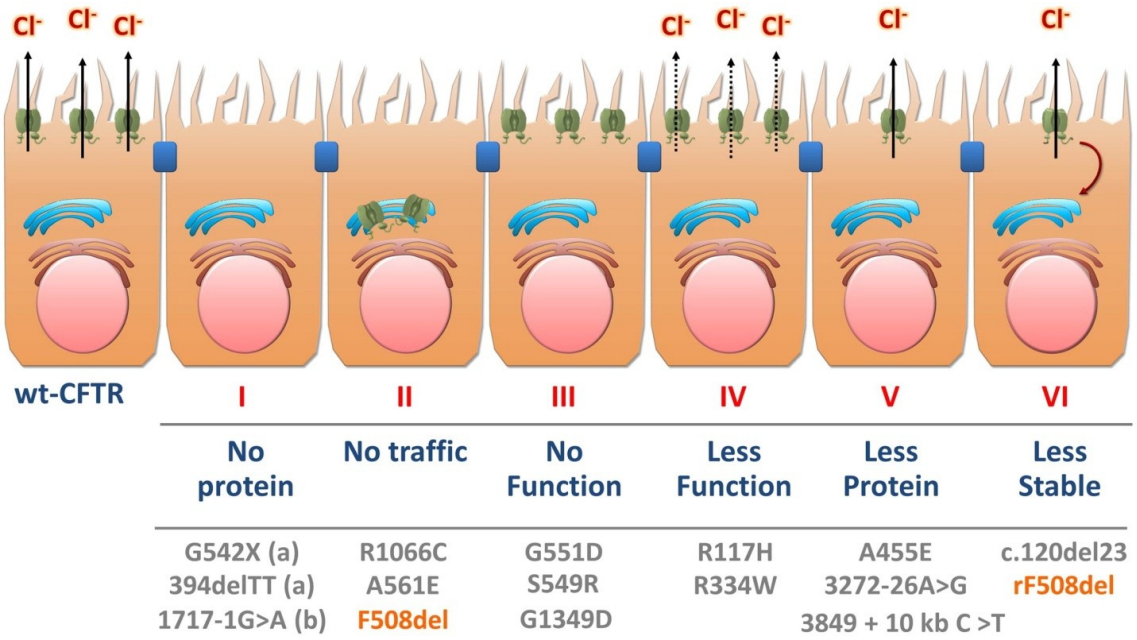

Figure 1. Classes of CFTR mutations compared to wild type CFTR. Adapted from (Bell et al., 2015)

1.2 Ion transport in airway epithelia

In healthy individuals, ion transport in airways is tightly regulated. Airways are composed of a layer of epithelial cells which are expressing variable ion channels.

CFTR is a cAMP dependent channel transporting Cl^- into the lumen and regulating Na^+ absorption through ENaC. Both secretion of Cl^- and absorption of Na^+ occur with water transport therefore CFTR regulates liquid volume on the surface of the airways. (Stutts et al., 1995) (Stutts et al., 1997)

In addition to CFTR, alternative Ca^{2+} activated Cl^- channels (CaCC) are also expressed in airway epithelia. These channels are activated as a result of

increased Ca^{2+} levels in the cells. Extracellular nucleotides act as an activator for CaCCs through purinergic receptor signaling (Knowles et al., 1991). TMEM16A has been identified as a transmembrane protein acting as a Cl^- channel. It is hypothesized to contribute to the Ca^{2+} activated Cl^- secretion in human and mouse airway epithelia (Galietta, 2009). Another alternative Cl^- channel identified is SLC26A9, which is highly expressed in airway epithelia (Anagnostopoulou et al., 2010). This channel is also shown in a genome wide association study (GWAS) to be a modifier in CF at meconium ileus. (Sun et al., 2012)

Even though Cl^- secretion through these alternative Cl^- channels is not sufficient to prevent the consequences of CFTR dysfunction in CF in humans, they could be used as target for therapeutic approaches for CF patients. On the other hand, in mice these channels are suggested to be one of the reasons for CFTR knockout mice not developing a lung disease. (Grubb and Boucher, 1999)

1.3 βENaC overexpressing mice as a CF mouse model

To study pathogenesis of CF disease, mouse models have been generated after the identification of *CFTR* gene related to CF disease. These mouse models contain mutations in the *Cftr* or have the knockout of *Cftr.* For example, *Cftr* knockout mouse model has the characteristics of CF disease in the intestine; however, it does not develop a CF-like lung disease (Grubb and Boucher, 1999).

As mentioned before, due to lack of functional CFTR channel, one abnormality in airways of CF patients is Na^+ hyperabsorption. To mimic Na^+ hyperabsorption in CF patients, a mouse model with airway specific overexpression of ENaC was developed (Mall et al., 2004). Club cell secretory protein (CCSP) promoter was used to express the ENaC only in the airways to prevent the phenotype in other organs. First studies have been performed by individually overexpressing all three subunits of ENaC, α, β and γ. It has been shown by measuring the bioelectrical properties of freshly excised tracheae of these mouse models that, the overexpression of β subunit of ENaC is sufficient to produce increase in

Amiloride (ENaC blocker) sensitive Na^+ transport (Figure 2). Further studies in βENaC-transgenic mouse model (βENaC-Tg) showed that both cAMP and Ca^{2+} activated Cl^- secretion remained unchanged compared to wild-type littermate controls.

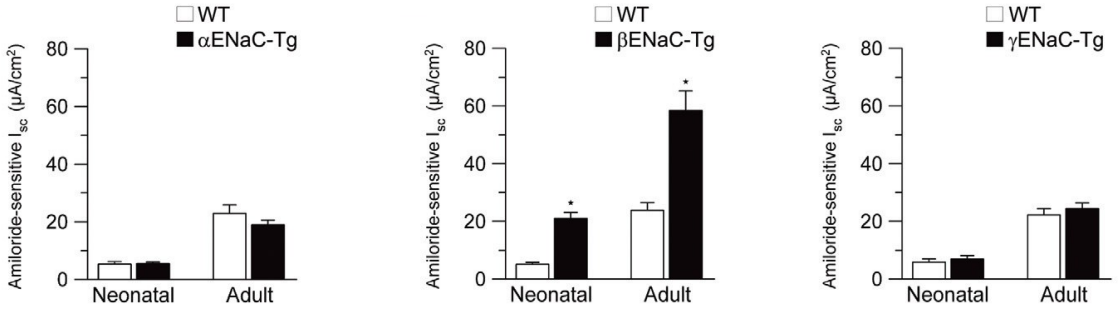

Figure 2. Amiloride-sensitive short-circuit current (I_{sc}) in neonatal and adult αENaC-, βENaC- and γENaC-Tg mice and wild-type (WT) littermates. Adapted from (Zhou et al., 2011).

Further characterization of βENaC-Tg mouse model displayed that increased Na^+ and fluid absorption results in ASL volume depletion and consequently reduced mucociliary clearance. Histological analysis revealed that βENaC-Tg mouse develops mucus plugging in the airways. These mucus plugs cause death in ~20% of βENaC-Tg (C57BL/6 background) mouse in the first 4 weeks of life (Figure 3). Histological analysis of βENaC-Tg mouse lungs showed increased levels of macrophage and neutrophil in the lumen of airways as well as goblet cell metaplasia.

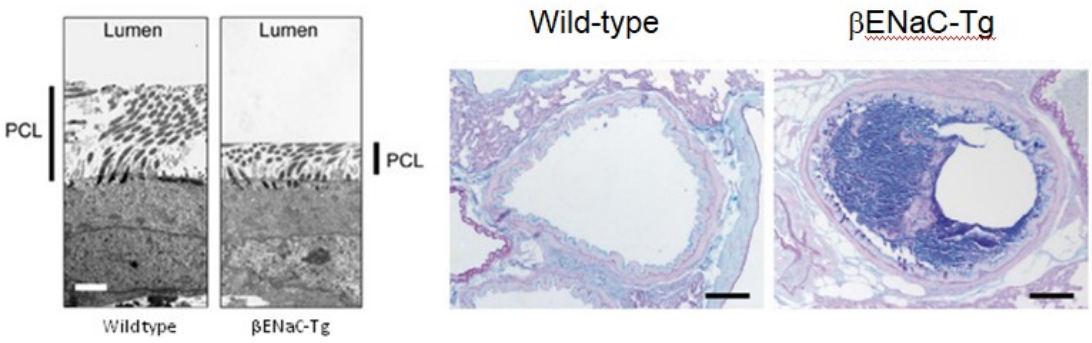

Figure 3. ASL volume depletion and mucus obstruction in βENaC-Tg mice. Adapted from (Mall et al., 2004) (Zhou et al., 2011)

In addition to histopathology, bronchoalveolar lavage (BAL) studies showed that βENaC-Tg mouse develops chronic airway inflammation. Due to reduced mucociliary clearance, βENaC-Tg was shown to have impaired bacterial pathogen clearance after infection.

To sum up, βENaC-Tg mouse model is developing a spontaneous lung disease which has the properties of CF lung disease. Therefore, this model has been intensively studied in the CF research field. Understanding the mechanism, studying the modifiers, evaluating the therapies of CF disease has been and continues to be the areas βENaC-Tg mouse model is used.

1.4 Regulation of airway surface liquid (ASL)

As shown in transgenic mice with airway specific overexpression of βENaC (βENaC-Tg), increased airway Na^+ absorption is sufficient to induce CF-like lung disease even in the presence of Cl^- secretion. However, the mechanism underlying the dysregulation of ENaC and the functional interaction between ENaC and CFTR is still incompletely understood. One approach to study epithelial dysfunction is to study airway surface liquid (ASL) homeostasis.

ASL is composed of two layers; one is the periciliary liquid layer among the surface of the epithelia, allowing cilia to beat, and on top is the mucus layer composed of secreted mucins such as MUC5AC and MUC5B, acting as a barrier for pathogens and particles (Hollenhorst et al., 2011). Ciliary beating is one of the most important mechanism for mucociliary clearance of the airways from inhaled pathogens and particles. Ion transport is also believed to be important for mucociliary clearance since it regulates the composition and hydration of the periciliary liquid which in turn allows the suitable environment for ciliary beating (Ghosh et al., 2015).

In order to understand the pathogenesis and to find out treatment strategies for respiratory diseases, ion transport and ASL homeostasis has been studied intensively in the last decades. Ussing chamber and patch clamp methods have been very useful to study ion transport properties of native or cultured tissues (Kansen et al., 1992). The height of the ASL layer in primary epithelial airway cultures prepared from murine and human airways is a valid parameter for studying ASL homeostasis. The height can be determined using confocal fluorescence microscopy by applying a volume of fluorescently labelled liquid (Tarran et al., 2005). This method has been useful to understand importance of ASL hydration in CF and COPD (Astrand et al., 2015). By using confocal fluorescent microscopy ASL height measurement method, different ENaC regulators such as channel-activating proteases (CAP) and their inhibitors, ATP, SPLUNC1 have been shown to have impact on ASL height regulation (Tarran et al., 2006b) (Garland et al., 2013) (Hobbs et al., 2012). Moreover, this method has been used to test pharmaceutical modulators of CFTR such as VX-770 and VX-809 *in vitro*. (Van Goor et al., 2009) (Van Goor et al., 2011)

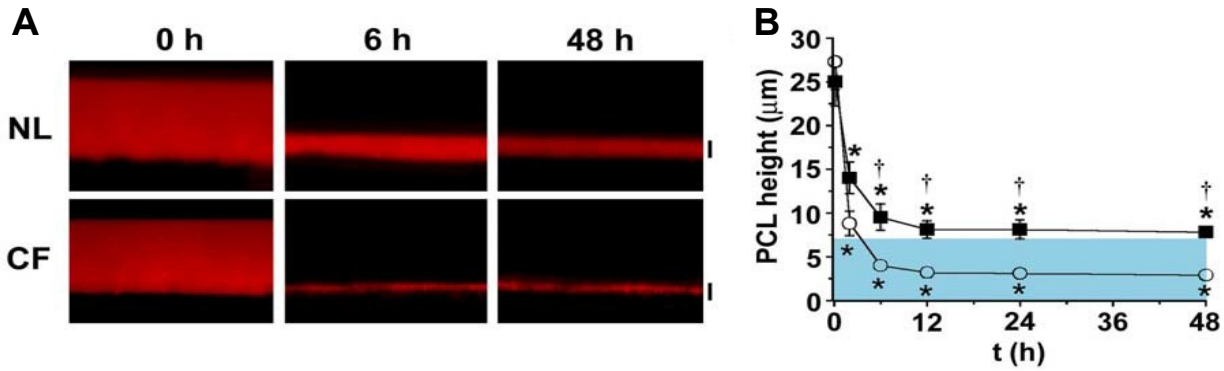

Figure 4. ASL height measurements of human bronchial epithelial cultures from healthy and CF cells. A) XZ confocal images of PCL at 0, 6, and 48 h after mucosal addition of 20 µl PBS containing Texas-red dextran to normal (NL) and CF bronchial epithelial cultures. B) Mean PCL height with time taken from NL (squares, n = 9) and CF (circles, n = 8) cultures. Adapted from (Tarran et al., 2005).

Although, it has been used intensively, this protocol requires fluorescence labeling and addition of liquid to the apical side of the epithelium resulting in non-physiological conditions. Sudden increase in ASL layer with additional liquid may cause dilution of epithelial channel regulators. Therefore, observation of ASL height regulation with this method is not under completely physiological conditions.

1.5 Treatment strategies for CF via pharmacological modulations of CFTR

There are around 2000 known mutations of *CFTR* and these mutations have different effects at RNA or protein level (Sosnay et al., 2013). After identifying the *CFTR* gene, there has been several drug screens performed to explore mutation-specific therapies. In the recent years there has been several CFTR corrector and potentiator compounds have been identified. Corrector compounds are chemical chaperons that improve folding and trafficking of class II mutant CFTR, while potentiators are compounds that increase the channel open probability of class III gating mutants. VX-770 (ivacaftor) is one of the approved drugs, which acts as a potentiator (Van Goor et al., 2009). When this compound is given orally

to G551D mutation carrying patients, there has been improvement in their lung function, increase in body weight and decrease in sweat chloride (Ramsey et al., 2011).

It has been previously shown that misfolding and trafficking defect of CFTR with ΔF508 mutation can be corrected by incubation of the cells at low temperature (27 ^{o}C) (Denning et al., 1992). To obtain the function there has been several drug screenings and VX-809 (lumacaftor) has been identified as correctors for this mutation, however initial clinical trials have not been successful (Van Goor et al., 2011) (Clancy et al., 2012). As ΔF508 constitutes a complex processing and channel defect, combination therapies have been considered. *In vitro* studies with human bronchial epithelial cells (HBE) which have ΔF508 mutation showed that, combination of VX-770 and VX-809 has increased Cl^- secretion by 25% (Van Goor et al., 2011). Clinical trials with the combination of VX-770 and VX-809 showed a modest improvement in lung function of CF patients.

1.6 Aim of the study

ASL height dehydration is a hallmark of cystic fibrosis lung disease. To study the effects of drugs, the individual contribution of ion channels and the interplay between the different cell types, the height of the ASL needs to be measured under the most physiological conditions. Studying primary airway epithelial cell culture is an established method and the current standard protocol for ASL height measurements is based on a volume challenge and fluorescent dye addition on the apical side of monolayer of airway epithelial cells, grown on air-liquid interface. This sudden increase in ASL layer with additional liquid may cause dilution of epithelial channel regulators. Therefore, observation of ASL height regulation with this method is not under fully physiological conditions. For this reason, we aim to establish confocal reflection microscopy as a novel method to study ASL homeostasis without the requirement of adding fluorescent labeling/volume. Reflection microscopy is a staining free method and this allows measuring ASL height without volume challenge and should have the potential to measure the height under the most physiological conditions during the observation period. With this method we aim to provide a tool to measure ASL height for preclinical testing. Newly found drugs can be tested on cell monolayers to test for effectiveness on ion transport at ASL level. Moreover, this method would support personalized medicine by testing patient specific drugs on patient derived primary airway epithelial cells to observe the effect on ASL height.

2. Materials and methods

All the materials were purchased from Sigma-Aldrich unless indicated otherwise.

2.1 Animal models

All animal studies were approved by the local animal welfare authorities (Regierungspräsidium, Karlsruhe, Germany). The generation of βENaC-overexpressing mice had been previously described (Mall et al., 2004). Mice were housed in a specific pathogen-free animal facility with free access to food and water.

2.2 Genotyping

Tissue lysis: Tail biopsies (tip of the tail) were obtained from each mouse and incubated at 95°C for 40 minutes in a thermomixer (thermomixer compact, Eppendorf) in 100 µL alkaline lysis buffer (25 mM NaOH, 0,2 mM EDTA in H_2O, pH=12). Then, 100µL of neutralizing buffer (40 mM Tris-HCl, pH=5) was added on the solution.

Polymerase Chain Reaction (PCR): AmpliTaq Gold 360 Master Mix (applied Biosystems) was used for the genotyping PCR. For 25 µL reaction 12,5 µL master mix was mixed with 1 µL of each forward and reverse primers (stock: 10 µM) and 1 µL of lysed DNA solution. Then, the volume was completed to 25 µL with H_2O.

Forward βENaC primer: CTTCCAAGAGTTCAACTACCG

Reverse βENaC primer: TCTACCAGCTCAGCCAGAGTG

PCR conditions: 95 °C 10 min

35 times 95 °C 20 sec

56 °C 20 sec

72 °C 90 sec

72 °C 7 min

Subsequently, amplified DNA fragments were separated by length using agarose gel electrophoresis. Forward and reverse primers had their specific binding sites on exon 12 and exon 13 of βENaC respectively. Exon 12 and exon 13 were separated by a ~100 bp intron. Therefore, amplification of wild-type allele produced 350 bp fragments whereas transgenic allele (cloned from cDNA) produced 254 bp fragments due to the absence of the ~100 bp intron. Thus, wild type and βENaC-Tg mice could be differentiated with the additional 254 bp fragment in samples from βENaC-Tg but not from wild type mice.

2.3 Primary murine tracheal epithelial cultures

Collection medium: 495 mL DMEM/F12 (1:1) and 5 mL Penicillin/Streptomycin (stock: 60 IU/mL) were mixed and sterile filtered through 0,22 µm filter.

Dissociation medium: 1.8 g $NaHCO_3$, 2.5 µL FeN_3O_9 (stock: 0,25 µM), 5 µL Sodium Pyruvate (stock: 100 µM) and 3 mL Penicillin/Streptomycin (stock: 60 IU/mL) were dissolved in 500 mL PBS (Ca^{2+}/Mg^{2+} free), sterile filtered through 0,22 µm filter and stored at 4°C. For each preparation into 20 mL dissociation medium 200 µL DNaseI (stock: 10 mg/mL in Aqua ad iniectabilia) and 1,4 mL ProteaseE (stock: 20 mg/mL in Aqua ad iniectabilia) were added freshly.

Culture medium: 475 mL DMEM/F12 (1:1), 25 mL HI FBS, 550 µL Insulin (human recombinant zinc, stock: 4mg/mL) and 1mL Primocin (stock: 50 mg/mL) were mixed and sterile filtered through 0,22 µm filter.

Coating transwell membranes: 20 µL acetic acid and 5 mg human placenta collagen were dissolved in 10 mL Aqua ad iniectabilia by vortexing. 340 µL is added on each transwell membrane (12mm inserts in 12 well plate, Costar/Corning) and let to air dry. Before using, membranes were washed 4 times with 400 mL PBS.

2.4 Isolation of murine tracheal epithelial cells:

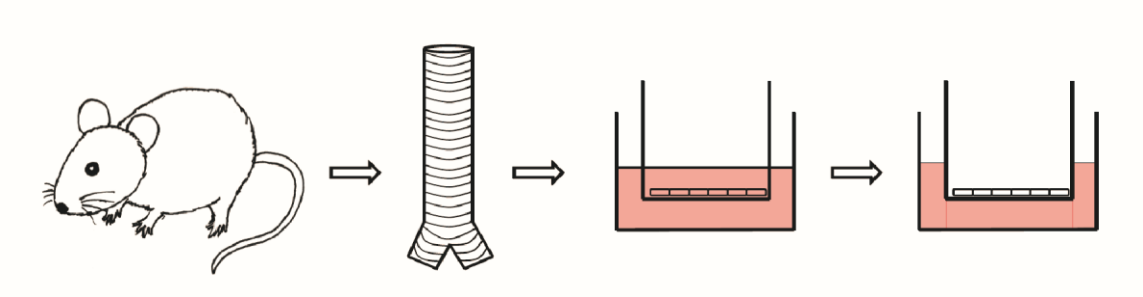

Figure 5. Schematic representation of primary murine tracheal epithelial cell preparation. Tracheae were excised from the mice and epithelial cells were isolated. First, cells were seeded in medium and after two days air liquid interface was started for differentiation.

For each individual experiment tracheae from 10 mice per group were freshly excised and pooled in collection medium and transferred to PBS to wash the tissue. Then, tracheae were transferred into dissociation medium which was freshly mixed with DNaseI and Protease E and incubated overnight on a shaker at 4°C, followed by 1 hour incubation on a shaker at 37 °C. To stop the dissociation reaction 5 mL HI-FBS was added and tubes were inverted 12-15 times. The solution was decanted and filtered through 100 µm cell strainer into a new 50 mL tube. The remaining tissue is washed with 10 mL culture medium and filtered through same cell strainer. The cell suspension in the new tube was centrifuged for 10 min at 1300 rpm at 4 °C (Megafuge 16R, Thermo Scientific). After careful aspiration of the supernatant, the cell pellet was dissolved in 5 mL culture medium and seeded on a 10 cm petri dish. Cells were incubated for 2 hours at 37 °C to separate the fibroblasts from epithelial cells. In this step,

fibroblasts attached to the dish and epithelial cells remain in the supernatant. Medium containing the epithelial cells was carefully transferred to a 15 mL tube and the cells were counted on Neubauer chamber by 1:10 dilution with Trypan blue. The cell suspension was centrifuged for 10 min at 1300 rpm at 4 ^{o}C and afterwards the cell pellet was resuspended in culture medium with the concentration of 1,2 million cells/mL. Then, the cells were seeded on transwell membranes (500µL cell suspension/membrane), 1 mL medium was added basolaterally in the plate. After 2 days air-liquid interface was started by carefully aspirating the medium from the apical side of the culture while cells were attached to the coated membrane. At the same time on the basolateral side medium was replaced with fresh medium. Then, every second day medium on the basolateral side was replaced until 14 days of culturing when the cells were fully differentiated.

2.5 Primary human nasal epithelial cultures

Dissociation medium: 1.8 g $NaHCO_3$, 2.5 µL FeN_3O_9 (stock: 0,25 µM), 5 µL Sodium Pyruvate (stock: 100 µM) and 3 mL Penicillin/Streptomycin (stock: 60 IU/mL) were dissolved in 500 mL PBS (Ca^{2+}/Mg^{2+} free), sterile filtered through 0,22 µm filter and stored at 4^{o}C. For each preparation into 20 mL dissociation medium 200 µL DNaseI (stock: 10 mg/mL in Aqua ad iniectabilia) and 1,4 mL ProteaseE (stock: 20 mg/mL in Aqua ad iniectabilia) were added freshly.

Culture medium: 500 mL Airway Epithelial Cell Growth Medium (PromoCell) was mixed with 12,3 mL Supplement Mix (PromoCell) and 1 mL Primocin (stock: 50 mg/mL).

Coating transwell membranes: 20 µL acetic acid and 5 mg human placenta collagen were dissolved in 10 mL Aqua ad iniectabilia by vortexing. 340 µL was added on each transwell membrane (12mm inserts in 12 well plate, Costar/Corning) and let to air dry. Before using, membranes were washed 4 times with 400 mL PBS.

Isolation of human nasal epithelial cells: Patient tissues were obtained from Heidelberg University Hospital after surgeries, and cells were isolated and cultured under air-liquid interface as previously described (Fulcher et al., 2005). From non-CF patients turbinate tissue and from CF patients nasal tissue from paranasal sinuses were obtained and the primary nasal cells were covered by the ethics approval S 136-2016.

Table 1. Tissue samples from non-CF patients for primary cell cultures.

Non-CF patients (n=7)	
Gender: 4 females, 3 males	Age (mean ± SD): 34 ± 14

Table 2. Tissue samples from CF patients for primary cell cultures.

CF patients (n=8)		
Gender: 4 females, 4 males	Age (mean ± SD): 20 ± 8	Genotype
F	24	ΔF508/ΔF508
F	20	ΔF508/ΔF508
M	15	ΔF508/ΔF508
F	21	ΔF508/ΔF508
M	9	ΔF508/Q220X
F	14	ΔF508/G542X
M	25	ΔF508/2183AA-G
M	34	ΔF508/R553X

Tissues were washed in PBS to remove blood and cut into pieces of ~3 mm^2. Then, tissue pieces were transferred into dissociation medium which was freshly mixed with DNaseI and Protease E and incubated overnight on a shaker at 4 ^oC, followed by 1 hour incubation on a shaker at 37 ^oC. To stop the dissociation reaction 5 mL HI-FBS was added and tubes are inverted 12-15 times. The solution was decanted and filtered through 100 µm cell strainer into a new 50 mL

tube. The remaining tissue was washed with 10 mL culture medium and filtered through same cell strainer. The cell suspension in the new tube was centrifuged for 10 min at 1300 rpm at 4 ^{o}C. In case of remaining blood cells supernatant was discarded carefully and cell pellet was dissolved in 5 mL RBC Lyse Buffer (eBioscience). After careful aspiration of the supernatant, cell pellet is dissolved in 5 mL culture medium and seeded on a 10 cm petri dish. Cells are incubated for 2 hours at 37 ^{o}C to separate the fibroblasts from epithelial cells. In this step, fibroblasts attached to the dish and epithelial cells remained in the supernatant. Medium containing the epithelial cells was carefully transferred to a 15 mL tube and the cells were counted on Neubauer chamber by 1:10 dilution with Trypan blue. The cell suspension was centrifuged for 10 min at 1300 rpm at 4 ^{o}C and afterwards the cell pellet was resuspended in culture medium with the concentration of 1,2 million cells/mL. Then, the cells were seeded on transwell membranes (500µL cell suspension/membrane), 1 mL medium was added basolaterally in the plate. After 2 days air-liquid interface was started by carefully aspirating the medium from the apical side of the culture while cells were attached to the coated membrane. At the same time on the basolateral side medium was replaced with fresh medium. Next day, 25 µL of Supplement Mix was added to the medium at the basolateral side. 2 days after seeding, air-liquid interface was started by aspirating the medium on the membrane carefully while cells were attached to the coated membrane, and replacing the medium at the basolateral side with fresh medium. Then, next day after medium change, 25 µL supplement mix was added to the medium and every second day medium at the basolateral side was replaced until 14 days of culturing when the cells were fully differentiated.

2.6 Immunofluorescence microscopy

The apical and basolateral side of the differentiated cell monolayers were washed with PBS. Then, the cells were fixed with ice-cold methanol for 10 min at -20 ^{o}C, and permeabilized with 0.2% (w/v) Triton X-100 for 8 min. After blocking

with 1% (w/v) BSA for 30 min, cells were incubated with polyclonal rabbit anti-βENaC (1:200) (Vermaelen and Pauwels, 2004) and monoclonal mouse anti-acetylated α-Tubulin (1:200, 322700, Life Technologies) or anti-ZO1 (zonula occludens protein 1, 1:200, 61-7300, Invitrogen) primary antibodies for 60 min. Cells were rinsed 3 times with PBS and then incubated with 1:200 dilution of respective Alexa Fluor labeled $F(ab')_2$ fragment (Invitrogen) together with 1:2000 dilution of Hoechst (1 mg/mL stock solution) to counterstain cell nuclei for 30 min. Cells were rinsed 3 times with PBS and mounted on coverslips with FluorSave medium (Merck Millipore). Images were obtained by using a confocal laser scanning microscope (Leica TCS SP8) with suitable settings for respective secondary antibody labels. Z-stack images were processed with the software Fiji (ImageJ).

2.7 Transepithelial Ussing chamber measurements

Ringer solution: 145 mM NaCl, 0.4 mM KH_2PO_4, 1.6 mM KH_2PO_4 x $3H_2O$, 5 mM Glucose, 1 mM $MgSO_4$ x $7H_2O$ and 1.3 mM Ca-Gluconat x $1H_2O$ are dissolved in H_2O and pH is adjusted to 7.4 with NaOH and HCl.

Ussing chamber is an electrophysiological method that allows studying ion flux across a tissue epithelium or a cell monolayer (Clarke, 2009). After culturing the primary airway epithelial cells for 2 weeks to form a proper polarized epithelium, experiments were performed in EasyMount Ussing chambers (Physiologic Instruments, San Diego, CA, USA). After mounting empty filters, chambers were filled with 5ml Ringer solution on both sides and the Ussing Chambers were adjusted for the electrode offset and bath solution resistance until a stability of both values was reached. For physiological conditions bath temperature was maintained at 37°C. The transepithelial voltage (V_{TE}) was clamped to 0 mV using a standard four Ag/AgCl electrode voltage clamp (VCC MC6; Physiologic Instruments) and the short circuit current (I_{sc}) was measured. Voltage pulses of 2 mV were applied every 60 s for 1 s for monitoring the transepithelial resistance

(*R*te) of cell monolayers. The I_{sc} was continuously recorded using LabChart7 for Windows (ADInstruments, Oxfordshire, UK) (Salomon et al., 2016). Then the filters with monolayer of cells are mounted in the Ussing chamber and the chambers are filled with Ringer solution. After an equilibration period of 20 min in the Ussing chamber, basal short circuit current was determined and amiloride (100 µM, luminal) was added to inhibit electrogenic ENaC-mediated Na^+ absorption for 5 min. Then, IBMX and forskolin (100 µM and 1 µM, luminal and basolateral, for 20 min) and UTP (100 µM, luminal, for 15 min) were added sequentially to induce cAMP-mediated and Ca^{2+}-activated Cl^- secretion. Then, bumetanide (100 µM, basolateral, for 20 min), an inhibitor of the basolateral Na^+-K^+-$2Cl^-$ cotransporter, was added as the last compound.

2.8 ASL height measurements with fluorescent microscopy

Primary tracheal epithelial cultures were washed with PBS, and 20 µl of PBS containing 2 mg/ml Rhodamine dextran (10 kDa; Molecular Probes) was added to the lumen to visualize the airway surface liquid layer. Images of the Rhodamine-labeled airway surface liquid were acquired by confocal microscopy (Leica TCS SP8). The height of the airway surface liquid was measured by averaging the heights obtained from xz scans of 15 predetermined positions on the culture as previously described. (Seys et al., 2015) Airway surface liquid height was measured 3 min following the addition of the Rhodamine dextran and at designated time points over a period of 24 h in primary tracheal epithelial cultures from βENaC-Tg mice and WT littermates.

2.9 ASL height measurements with reflection microscopy

All imaging was conducted at 37°C and 5% CO_2 using a microscope incubator (EMBL, Heidelberg) enclosing the entire stage, nosepiece and upper part of the microscope. To ensure humidity under typical cell culture conditions (>98%), a

custom-made humidity chamber for the transwells was developed. In brief, an anodized aluminum holder in the size of a typical 96-well plate with a water reservoir and a central position to accommodate a 35mm sample-holder was manufactured and closed by a humidity tight lid. Exchange of gas and brightfield microscopy was enabled by a 50mm lumox dish (using the bottom part) with its gas permeable clear membrane (Sarstedt, Nümbrecht, Germany) placed in the center of the lid.

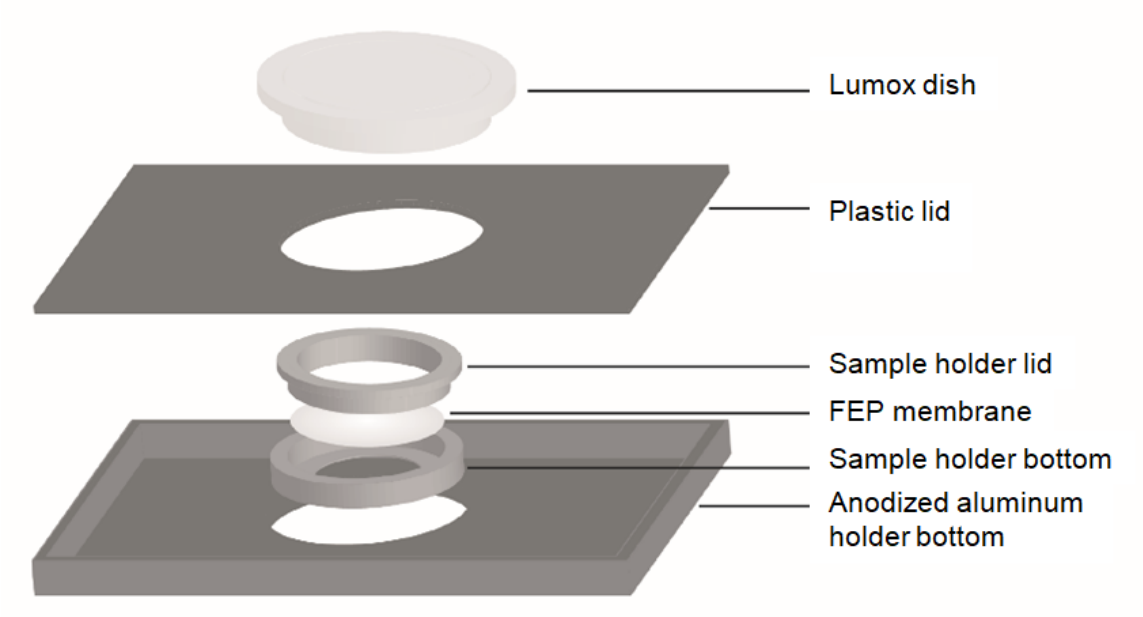

Figure 6. Schematic representation of reflection microscopy set up. Transwell sample was placed on FEP membrane and sample holder was closed. Sample holder was placed in Aluminum holder with water reservoir in the bottom and closed with the plastic lid and Lumox dish.

On a Leica PlanApo CS20 20X / NA 0.75 multi-immersion objective, Immersol W 2010 (Zeiss) was used as immersion liquid and correction collar was set to 0µm coverslip thickness and manually optimized to minimize distortion of the signal of the lower reflection signal of the transwell membrane. As a substitute for a glass coverslip, an FEP (fluorinated ethylene propylene) membrane was used. The FEP membrane was stamped out to the same size as the glass coverslip (24 mm diameter). FEP membrane has the practically the same refractive index as water

and therefore no optical correction was necessary compared to a glass coverslips and the correction collar could be set to or close to 0µm glass coverslip thickness. On the FEP membrane 2 pieces of parafilm (2mm width, 10mm length) were placed on two opposite sides to leave sufficient space for growth medium under the membrane.

The reflection signal was recorded in XZ-scans using a 488 nm laser and a PMT as detector. For long term experiments, sample was kept in the microscope for the time period and images were taken every 15 minutes at 15 defined positions.

2.10 Pharmacological treatment

To measure the effect of pharmacological modulations on human nasal epithelial cells, cells were placed in the microscope and first, steady state ASL height was measured. Then, Benzamil, IBMX/Forskolin and Bumetanide (final concentrations in growth medium: 100µM, 100µM/1µM, 100µM) were added subsequently to the cells basolateral. In this process ASL height was measured continuously by taking images at every 15 minutes and for 45 minutes after each chemical.

2.11 Statistical analysis

Data were analyzed with GraphPad Prism software (GraphPad Software, La Jolla, CA) and presented as mean ± SEM. Statistical analysis were performed using paired and unpaired, two tailed Student's t-test as appropriate, and $p<0.05$ was accepted as statistically significant. All data were obtained from at least three independent experiments.

3. Results

3.1 Establishing the murine primary airway epithelial cell culture and its basic properties

In order to study ASL homeostasis in chronic lung diseases, murine primary airway epithelial cell isolation and culturing was optimized. The cells were isolated both from βENaC-Tg mice and their littermate wild-type controls. Cells were grown on air-liquid interface to mimic the physiological conditions. In order to characterize the cell types in the primary airway epithelial cultures, immunofluorescence staining of the cells was performed. Cells were stained for cilia marker (acetylated α-tubulin antibody) (green) and quantifications showed that monolayer of the cells from both βENaC-Tg and wild-type mice were about 50% ciliated similar to the native trachea tissue. In parallel, cells were stained with βENaC antibody (red) to confirm the overexpression in the differentiated cells. With βENaC staining both endogenous and overexpressed protein can be observed. As expected overexpression of βENaC was observed in the stained tissue in the non-ciliated cells, since the overexpression is under control of the CCSP (club cell secretory protein) promoter (Mall et al., 2004). In order to do the quantifications cells were counterstained for nuclei which is shown as blue in the images. As can be seen in the graph about 50% of the βENaC expressing cells had overexpression of the protein in βENaC-Tg mice derived cultures. Moreover, to identify the tight junctions between the monolayer of airway epithelial cells, tight junction protein ZO-1 (zonula occludens-1) staining was performed. Immunofluorescence staining confirms that cells were forming tight junctions in the monolayer cultures as in native epithelia.

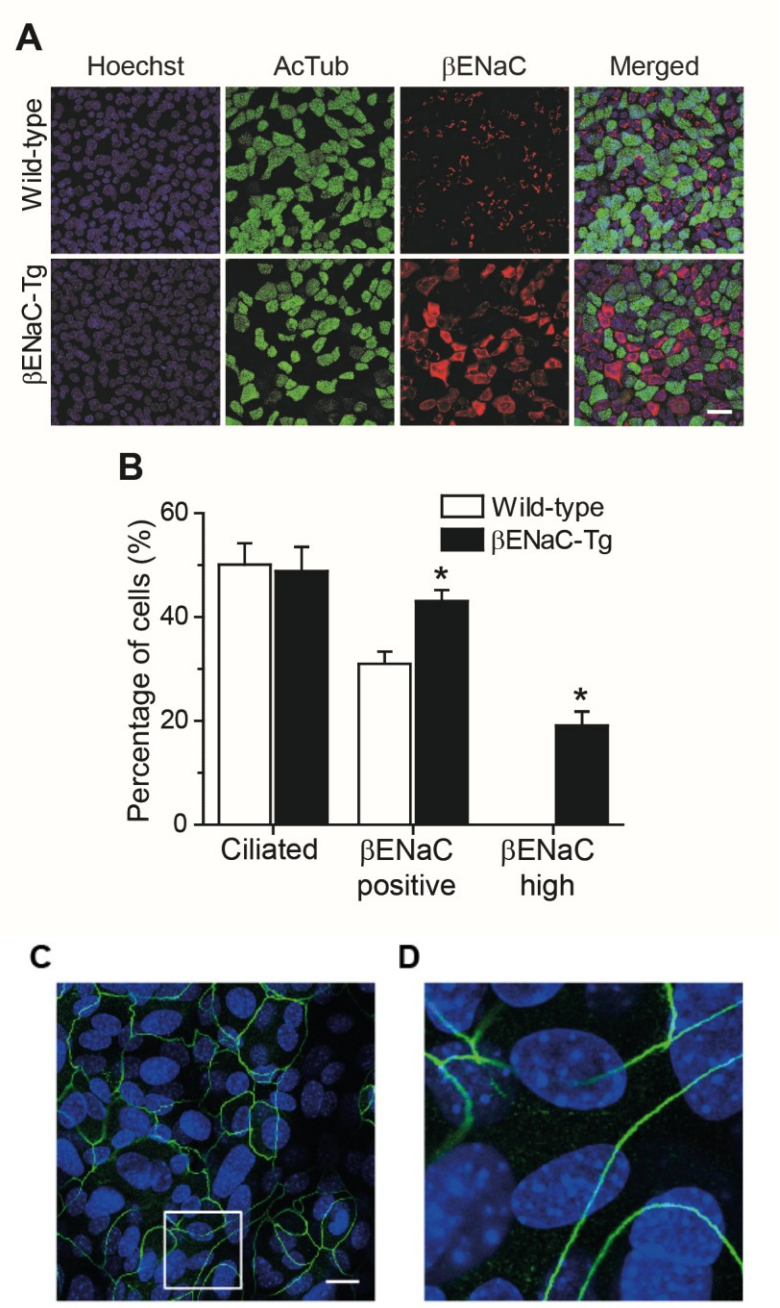

Figure 7. Immunofluorescence staining of murine primary airway epithelial cultures. A) Immunofluorescence staining of wild-type and βENaC-transgenic cells using antibodies against acetylated α -tubulin (green) and βENaC (red) to depict ciliated cells and to confirm overexpression. Cells were counterstained with Hoechst (blue) for nuclei. B) Numeric densities of ciliated, βENaC expressing non-ciliated cells and βENaC overexpressing cells (βENaC high) in primary airway epithelial cell cultures from βENaC-transgenic mice and wild-type littermate controls. C) Immunofluorescence staining of

wild-type cells with antibody against ZO-1 (green). Cells were counterstained with Hoechst (blue) for nuclei. D) Close up image of marked region in C. (n=5, * $p<0.005$) (error bars represent SEM) (scale bars: 20µm)

Ion transport properties, as a prerequisite of liquid handling, were studied by Ussing Chamber measurements. After mounting, the membrane with airway epithelial cells divides the chamber in two halves, separating the luminal from the basolateral bath. For measurement, voltage was applied and short circuit current was recorded. During the experiments, after an equilibration period of 20 min in the Ussing chamber, basal short circuit current was determined and amiloride (100 µM, luminal) was added to inhibit electrogenic ENaC-mediated Na^+ absorption for 5 min. Then, IBMX and forskolin (100 µM and 1 µM, luminal and basolateral, for 20 min) and UTP (100 µM, luminal, for 15 min) were added sequentially to induce cAMP-mediated and Ca^{2+}-activated Cl^- secretion. Then, bumetanide (100 µM, basolateral, for 20 min), an inhibitor of the basolateral Na^+-K^+-$2Cl^-$ cotransporter, was added as the last compound. Due to the overexpression of ENaC basal current in βENaC-Tg cultures was higher than wild-type cultures and similarly blocking the ENaC with Amiloride results in higher difference in βENaC-Tg cultures. With other compounds activation or inhibition of ion channels did not show difference between βENaC-Tg and wild-type cultures (Figure 8). These data showed that primary airway epithelial cultures were mimicking the native tissue and produce functional ion channels. (Mall et al., 2004)

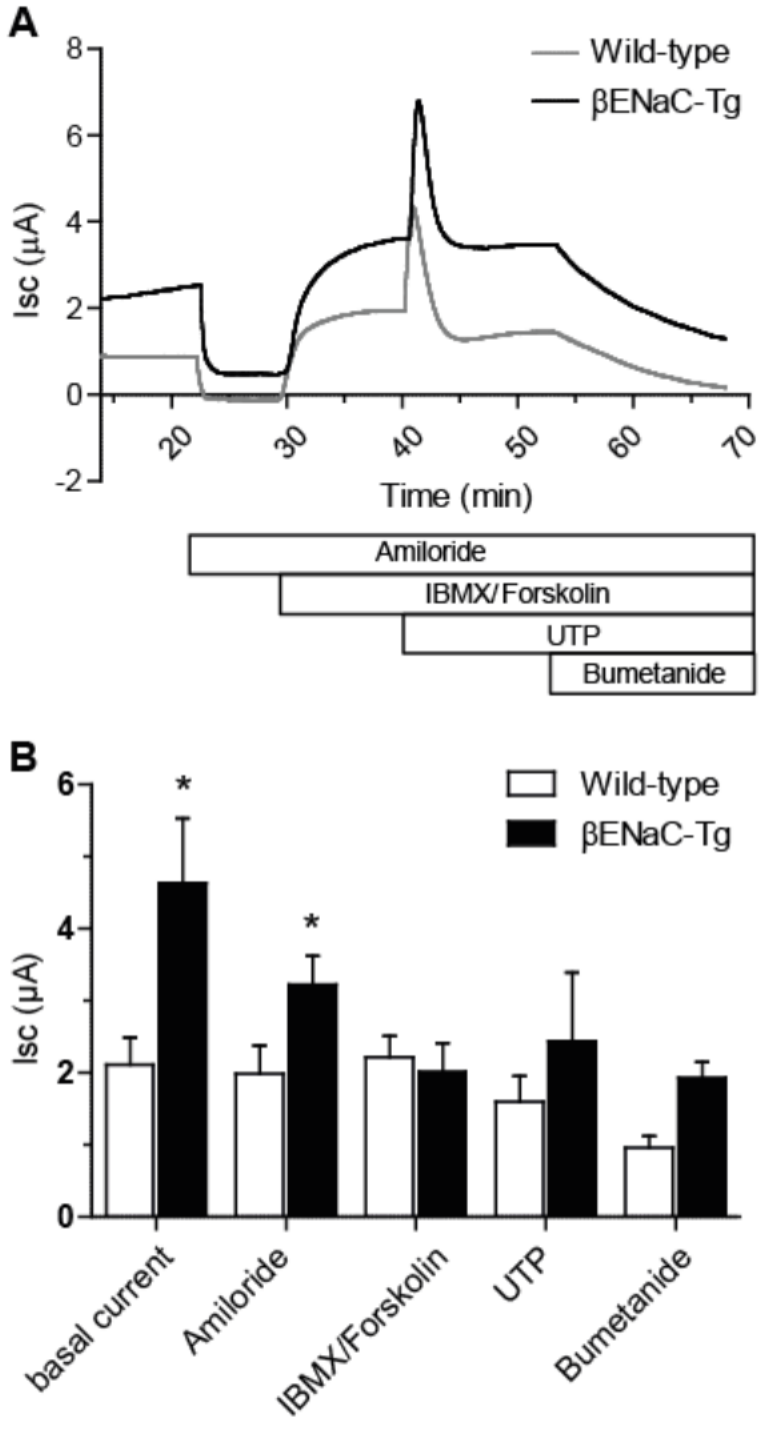

Figure 8. Ussing chamber measurements of βENaC-Tg and wild-type murine primary airway epithelial cultures. A) Representative recordings of βENaC-Tg and wild type cultures. B) Summary of basal current and differences after addition of ion channel modulators. (n=4, * $p<0.05$)

3.2 Design of a novel ASL height measurement method using confocal reflection microscopy (CRM)

ASL dehydration is one of the most important hallmarks of CF disease. Therefore it has been studied exclusively in the recent years. Measuring ASL height by confocal microscopy gives us the opportunity to study differences in airway ion transport and ASL homeostasis under physiological thin film conditions. Commonly used ASL height measurement method has been very helpful to study the pathogenesis of CF disease in the past decade however the method has some drawbacks. In this method it is necessary to add a fluorescent dye to the apical surface of air-liquid interface grown airway cells. Addition of this volume causes volume challenge to the cells and interferes with the physiological conditions. We optimized a method by using reflection confocal microscopy to measure the ASL height without volume addition and under more physiological conditions. Reflection microscopy measures the reflected light at each interface due to refractive index differences between layers. For the optimization experiments primary tracheal epithelial cells from mice which were grown on membrane supports was used. To mimic the physiological conditions and to promote full differentiation cells were grown on air-liquid interface as explained in 'Materials and Methods' part. When the sample was placed on the inverted commercially available confocal microscope (Leica SP8) laser light was sent and reflected light was recorded at three interfaces which are the interfaces between (1) the medium and the permeable membrane, (2) the permeable membrane and the cells, (3) ASL layer and the air (Figure 9). To keep the cells under most physiological conditions during measurements, we used a temperature, CO_2 and humidity controlled chamber and we measured these parameters over a time period. As can be seen on the graph (Figure 10) temperature was stable at 37^{o}C and humidity was stable at ~97%. The sudden decreases in the humidity measurement were caused by opening the lid to and closing again. After closing the lid the humidity was increasing back to ~97% which was important to avoid evaporation of ASL on the monolayer of cells.

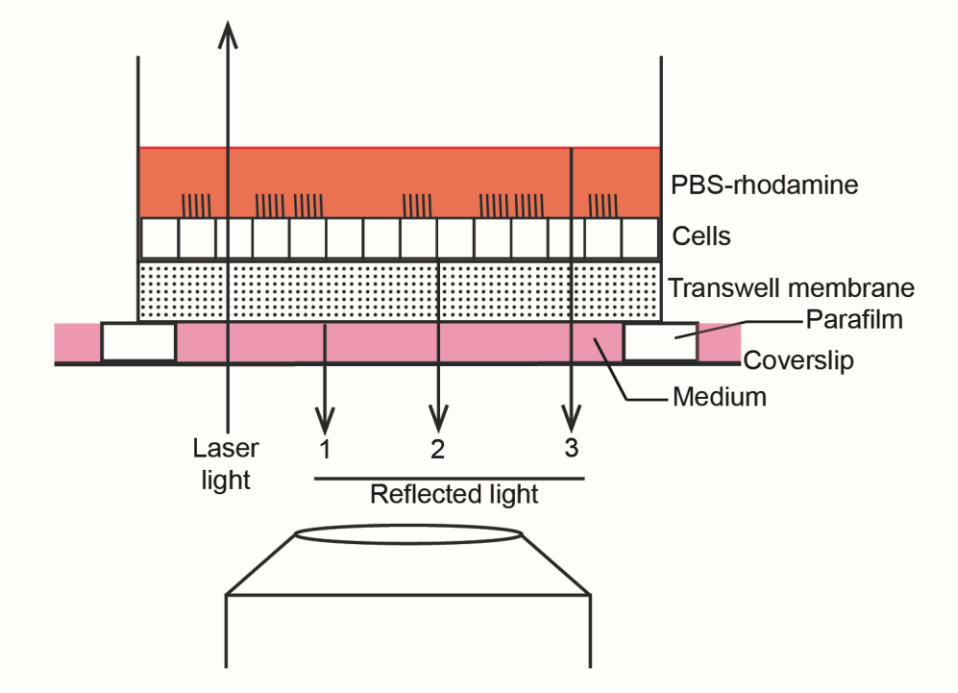

Figure 9. Principle of ASL height measurements using an inverted microscope. Cells were placed on the coverslip on a spacer of parafilm to ensure cells are supplied with medium. Set up was depicted after the rhodamine-PBS addition. Arrows represent the laser light sent by the microscope and reflected light (1, 2 and 3) at each interface due to refractive index differences.

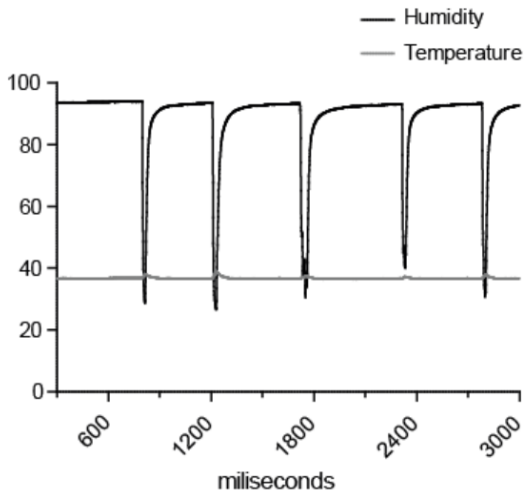

Figure 10. Humidity and temperature measurements of the microscope chamber to provide physiological conditions for the airway epithelial cells during ASL height measurements.

Next step was to compare our novel method to the commonly used ASL height measurement method. Comparison of fluorescent and reflection microscopy images of the same position on wild type cells immediately after fluorescent dye addition shown on the line profile graph that both measurements showed similar results (Figure 11). There are three clear peaks on the reflection microscopy line profile, first of which corresponds to lower surface of the permeable membrane due to refractive index differences between membrane and the cell growth medium underneath. Medium was added into the chamber to maintain their culture conditions. The second peak is observed where the permeable membrane finishes and the cells are grown. The third peak is where the liquid layer on the cells finishes, which corresponds to the surface of ASL. For calculations, the distance between the peaks number 2 and 3 is measured, which gives the height of cells and ASL layer. In comparison, for the fluorescent image 60% of the peak is taken, which would be the distance between the points a and b for the cell height and the distance between x and y for the fluorescently labeled ASL on the graph.

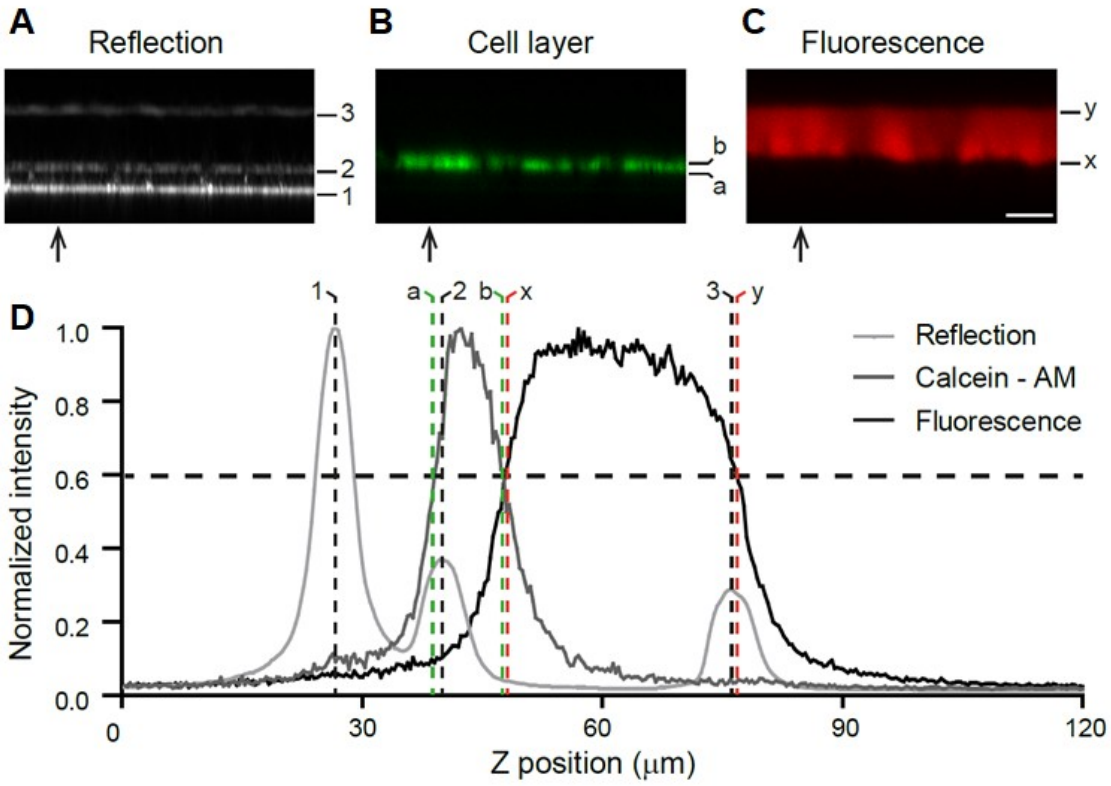

Figure 11. Description of reflection and fluorescent ASL height measurement calculations. A) Representative image of reflection signals recorded with 488 laser light by XZ-scanning. B) Fluorescence image of the cells stained with Calcein-AM. C) Fluorescence image of ASL stained with Rhosamine-dextran recorded in parallel. D) Line profiles recorded at the indicated positions (arrow) in A, Band C. For the reflection signal distance between peaks 2 and 3 corresponded to cells and ASL. For the fluorescence signals distance between a and b was accepted as cell height which was ~60% of the fluorescence and x and y is accepted as ASL height which was ~60% of the fluorescence.

3.3 Confocal reflection microscopy correlates with confocal fluorescence ASL height measurements

Like proximal airways in vivo, primary airway epithelial cultures are predominantly Na^+/volume absorbing over time after volume addition until steady state conditions are reached, which corresponds to the stretched cilia height. To standardize the starting ASL height, cultures were washed with PBS 48h prior to the experiment to remove excess mucus layer. To observe the liquid layer with confocal fluorescent microscope, rhodamine - dextran (10 kDa) fluorescent dye was added on the cells, which is freely permeable in the ASL but impermeable across the epithelium. The measurements were started immediately after addition of the dye and repeated after 2, 6 and 24 hours, to record the liquid absorption rates of airway epithelial cultures. Imaging was performed on an inverted confocal microscope (Leica SP8) using a 20x objective with water immersion (similar to (Tarran et al., 2005)). XZ scans were recorded for each time point, images were taken at 15 positions per well and time point and average values were calculated.

First measurements were performed in wild-type mouse derived pAECs by using the classical ASL height measurement method. In these experiments in addition to fluorescent measurements an additional channel was added for reflection and all the measurements were performed in parallel. The results were compared and showed that both fluorescent and reflection microscopy images were giving similar results (Firgure 12). To separate the cell layer from ASL layer and to calculate the ASL height in reflection microscopy images, cells were stained basolaterally with a fluorescent dye (Calcein AM) prior to microscopy without perturbation of ASL layer. With fluorescent cell staining more robust calculation of the ASL height was aimed.

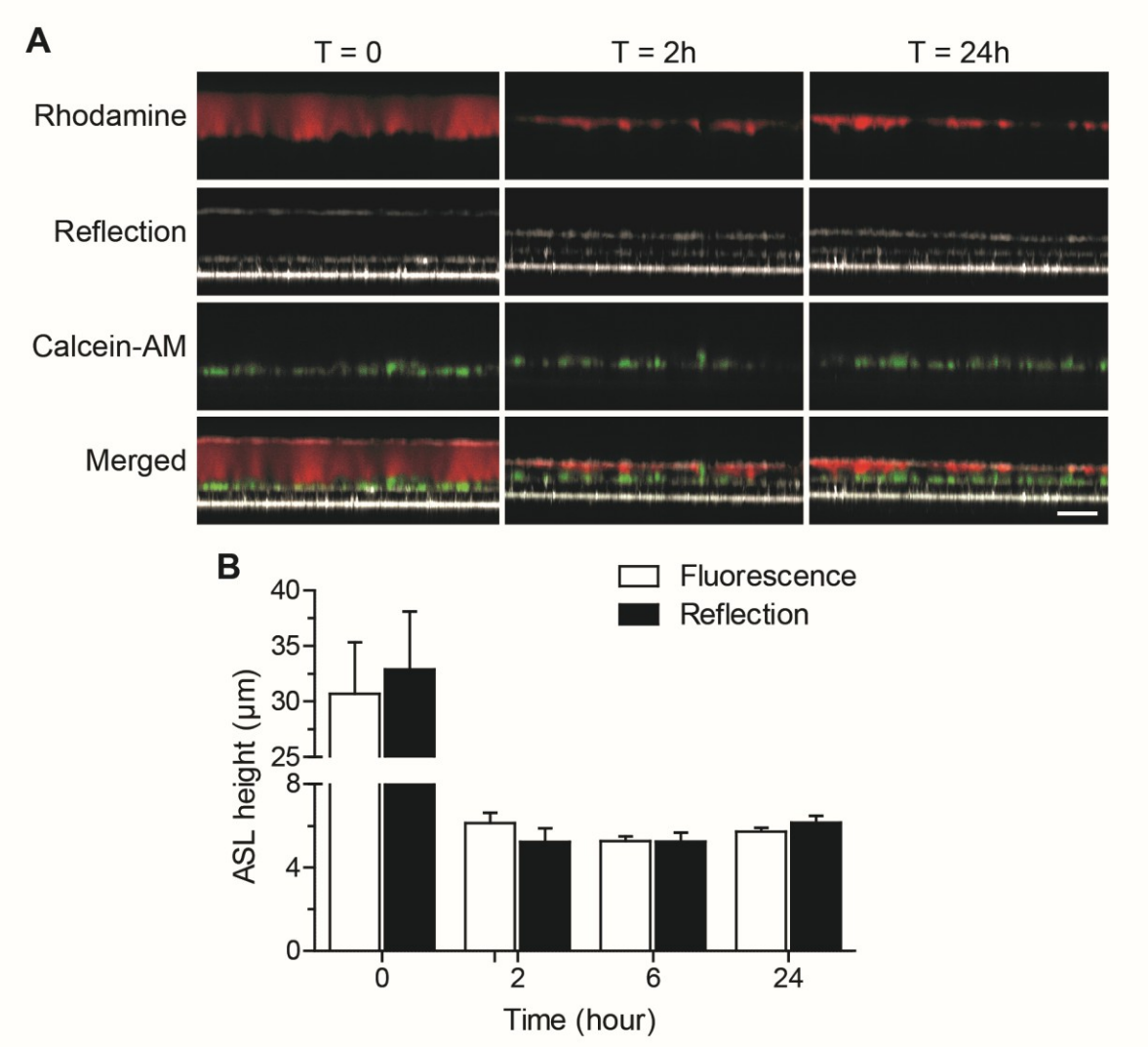

Figure 12. Comparison of reflection and fluorescence confocal microscopy after volume addition. A) Representative images taken for different channels at defined time points after apical fluorescent dye addition to the wild-type mice derived primary airway epithelial cells following basolateral calcein-AM staining. (scale bars: 25µm) B) Summary of fluorescent and reflection microscopy images at defined time points showed that height of the ASL was measured similarly for both techniques at all time points after volume addition. (n=4) (error bars represent SEM)

3.4 Confocal reflection microscopy detects lower ASL height in βENAC transgenic mouse model

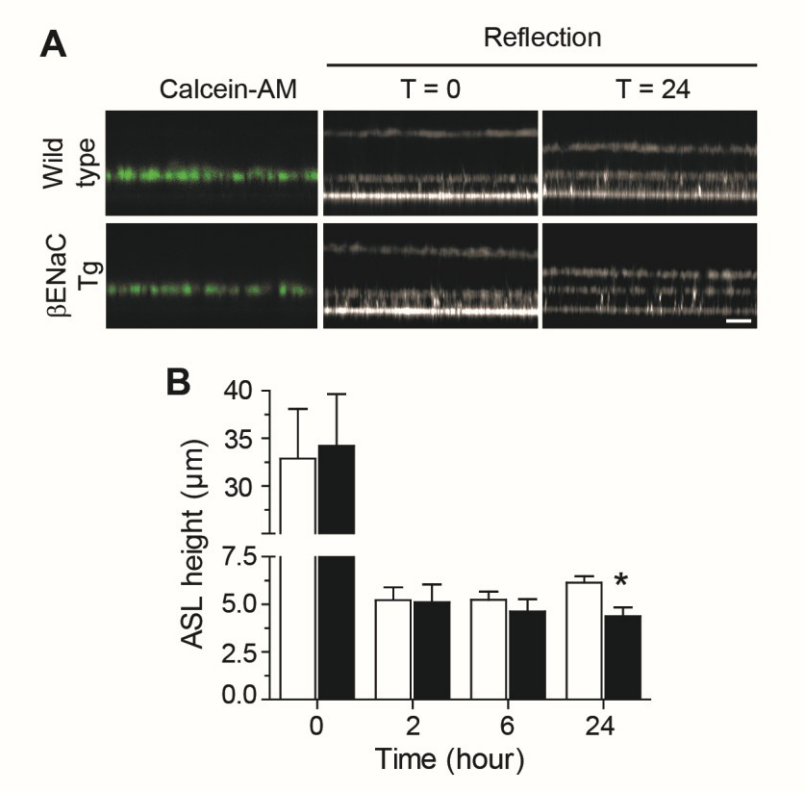

Figure 13. ASL height measurements of wild-type and βENaC-Tg airway epithelial cells with reflection confocal microscopy after volume addition. A) Representative images taken for reflection and Calcein-AM staining at defined time points after apical fluorescent dye addition to wild-type and βENaC-Tg cells. (scale bar: 12μm) B) Summary of reflection measurements at defined time points after volume addition showed that height of ASL was similar in wild-type and βENaC-Tg cells in early time points. However, 24 hours after volume addition height of the ASL layer of βENaC-Tg cells remained lower than wild-type cells due to increased Na^+ absorption. (n=4, * $p<0.01$) (error bars represent SEM)

Experiments on ASL height measurements with reflection microscopy after volume addition showed that liquid absorption is similar in wild-type and βENaC-Tg cells at early time points; however, 24 hours after volume addition height of

the ASL layer of transgenic cells remained lower than the height in wild-type cells, which supported the previously published data (Mall et al., 2010). Overexpression of βENaC, and thus increased levels of Na^+ absorption resulted in a decreased height (~4,5 μm) of the ASL layer when compared to wild-type littermate controls (~7 μm). These experiments confirmed the suitability of reflection confocal microscopy for ASL height measurements.

In order to keep the cells in most physiological conditions during the measurements, in the microscope, cells were kept at 37 ^oC with 5% CO_2 and 98% humidity in dark using a custom-made humidifying chamber and a standard environmental control box for temperature and CO_2.

3.5 Confocal reflection microscopy is able to measure ASL height in physiological conditions

To compare the steady state ASL height of βENaC-Tg and wild-type mice derived pAECs long term measurements with reflection microscopy were performed without any volume addition. For long term measurements microscope was set to take images at defined positions every 15 minutes for 5 hours. After images were taken with reflection confocal microscopy, ImageJ and Matlab programs with custom-written scripts were used for the analysis and calculation of ASL height from different positions and time points. The measurements revealed that when the ASL height was measured without any fluorescent dye addition to the apical surface, under unperturbed conditions, βENaC-Tg cells had ~5μm ASL height whereas wild-type cells had ~8.5 μm. The steady state long term measurements showed that with reflection confocal microscopy ASL height can be measured under more physiological conditions without any volume challenge for the air-liquid interface grown cells.

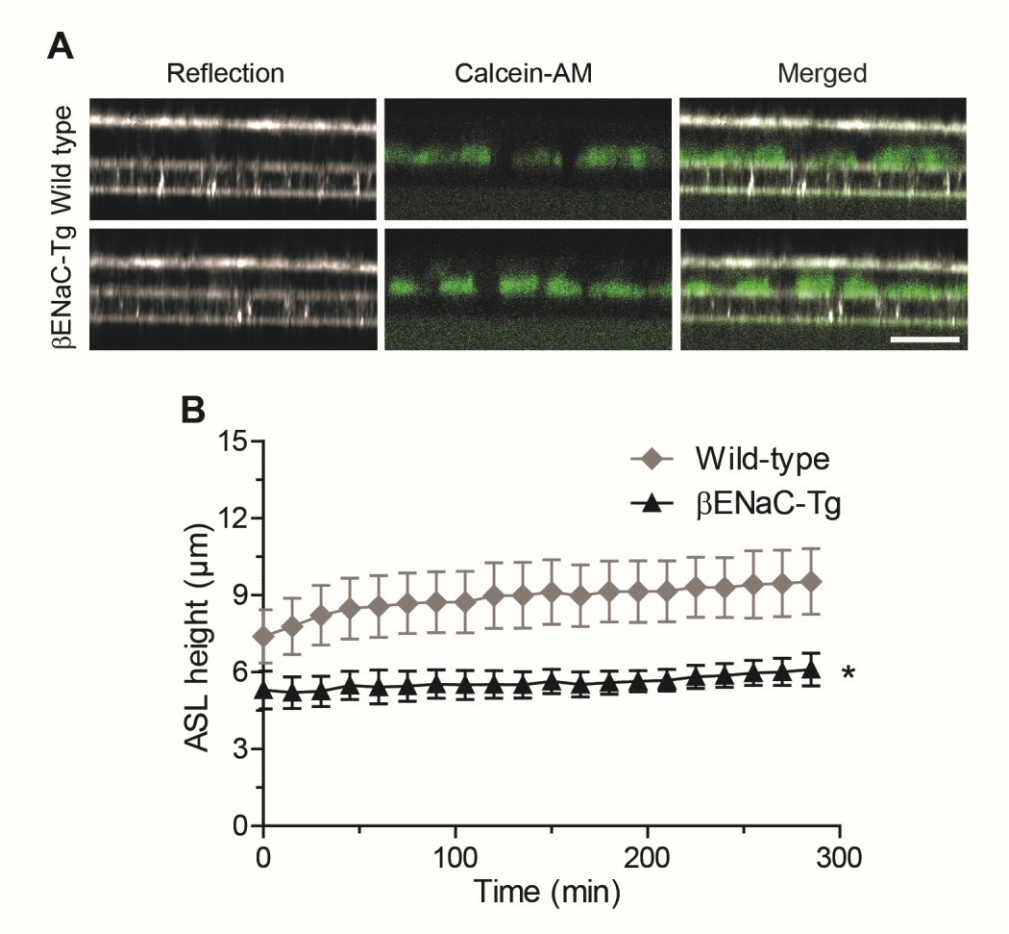

Figure 14. ASL height measurements of wild-type and βENaC-Tg airway epithelial cells with reflection confocal microscopy under physiological conditions. A) Representative images taken during steady state measurements for wild-type and βENaC-Tg mice derived primary airway epithelial cells under temperature, humidity and CO_2 controlled conditions. (scale bar: 30 μm) B) Summary of steady state measurements under physiological conditions for 5 hours showed that ASL height of βENaC-Tg cells was lower than wild-type cells and reflection confocal microscopy is a novel method to measure ASL height in physiological conditions. (n=5, * $p<0.01$) (error bars represent SEM)

3.6 Confocal reflection microscopy detects lower ASL height in CF patients

After optimizing and confirming the suitability of the reflection microscopy in murine tracheal epithelial cells, experiments with primary human nasal epithelial cells from CF and non-CF patients were performed. It was clearly seen in steady state conditions that CF cells had lower ASL height than non-CF cells due to CFTR channel deficiency. This ASL height dehydration could be recovered to non-CF levels when the cells were grown in 27^{o}C for 48h before the measurements. Temperature correction for CF cells helped them to stabilize CFTR protein, therefore resulting in Cl^- secretion and accordingly normal ASL height levels. Similarly, when the CF cells were treated chronically with VX-809 and VX-770 ASL height was recovered close to non-CF ASL height levels. Treatment with VX-809 and VX-770 together resulted in correction and potentiation of CFTR channel in ΔF508 homozygous cells.

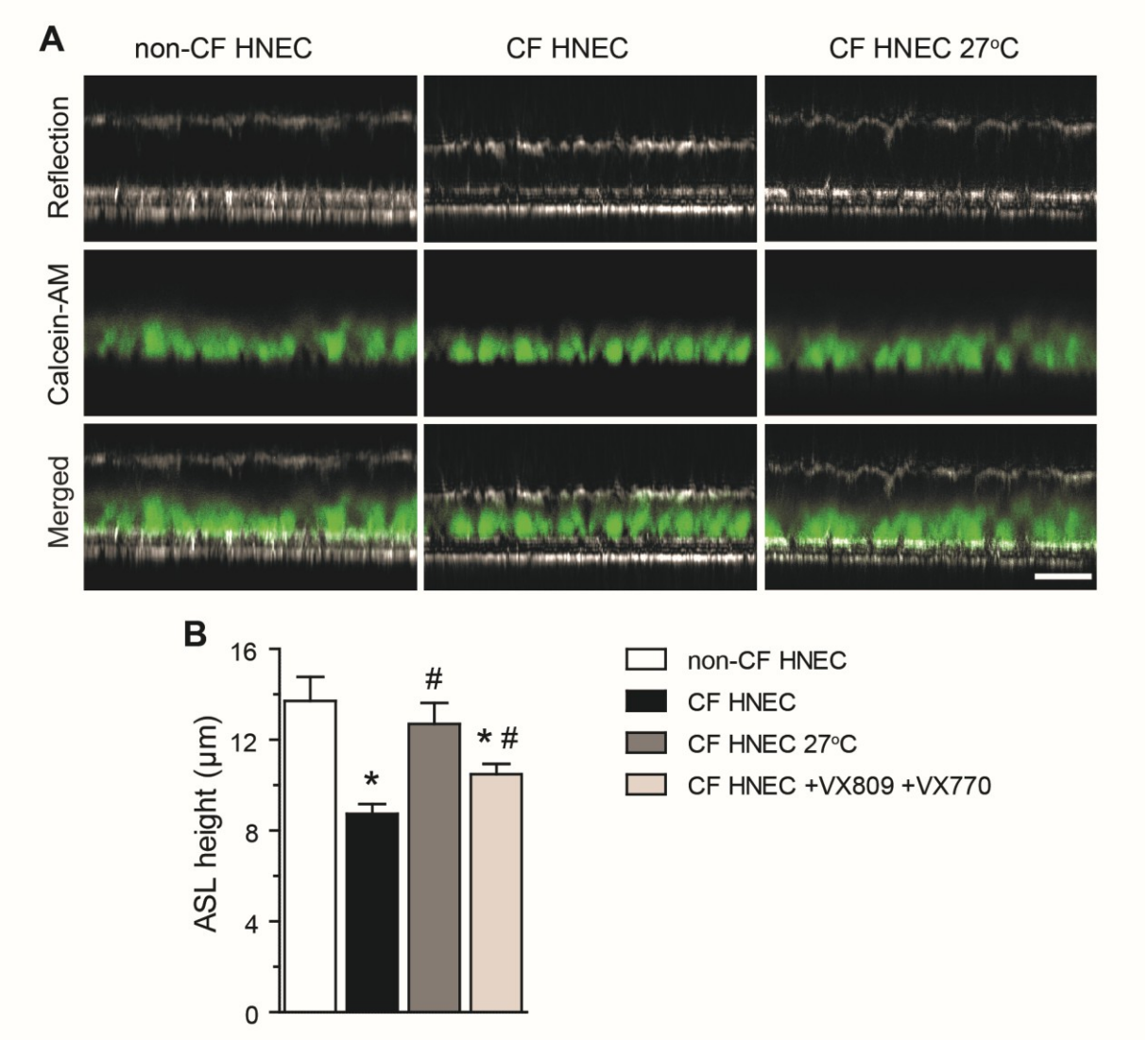

Figure 15. ASL height measurements of primary human nasal epithelial cells derived from CF and non-CF patients. A) Representative images taken for non-CF, CF and temperature corrected CF cells under physiological conditions. B) Reflection confocal microscopy showed that ASL height of CF HNECs was lower than non-CF patient derived cells under steady state conditions. However with temperature correction of ΔF508 homozygous patient derived cells ASL height was recovered to non-CF levels. Chronic treatment of CF cells with VX-809 and VX-770 increased the ASL height compared to non-treated CF cells. (n=5-7, * $p<0.01$) (error bars represent SEM)

3.7 Confocal reflection microscopy can detect small changes in ASL height

Moreover, ASL height of non-CF and CF cells were measured with reflection confocal microscopy under physiological conditions undergoing pharmacological modulations. After measuring the steady state ASL height of the cells, benzamil was added to inhibit ENaC mediated Na^+ absorption. Then, 3-isobutyl-1-methylxanthine (IBMX) and forskolin were added to induce cAMP-mediated Cl^- secretion. Then, bumetanide was added as an inhibitor of the basolateral Na^+-K^+-$2Cl^-$ cotransporter. Addition of benzamil resulted in increase of ASL height in non-CF epithelial cells due to lack of liquid absorption and on the other hand continuous secretion, however no further increase was observed after addition of IBMX/Forskolin. Then, with the addition of bumetanide, blocking the Na^+-K^+-$2Cl^-$ cotransporter resulted in decrease of the ASL height in non-CF cells back to steady state levels. All the chemicals were added basolaterally when the cells were kept in the microscope and ASL height was measured at the same positions after each chemical. These experiments revealed that reflection confocal microscopy was capable of measuring even the small ASL height changes. Same chemicals were added to the CF patient derived epithelial cells however no further change in ASL height was observed due to lack of functional CFTR channel and as a result lack of proper secretion.

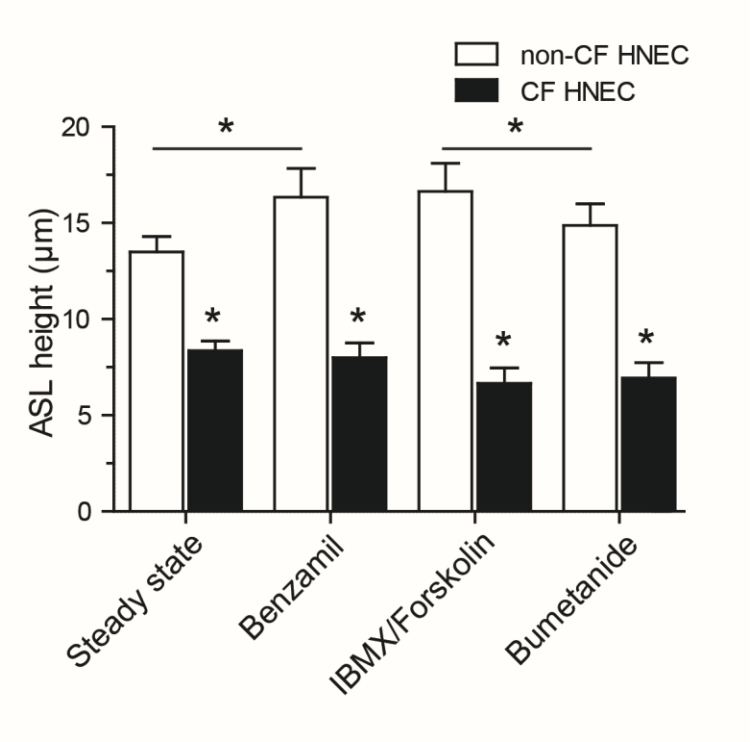

Figure 16. Measurements of ASL height changes in response to ion channel modulators in human nasal epithelial cells derived from CF and non-CF patients. Continuous ASL height measurements with subsequential pharmacological modulations revealed that in non-CF cells addition of Benzamil resulted in increase of ASL height due to blocked Na^+ absorption. Blocking NKCC cotransporter with Bumetanide resulted in decrease of ASL height back to steady state levels. ASL height didn't change for CF cells due to lack of functional CFTR channel. (n=4-6, * $p<0.01$) (error bars represent SEM)

4. Discussion

Cystic fibrosis affects many epithelial organs and it is known that ion transport properties differ in epithelia from different organs. However, lung has the highest contribution to morbidity and mortality of CF patients due to ASL dysregulation resulting in mucus obstruction, airway inflammation, bacterial infection and finally leading to respiratory failure. Therefore, it is critical to test potential drugs in *in vitro* systems derived from various organs especially the airways in order to reliably predict treatment success (Haggie et al., 2017; Pedemonte et al., 2010; Rowe et al., 2010). In recent years, two CFTR modulators, ivacaftor (VX-770, Kalydeco) and lumacaftor/ivacaftor (VX-770/VX-809, Orkambi), have been approved by the U.S. Food and Drug Administration to treat CF patients with certain CFTR mutations. The approval has been expanded based on *in vitro* testing in FRT (Fischer rat thyroid) cells expressing recombinant CFTR with the examined mutations (Van Goor et al., 2014). Recent reports could demonstrate that prediction based on experiments in heterologous cell systems may sometimes be inaccurate (Haggie et al., 2017; Pedemonte et al., 2010; Rowe et al., 2010). Therefore, there is a need to refine these predictions using homologous cell model systems. Further, it is important that these *in vitro* systems mimic the *in vivo* situation as closely as possible.

In order to model the airway epithelium, we used air-liquid interface (ALI) culture conditions to promote full differentiation of cultured mouse tracheal epithelial and human nasal epithelial cells to ciliated and non-ciliated airway cells forming a tight monolayer maintaining an ASL. To preclinically test drugs for their potential to improve ASL hydration and thus mucociliary clearance (Tarran et al., 2006a), ASL height can be directly measured using confocal microscopy (Matsui et al., 1998a; Matsui et al., 1998b).

4.1 Reflection confocal microscopy is a novel method to measure ASL height

Conventional confocal fluorescence microscopy is performed with addition of a volume of liquid different to what is normally experienced by the airways under steady state conditions. Even though the current protocol to measure ASL height has been very helpful to study ASL regulation, due to the volume addition on the apical surface of the cell monolayers it is not performed under physiological conditions. Therefore, in this study reflection confocal microscopy method was developed to avoid volume addition on the cells and was shown to be comparable to the gold standard of ASL height measurements adding important additional parameters that can only be determined in the absence of volume addition as base line steady state and effects of acute and chronic modulation of ASL regulation on ASL height.

The most important advantage of reflection confocal microscopy ASL height measurement is that, it is not necessary to label the ASL fluorescently. However, due to little refractive index differences between the cells and ASL layer it is not always possible to see the signal between these two layers with reflection microscopy. Therefore, it is necessary to label the cells fluorescently. As explained in the methods part this labeling was performed with Calcein-AM dye basolaterally without interfering with the ASL layer. This provided us robustness for the measurements and calculations.

4.2 βENaC-Tg cells have lower ASL height than wild-type cells under steady state conditions

Direct comparison studies of ASL height measurements showed that reflection confocal microscopy measurements recapitulate the results from current standard protocol with fluorescent confocal microscopy. ASL height measurements with reflection microscopy of βENaC-Tg and wild-type mouse derived tracheal epithelial cells were also comparable to previous research performed by current standard fluorescent protocol. (Seys et al., 2015) These

findings confirm the suitability of confocal reflection microscopy to measure ASL height.

It can be speculated that, the applied perturbations, by apically adding extra liquid might affect volume regulation more prolonged than previously assumed. It also might take longer then assumed to re-balance the soluble endogenous regulators of ASL (Myerburg et al., 2006; Tarran et al., 2006b) and return to equilibrium. Further, mucus layer-cilia interactions have been reported to also affect airway surface volume regulation (Button et al., 2013), which might also be unsettled when using conventional confocal fluorescence microscopy compared to the presented confocal reflection microscopy. Steady state ASL height measurements of βENaC-Tg and wild-type mouse derived tracheal epithelial cells further confirmed the suitability of this novel method. Cells showed constant steady state ASL height after 30 min of equilibration time under physiological conditions, where they were kept in humidity, temperature and CO_2 controlled chamber.

4.3 Reflection confocal microscopy is suitable to measure ASL height in human pAECs

ASL volume is a complex system and challenging to study since numerous factors are contributing. So far, ASL height measurements presented a good approximation to predict effects of drugs on ASL volume regulation but a direct measurement has not been presented yet. Further experiments with patient derived airway epithelial cells showed that this method is not only applicable to mouse models but also to clinical study with human cells. Since, there is no perturbation of ASL layer is necessary, most physiological conditions are approached to preclinically test new compounds. These tests would mimic the *in vivo* situation best, as the drugs are easily added basolaterally similar to the natural route of delivery in the human body.

To study ion transport on the epithelia Ussing chamber and confocal reflection microscopy ASL height measurements have being used, however both these

methods involve dilution or even wash out of the surface regulators on the epithelia which interferes with the physiological conditions. With confocal reflection microscopy, compared to Ussing chamber and fluorescent confocal microscopy measurements, actual effects on ASL height can be detected. Due to dilution of regulators in these two methods activation or inhibition of channels can be measured but not the real effect on ASL. On the contrary in reflection measurements since the ASL layer is kept intact, real effect on ASL height is observed.

4.4 Small changes in ASL height can be detected by confocal reflection microscopy

Another advantage of reflection confocal microscopy would be the possibility of observing also acute responses of modulators in addition to the chronic exposure. In the presence of transepithelial ion transport modulation starting with benzamil lead to an increase in ASL height due to blockage of Na^+ and coupled volume absorption. However, subsequent stimulation of CFTR-mediated Cl^- secretion did not result in further increase of ASL height in nasal epithelial cells from non-CF subjects. Benzamil-induced apical membrane hyperpolarization and the reduced mucosal chloride concentration might have already stimulated fluid secretion (Boucher, 1994a, b). Subsequent inhibition of the Na^+-K^+-$2Cl^-$ co-transporter, resulting in a reduced driving force for Cl^- secretion lead to net fluid absorption. Since benzamil block of Na^+ absorption was still active (Hofmann et al., 1998), ASL height did not come back to base line levels. Acute pharmacological treatment of nasal epithelial cells from CF patients did not show any effect on ASL height. Acute Benzamil block was not enough to restore ASL volume although CF cultures are characterized by increased amiloride-sensitive Na^+ currents when measured under thick-film conditions (Boucher et al., 1986). At the same time CF cultures exhibited loss of constitutive chloride secretion in addition to loss of cAMP-stimulated CFTR currents (Bertrand et al., 2017) which might be necessary to induce net fluid secretion and restoration of ASL volume with acute block of ENaC mediated Na^+ absorption.

4.5 Chronic treatment of CF pAECs with VX-809 and VX-770 restrores ASL

CFTR correction in ALI cultures from CF patients by confocal reflection microscopy was also studied in this thesis. Indeed, restoration of CFTR function of the cells by temperature correction exerted a considerable effect on steady state ASL height from CF cultures. The effect of a pharmacological rescue of CFTR by combination of VX-770 and VX-809 (ivacaftor-lumacaftor) on ASL steady states was also tested. Van Goor and colleagues could show ΔF508-CFTR-mediated chloride transport reached levels equivalent to approximately 25% of that measured in non-CF human bronchial epithelial cells when VX-770 was acutely applied to cultured ΔF508-human bronchial epithelial cells pretreated with VX-809 for 48 h (Van Goor et al., 2011). However, two following studies showed a destabilizing effect of VX-770 on VX-809 when applied chronically (Cholon et al., 2014; Veit et al., 2014). Meanwhile first data on observational studies to determine actual patient benefit of the treatment showed partial rescue of *in vivo* CFTR function determined by intestinal current measurement and nasal potential difference in response to lumacaftor-ivacaftor treatment to levels of 18% and 10% of normal, respectively (Graeber et al., 2018). Here it was shown that chronic exposure of human nasal epithelial cells of CF patients (Table 2) to VX-809 and VX-770 restored ASL height to 80% of normal under base line steady state conditions indicating that the level of functional rescue achieved with lumacaftor-ivacaftor in the nasal cavity may improve long-term clinical outcomes of chronic rhinosinusitis in ΔF508 homozygous patients. Furthermore, comparison of ion transport properties of human nasal epithelial cells and human bronchial epithelial cells have been shown to be highly similar (Pranke et al., 2017) suggesting to maybe also predict clinical outcome of the lower respiratory tract based on results obtained with human nasal epithelial cell cultures.

4.6 Conclusions and future perspectives

This study showed for the first time that ASL height measurement by confocal reflection microscopy is a novel sensitive read out of ASL regulation under near-

physiological steady state conditions to study airway surface function in healthy and diseased airways and acute as well as chronic effects of new pharmacological compounds. In times of precision medicine in CF, ASL height measurement by confocal reflection microscopy will be a valuable tool to add to prediction of treatment responses and patient outcome. Possibility of long-term measurements of ASL height with reflection microscopy gives us the opportunity to study dynamics and kinetic of the ASL height regulation, which is important for chronic lung diseases. With the possibility of long-term continuous measurements, sequential addition of modulators is also possible with this method. Since airway epithelial cultures are routinely available and an important tool in respiratory research combined with reflection confocal microscopy ASL height measurements a new tool for personalized medicine would be available. Patient specific drugs can be easily tested, dosage can be adjusted and effects can be observed directly on ASL height of the patient materials. There are many different CFTR mutations causing CF in patients, which still need to be investigated for novel drugs and drug testing would be easily done with this novel method.

In summary, the results demonstrate that ASL height measurements with reflection confocal microscopy can be performed without perturbation of ASL layer and eliminates the step to wait for 24 hours to reach steady state conditions. Moreover, with this method it is possible to observe results of sequential drug addition and long term measurements. During measurements basolateral drug delivery as *in vivo* conditions makes this method closer to physiological conditions. Taken together, this study shows that reflection confocal microscopy is a novel method to study ASL dysregulation in chronic airway diseases, opens a new route for personalized medicine and provides a new tool for preclinical testing.

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6. Publications from PhD studies

6.1 Original articles

Seys LJ, Verhamme FM, Dupont LL, Desauter E, Duerr J, Seyhan Agircan A, Conickx G, Joos GF, Brusselle GG, Mall MA (2015) Airway Surface Dehydration Aggravates Cigarette Smoke-Induced Hallmarks of COPD in Mice, PLoS One. 2015 Jun 12;10 (6):e0129897

Seyhan Agircan A, Lampe M, Scheuermann H, Albrecht T, Graeber SY, Pepperkok R, Duerr J, Mall MA. Confocal reflection microscopy as novel method to study airway surface liquid dysregulation in cystic fibrosis under physiological conditions (*manuscript in preparation*)

Duerr J, Leitz DHW, Szczygiel M, Dvornikov D, Fraumann SG, Kreutz C, Seyhan Agircan A, Konietzke P, Engelmann TA, Hegermann J, Kawabe H, Knudsen L, Ochs M, Rotin D, Muley T, Kreuter M, Herth FJF, Wielpütz MO, Beers MF, Klingmüller U, Mall MA. Conditional deletion of Nedd4-2 in lung epithelial cells causes progressive pulmonary fibrosis in adult mice (*under revision*)

6.2 Peer-reviewed abstracts:

A Seyhan Agircan, M Lampe, J Duerr, R Pepperkok, MA Mall (2015) Development of Confocal Reflection Microscopy for the Study of Airway Surface Liquid Dysregulation in Cystic Fibrosis. Pneumologie 2015; 69 - A15 DOI: 10.1055/s-0035-1556607

Seyhan Agircan A., Lampe M., Duerr J., Pepperkok R., Mall M.A. (2016) Confocal reflection microscopy is a promising novel method to study airway surface liquid dysregulation in cystic fibrosis. Pediatr Pulmunol 51, S228 doi:10.1002/ppul.23576

6.3 Non-peer-reviewed abstracts:

D Leitz, J Duerr, A Seyhan Agircan, J Schatterny, MF Beers, MA Mall. Conditional Deletion of the Ubiquitin Ligase Nedd4-2 from Lung Epithelia Results

in Diffuse Parenchymal Lung Disease in Mice. (2014) DZL Annual Meeting, Heidelberg, Germany

Ayca Seyhan Agircan, Marko Lampe, Julia Duerr, Reiner Pepperkok, Marcus A. Mall. (2015), Investigation of airway surface liquid handling in primary airway epithelial cultures of CF mouse models by confocal reflection microscopy, 9th European CF Young Investigator Meeting, Paris, France

D Leitz, J Duerr, A Seyhan Agircan, J Hegermann, J Schatterny, S Mulugeta, M Ochs, MF Beers, MA. Mall. (2015) Conditional Deletion Of Nedd4-2 in Alveolar Type II Cell Produces Pro-Surfactant Protein C Mistrafficking, Ultrastructural Abnormalities and Diffuse Parenchymal Lung Disease (DPLD) In Mice. DZL Annual Meeting, Hamburg, Germany

Ayca Seyhan Agircan, Marko Lampe, Julia Duerr, Reiner Pepperkok, Marcus A. Mall. (2016), Confocal reflection microscopy is a promising novel method for the study of airway surface liquid dysregulation in cystic fibrosis, ECFS Basic Science Conference, Pisa, Italy

Ayca Seyhan Agircan, Marko Lampe, Julia Duerr, Reiner Pepperkok, Marcus A. Mall. (2016), Confocal reflection microscopy is a promising novel method for the study of airway surface liquid dysregulation in cystic fibrosis, NACF Conference, Orlando, USA

Ayca Seyhan Agircan, Marko Lampe, Julia Duerr, Reiner Pepperkok, Marcus A. Mall. (2016), Development of confocal reflection microscopy for the measurements of airway surface liquid dysregulation in cystic fibrosis, DZL Annual Meeting, Hannover, Germany

Ayca Seyhan Agircan, Marko Lampe, Julia Duerr, Reiner Pepperkok, Marcus A. Mall. (2017), Confocal reflection microscopy is a promising novel method for the study of airway surface liquid dysregulation in cystic fibrosis, ECFS Basic Science Conference, Albufeira, Portugal

7. Acknowledgements

First of all, I would like to thank my supervisor Prof. Marcus Mall for giving me the opportunity to do my PhD in his group. His scientific guidance and immense knowledge helped me greatly throughout this project.

I would also like to thank Prof. Ursula Klingmüller for agreeing to be my co-supervisor and her input during my TAC meetings and Prof. Stephan Frings and Prof. Elmar Schiebel for agreeing to be in my thesis committee.

I would like to express my sincere gratitude to Dr. Julia Dürr. I would like to thank her for all the help both science related and unrelated fields and her patience to all my questions.

I would also like to thank Dr. Marko Lampe for all his help throughout my PhD project, without his technical knowledge and support this project wouldn't be successful.

My special thanks go to Pamela Millar Büchner, Claudius Wagner, Stella Jianghui Zhu, and Anita Balazs for the fruitful environment in the lab and for lots of laugh together. I appreciate your support and advices. I am grateful to all the members of AG Mall for providing such a pleasure atmosphere during my PhD studies. I would like to thank especially to Heike Scheuermann, Jolanthe Schatterny and Simone Schmidt for their technical help with my experiments.

Big thanks to my dearest friends in Heidelberg especially Malwina Michalak and Chaitali Chakraborty for their patience, encouragements and lots of fun we had together. I would like to thank also the 'Turkish community' in Heidelberg especially Yagmur, Selcen, Deniz, Alper, Bahtiyar, Kevser and Irem for making me fell at home.

Last but not least I would like to thank my parents Gülcan and Mustafa Seyhan, my brother Alp Arda Seyhan and my husband Fikret Gürkan Agircan for always being there for me and giving me the spirit to achieve my goals and always supporting me. Teşekkürler…