

Institute of Veterinary Pathology, Department of Veterinary Medicine
Freie Universität Berlin

**Candidates for Alternative Chloride Conductance
in Cystic Fibrosis:
Localization of mCLCA5 and mCLCA6 and
Quantitative mRNA Expression Analyses of
15 Candidate Genes**

Thesis submitted for the fulfillment of a
doctoral degree in Veterinary Medicine
(Dr. med. vet.)
at the
Freie Universität Berlin

submitted by
Josephine Braun
Veterinarian from Groß-Gerau

Berlin 2010
Journal-No.: 3423

**Printed with permission of the Department of Veterinary Medicine
of the Freie Universität Berlin**

Dean: Univ.-Prof. Dr. Leo Brunnberg

Supervision: Univ.-Prof. Dr. Achim Gruber, Ph.D._(Cornell Univ.)

First Reviewer: Univ.-Prof. Dr. Achim Gruber, Ph.D._(Cornell Univ.)

Second Reviewer: Univ.-Prof. Dr. Holger Martens

Third Reviewer: Univ.-Prof. Dr. Ortwin Simon

Descriptors (according to CAB-Thesaurus):

Animal models, chloride, cystic fibrosis, gene expression, ion channels, mice,
microdissection (MeSH), polymerase chain reaction

Day of Doctorate: 30 November 2010

Aus dem Institut für Tierpathologie
des Fachbereichs Veterinärmedizin der Freien Universität Berlin

**Kandidaten der alternativen Chloridleitfähigkeit
bei Zystischer Fibrose:
Lokalisierung von mCLCA5 und mCLCA6 und
quantitative mRNA Expressionsanalysen von
15 Kandidatengen**

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Josephine Braun
Tierärztin aus Groß-Gerau

Berlin 2010
Journal-Nr.: 3423

**Gedruckt mit Genehmigung des Fachbereichs Veterinärmedizin
der Freien Universität Berlin**

Dekan: Univ.-Prof. Dr. Leo Brunnberg

Betreuung: Univ.-Prof. Dr. Achim Gruber, Ph.D.(Cornell Univ.)

Erster Gutachter: Univ.-Prof. Dr. Achim Gruber, Ph.D.(Cornell Univ.)

Zweiter Gutachter: Univ.-Prof. Dr. Holger Martens

Dritter Gutachter: Univ.-Prof. Dr. Ortwin Simon

Deskriptoren (nach CAB-Thesaurus):

Animal models, chloride, cystic fibrosis, gene expression, ion channels, mice,
microdissection (MeSH), polymerase chain reaction

Tag der Promotion: 30.11.2010

Für meine Eltern

Contents

List of Abbreviations.....	3
1. Introduction	5
1.1 Cystic Fibrosis	5
1.1.1 Disease and Pathogenesis	5
1.1.2 Heterogeneity of Phenotype and Modulation of Disease Severity by Modifier Genes	6
1.1.3 CaCC as Modifiers in Cystic Fibrosis.....	6
1.2 Candidate CaCCs and a Chloride Channel as Mediators of Alternative Chloride Conductance	7
1.2.1 Bestrophins	8
1.2.2 CLC	8
1.2.3 Tweety.....	9
1.2.4 TMEM16.....	10
1.2.5 SLC26A.....	10
1.2.6 CLCA.....	11
1.3 Mouse Models and Strains.....	14
1.4 Aims and Hypotheses	17
2. Research Publications in Journals with Peer-Review.....	19
2.1 Murine mCLCA5 Is Expressed in Granular Layer Keratinocytes of Stratified Epithelia	20
2.2 Murine mCLCA6 Is an Integral Apical Membrane Protein of Non-goblet Cell Enterocytes and Co-localizes with the Cystic Fibrosis Transmembrane Conductance Regulator.....	36
2.3 Quantitative Expression Analyses of Candidates for Alternative Anion Conductance in Cystic Fibrosis Mouse Models	52
3. Declaration of Own Portion of Work in the Research Publications.....	67
3.1 Murine mCLCA5 Is Expressed in Granular Layer Keratinocytes of Stratified Epithelia	67
3.2 Murine mCLCA6 Is an Integral Apical Membrane Protein of Non-goblet Cell Enterocytes and Co-localizes with the Cystic Fibrosis Transmembrane Conductance Regulator.....	67
3.3 Quantitative Expression Analyses of Candidates for Alternative Anion Conductance in Cystic Fibrosis Mouse Models	68
4. Concluding Discussion.....	69
4.1 Validation of Methods.....	69

CONTENTS

4.2	Cellular Expression of mCLCA5 and mCLCA6	71
4.2.1	Cellular Expression of mCLCA5	72
4.2.2	Cellular Expression of mCLCA6	72
4.2.3	Do mCLCA5 and mCLCA6 Occupy Specific Functional Niches? ..	73
4.3	Extrapolation from Mouse to Human.....	75
4.4	Quantitative mRNA Expression Comparison of Candidate CaCCs	76
4.4.1	Comparison of the Expression Levels between the Wild Type and <i>cftr</i> Mutant Mouse Strains	76
4.4.2	Comparison of the Expression Levels between the Four Wild Type Mouse Strains of Different Genetic Background: NMRI, BALB/c, DBA/2 and C57BL/5.....	78
4.4.3	Comparison of the Expression Levels between the Two <i>cftr</i> Mutant Mouse Models: <i>cftr</i> ^{TgH(neoim)Hgu} and <i>cftr</i> ^{Im1Cam}	79
4.4.4	Comparison of the Expression Levels between the Three <i>cftr</i> ^{TgH(neoim)Hgu} Mutant Mouse Strains of Different Genetic Background: BALB/c, DBA/2 and C57BL/5	80
4.4.5	Comparison of Results with Previous Studies	80
4.5	Do the Candidate Genes Play a Role in CF?.....	81
4.6	Conclusion.....	83
4.7	Outlook	84
5.	Summary.....	87
6.	Zusammenfassung	89
7.	References.....	91
8.	Talks and Poster	104
9.	Attachments	105
9.1	Quantification of mCLCA6 and mCLCA3 in Luminal and Basal Crypt Epithelial Cells of the Caecum	105
9.2	Relative Quantification of mCLCA1, -2, -3, -4 and -6 in the <i>cftr</i> ^{TgH(neoim)Hgu} Mouse Model Inbred on C57BL/6 and DBA/2, BALB/c and NMRI Genetic Backgrounds and Their Respective Wild Types	106
10.	Acknowledgments / Danksagung.....	115
	Selbständigkeitserklärung.....	116

List of Abbreviations

4T1	A murine mammary epithelial tumor cell line
ANO	An octamin
ATP	A denosine T riphosphate
BEST	B estrophin
BLAST	B asic L ocal A lignment S earch T ool
CaCC	C alcium-activated C hloride C urrents
CaMKII	C almodulin-dependent protein K inase I I
cAMP	C yclic A denosine M onophosphate
cDNA	C omplementary D eoxyribonucleic A cid
CF	C ystic F ibrosis
CFTR	C ystic F ibrosis T ransmembrane conductance R egulator
cGMP	C yclic G uanosine M onophosphate
CLC	C hloride C hannel
CLCA	C hloride channel, C alcium-activated
CPAE	C alf P ulmonary A rtery E ndothelial cells
DIDS	4,4' d iisothiocyanatostilbene-2,2'- d isulfonic acid
DNA	D eoxyribonucleic A cid
EF1a	E longation F actor 1 α
ENaC	E pithelial Sodium (Na) C hannel
et al.	et alii (latin for "and others")
GOI	G ene(s) O f I nterest
HC11	A clonal mammary epithelial cell line
HEK293	H uman E mryonic K idney 293 cells
IFRD1	I nterferon- R elated D evelopmental R egulator 1
IL-4	I nterleukin- 4
LCM	L aser C apture M icrodissection
miRNA	M icro R ibonucleic A cid
mRNA	M essenger R ibonucleic A cid
PCR	P olymerase C hain R eaction
RNA	R ibonucleic A cid
RT-qPCR	R everse T ranscriptase q uantitative P olymerase C hain R eaction
siRNA	s mall i nterfering R NA
SLC26A9	S olute C arrier F amily 26 , M ember 9
STAS	S ulfate T ransporters and A nti- s igma-factor

LIST OF ABBREVIATIONS

TGFB1	T ransforming G rowth F actor B eta 1
TMEM16	T rans m embrane Protein 16
TTYH	T weety H omolog
UV-A	U ltra V iolet A , long wave (400 nm-320 nm), or black light
VRAC	V olume R egulated A nion C hannel

1. Introduction

1.1 Cystic Fibrosis

1.1.1 Disease and Pathogenesis

Cystic Fibrosis (CF), also called mucoviscidosis, is an autosomal recessive inherited disorder caused by mutations in the gene named cystic fibrosis transmembrane conductance regulator (*CFTR*; Ratjen and Doring 2003). It is a fatal genetic disease affecting primarily the Caucasian population. Approximately one in 25 Europeans (4%) carry one mutant *CFTR* allele and approximately one in every 3,000 newborn Europeans and Americans (0.03%) is affected, making it the most common fatal genetic disorder in these populations (Jonsdottir et al. 2008; Pearson 2009). The incidence is less frequent in African-Americans (1:17,000) and Asian populations (<1:100,000; McIntosh and Cutting 1992).

The *CFTR* protein belongs to a family of ATP (adenosine triphosphate)-binding proteins encoded on chromosome 7. It encodes for a cAMP (cyclic adenosine monophosphate)-activated Cl⁻ channel. More than 1,500 mutations of *CFTR* are known so far (Jonsdottir et al. 2008). The mutations can be grouped into six classes: (1) *CFTR* protein is premature and truncated; (2) defective processing and trafficking; (3) defective regulation; (4) defective conductance; (5) partly defective production or processing; and (6) defective regulation of other channels (Buchanan et al. 2009; Ratjen and Doring 2003). The most frequent mutation affecting approximately 70% of patients belongs to class two and is named $\Delta F508$ due to a phenylalanine deletion at position 508 (Buchanan et al. 2009; Ratjen and Doring 2003).

The disease presents as a multiple exocrinopathy based on defective Cl⁻ secretion and hyperactivated sodium absorption in several exocrine epithelia like airways, pancreas, intestine, liver, vas deferens and sweat glands (McIntosh and Cutting 1992; Schwiebert et al. 1998). Above all, cystic fibrosis causes severe pulmonary disease in infants and young adults, limiting life expectancy. The altered ion conductances result in lack of fluid secretion and thus development of stringy mucous. The pulmonary disease is characterized by mucous plugging, decreased mucociliary clearance, secondary bacterial infection especially by *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Burkholderia cepacia* (Drumm 2001), and fibrosis. Further frequent complications are pancreatic enzyme insufficiency, constipation, biliary cirrhosis, and infertility in males (>95%) due to obstruction or

INTRODUCTION

absence of the vas deferens (Rowntree and Harris 2003). 15 % of CF patients are affected by meconium ileus at birth, a complication with high mortality (Zielenski et al. 1999).

1.1.2 Heterogeneity of Phenotype and Modulation of Disease Severity by Modifier Genes

The cystic fibrosis phenotype shows large variations between affected individuals. These can partly be explained by the diversity of *CFTR* gene mutations. However, patients with the same *CFTR* genotype and even siblings or twins sharing most environmental factors show clinical variations (Kerem et al. 1990). This suggests that the CF phenotype is modulated by other genetic and environmental factors (Rozmahel et al. 1996; Santis et al. 1990).

Several studies imply that genes (modifier genes) other than *CFTR* can significantly modify the CF manifestation of the basic defect and the clinical picture (Bronsveld et al. 2001). Genetic variations of alleles outside the *CFTR* locus may even influence disease outcome. Although these allelic variations may themselves fail to present an apparent affect in a healthy person, they could act as a modifier when accompanied by a mono-genetic disorder such as CF (Bremer et al. 2008; Cutting 2005; Drumm 2001).

1.1.3 CaCC as Modifiers in Cystic Fibrosis

Several groups have demonstrated strong evidence that a Ca^{2+} -activated Cl^- conductance ameliorates disease severity in the intestine (Gray et al. 1994; Rozmahel et al. 1996), lung (Clarke et al. 1994; Grubb et al. 1994) and pancreas (Clarke et al. 1994). It may present a potential target for CF disease intervention but its molecular identity is unknown. Moreover, in *cftr*^{-/-} mouse models Ca^{2+} -activated Cl^- currents (CaCC) have been detected in airways and pancreas (Clarke et al. 1994) as well as intestinal tissue of *cftr*^{-/-} mice with prolonged survival (Rozmahel et al. 1996). This *CFTR*-independent Cl^- conductance has been observed in roughly 20% of CF patients homozygous for ΔF508 mutation on rectal biopsies (Bronsveld et al. 2000). In contrast to human CF patients, most *cftr*^{-/-} mouse models do not acquire the characteristic pulmonary phenotype but develop severe intestinal obstruction (Ratcliff et al. 1993). Clarke and coworkers therefore proposed that the Ca^{2+} -activated Cl^- conductance observed in the airways could compensate for the lack of *CFTR* Cl^- channel function. On the other hand, its absence from the intestine could

explain the severe pathology in this tissue (Clarke et al. 1994). Wilschanski and coworkers further found a Ca^{2+} -activated Cl^- conductance in the intestine of Class III *Cftr*^{m1HSC/m1HSC} mouse models, characterized by prolonged survival, which again could be linked to the milder intestinal phenotype (Wilschanski et al. 1996).

Ca^{2+} -activated Cl^- channels are one of five subgroups of anion channels found in excitable and non-excitable cells and may account for these currents. Their biophysiological characteristics have been analyzed in some detail, however, their molecular identity remains unknown. Ca^{2+} -activated Cl^- channels are activated by an increase in cytosolic Ca^{2+} : (1) Some are activated directly by micromolar or submicromolar Ca^{2+} concentrations and direct Ca^{2+} binding; (2) others are activated indirectly by calmodulin-dependent protein kinase II (CaMKII) protein phosphorylation; and (3) others are activated by Ca^{2+} through cGMP (cyclic guanosine monophosphate)-activated channels (Hartzell et al. 2005; Jentsch et al. 2002; Pusch 2004). Dependent on their single channel conductance they are grouped into (1) low (1-3pS); (2) intermediate (8pS and 15pS); and (3) higher (40-50pS) conductance channels (Eggermont 2004; Gruber et al. 2000; Kuruma and Hartzell 2000). Ca^{2+} -activated Cl^- channels are time and voltage dependent whereby activation is voltage independent and inactivation is voltage dependent. Furthermore, they have a characteristic anion selective permeability sequence and they are sensitive to 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) and niflumic acid (Eggermont 2004; Gruber et al. 2000; Kuruma and Hartzell 2000).

1.2 Candidate CaCCs and a Chloride Channel as Mediators of Alternative Chloride Conductance

CaCC are present in numerous cells, however, the molecular identity responsible for these currents is not well understood (Eggermont 2004).

A number of genes encoding for Ca^{2+} -activated Cl^- channels or Ca^{2+} -activated transporter proteins as well as mediators of Ca^{2+} -activated Cl^- currents with similar electrophysiological properties have been discovered, some of which are better characterized than others. These all could account for the observed alternative Cl^- currents described in several secretory epithelia affected by CF.

Candidates forming putative Ca^{2+} -activated Cl^- channels or transporters are bestrophins, CLC (chloride channels), tweety and TMEM16 (transmembrane protein 16), also known as anoctamins. Candidate Ca^{2+} -activated Cl^- current mediating

INTRODUCTION

proteins include a gene family named Cl⁻ channels, calcium-activated, short CLCA (Gruber et al. 2002). Unlike CaCC, SLC26A9 (solute carrier family 26, member 9) is a Cl⁻ channel inhibited by Ca²⁺ (Loriol et al. 2008). However, its association with CFTR and transepithelial Cl⁻ channel activity could also make it an interesting target as alternative Cl⁻ channel (Loriol et al. 2008).

1.2.1 Bestrophins

The family of bestrophins comprises four members in humans and three functional genes in mice (Barro Soria et al. 2006; Kramer et al. 2004). They function both as Cl⁻ channels and as regulators of voltage-gated Ca²⁺ channels (Hartzell et al. 2008). In humans, several mutations in *hBEST1* have been linked to the disease Best Vitelliform Macular Dystrophy (Hartzell et al. 2008). Bestrophins are multimeric proteins of which two contradictory topology models exist describing six (Milenkovic et al. 2007) or four (Tsunenari et al. 2003) transmembrane domains respectively. Bestrophins are directly activated by physiological concentrations of intracellular Ca²⁺ (Abdel-Ghany et al. 2003; Barro-Soria et al. 2009) and they are sensitive to DIDS and niflumic acid (Kunzelmann et al. 2007). Thus they show characteristic electrophysiological properties of CaCC. In particular the two murine family members mBEST1 and mBEST2 are interesting potential modifiers of CF disease severity as both account for Ca²⁺-dependent Cl⁻ currents in mouse respiratory epithelium (Barro-Soria et al. 2008). Furthermore, the mRNA (messenger ribonucleic acid) of murine *mbest1* and *BEST2*, the human ortholog of *mbest2*, are expressed in the CF relevant tissues colon, liver, lungs and kidney (Hartzell et al. 2008) making them interesting targets.

1.2.2 CLC

Cl⁻ Channels (CLC) are a family of nine Ca²⁺-activated and voltage gated Cl⁻ transport proteins subdivided into two groups. Four CLC proteins (CLC-1, -2, -Ka, and -Kb) are voltage gated Cl⁻ anion channels found at the plasma membrane and five (CLC-3 to -7) are Cl⁻-H⁺ exchangers found on intracellular membranes (Devuyst and Guggino 2002; Duran et al. 2009). They are predicted to have 10-12 transmembrane domains and the functional unit consists of a homodimer (Duran et al. 2009; Jentsch et al. 1999). Their channel structure model is based on crystallization data, reviewed in (Nilius and Droogmans 2003).

CLC proteins have been linked to several inherited diseases including Myotonia Congenita (CLC-1), Bartter's Syndrome (CLC-Kb) and Dent's Disease (CLC-5), reviewed in (Jentsch et al. 2002). CLC-2 and CLC-4 have both been discussed as candidates for alternative Cl⁻ conductance in the absence of functional CFTR (Fuller and Benos 2000a; Mohammad-Panah et al. 2002; Nilius and Droogmans 2003).

However, for CLC-2 in mice Zdebik and coworkers recognized that mice with disrupted *cftr* gene survived longer when the *clc-2* gene was additionally disrupted (Zdebik et al. 2004). In mice, *clc-2* was therefore concluded unlikely to be a key modifier gene in the lung and intestine (Blaisdell et al. 2004; Zdebik et al. 2004).

CLC-4 on the other hand was found on human chromosome 19q13, a region containing putative modifier genes for CF (Gyomorey et al. 2001), which makes it an interesting candidate. Murine *clc-4* has a broad tissue expression pattern including tissues relevant to CF like airways and intestine (Gyomorey et al. 2001; Mohammad-Panah et al. 2002; Murray et al. 1995; Wang and Weinman 2004). Both its electrophysiological properties as a CaCC and its expression in CF relevant tissues make CLC-4 a putative alternative Cl⁻ conductance pathway.

The splice variant of human CLC-3, CLC-3B, co-localizes with CFTR-interacting PDZ proteins in the Golgi. Both CLC-3B and CFTR also bind ERM-binding phosphoprotein 50 and PDZK1, which are localized at the plasma membrane (Gentsch et al. 2003). Little is known about CLC-3 and many laboratories had difficulties to measure CLC-3 currents (Jentsch et al. 1999). Due to a potential link between CLC-3B and CF due to its colocalization with CFTR, its murine ortholog was included in the studies.

1.2.3 Tweety

Of the three human homologs of tweety, *hTTYH1* to *-3*, *hTTYH3* resembles a promising modifier gene for CF because it encodes for a large conductance Ca²⁺ activated and DIDS-sensitive Cl⁻ channel with five to six transmembrane segments (Suzuki 2006; Suzuki and Mizuno 2004). It is expressed in excitable tissues including the colon and kidney. Its murine ortholog mTTYH3 has additionally been located in the pancreas. Human hTTYH1 and hTTYH2 on the other hand are primarily expressed in the brain and hTTYH1 is a Ca²⁺-independent volume sensitive Cl⁻ channel (Rae et al. 2001; Suzuki 2006; Suzuki and Mizuno 2004). Thus, *hTTYH1* and *hTTYH2* are unlikely candidate modifier genes. Its localization in the CF relevant tissues colon, kidney and pancreas makes mTTYH3 an interesting target to investigate.

1.2.4 TMEM16

TMEM16 have recently been discovered as Ca^{2+} -activated Cl^- channels or CaCC subunits (Caputo et al. 2008; Schroeder et al. 2008; Yang et al. 2008). Hydropathy analyses predicted eight putative transmembrane domains. Electrophysiological analyses suggested typical anionic channel properties, which led to them also being called anoctamins (Yang et al. 2008). There are 10 family members known to date. TMEM16A (ANO1) and TMEM16B (ANO2) were shown to generate voltage dependent Cl^- currents activated by physiological Ca^{2+} signals in multiple expression systems including Axolotl oocytes and mammalian HEK293 (human embryonic kidney 293 cells) cells (Caputo et al. 2008; Duran et al. 2009; Hartzell et al. 2009; Schroeder et al. 2008; Yang et al. 2008). Murine mTMEM16A is expressed in various secretory epithelia including tissues relevant to CF such as pancreas, colon, liver, lungs, kidneys and stomach (Caputo et al. 2008; Rock et al. 2009; Schroeder et al. 2008; Yang et al. 2008). TMEM16F (ANO6) and TMEM16K (ANO10) are of special interest in the respiratory tract where they were shown to be expressed in tracheal epithelium and mesenchyme and display candidates for residual CaCC in *tmem16a*^{-/-} mice (Rock et al. 2009). Its electrophysiological properties and expression pattern make *mtmem16a* a likely candidate modifier gene and alternative target in CF (Yang et al. 2008).

1.2.5 SLC26A

Although it does not evoke a characteristic CaCC, SLC26A was included in the studies. The SLC26 transporters consist of 11 family members known to date. Based on functional similarities some can be grouped into three groups: Group (1) are sulfate transporters including SLC26A1 and SCL26A2; Group (2) are $\text{Cl}^-/\text{HCO}_3^-$ exchangers including SLC26A3, SLC26A4 and SLC26A6; Group (3) are selective Cl^- channels including SLC26A7 and SLC26A9, reviewed in (Dorwart et al. 2008).

Several family members have been associated with genetic disease: *SLC26A2* with chondrodysplasias; *SLC26A3* with Cl^- -losing diarrhea; *SLC26A4* in Pendred syndrome and hereditary deafness (Mount and Romero 2004).

Membrane-spanning prediction algorithms predicted between eight and 14 transmembrane segments (Bertrand et al. 2009). An important structural component seems to be the STAS (sulfate transporters and anti-sigma-factor) domain, a protein-protein interaction domain involved in transport function (Bertrand et al. 2009).

In both human and murine SLC26A3 and SLC26A6 the STAS domains interact with CFTR R-domains. However, when expressed in HEK293 cells these two family members did not generate measurable Cl⁻ currents (Bertrand et al. 2009).

SLC26A9 is also associated with CFTR and contributes significantly to anion secretion in airway gland and surface epithelia (Bertrand et al. 2009). Interaction with CFTR regulates epithelial fluid and electrolyte secretion and thus modulated the airway surface liquid volume (Bertrand et al. 2009), reviewed in (Dorwart et al. 2008). Electrophysiologically the Cl⁻ channel SLC26A9 was inhibited by DIDS and is insensitive to intracellular cAMP. Ca²⁺ also inhibited SLC26A9 associated currents (Loriol et al. 2008).

Human SLC26A family members are found in all of the epithelia where CFTR has been found (Bertrand et al. 2009). Of these, SLC26A9 is predominantly expressed in the lung where it is found in the luminal membrane of ciliated airway bronchial and alveolar epithelial cells, gastric surface and lesser in glandular cells, reviewed in (Dorwart et al. 2008).

SLC26A9 does not outline a Ca²⁺-activated Cl⁻ channel. However, its contribution to transepithelial Cl⁻ channel activity in bronchial epithelia and its association with CFTR could make it an interesting target as alternative Cl⁻ channel (Loriol et al. 2008).

1.2.6 CLCA

The *CLCA* gene family encodes a class of proteins, some of which mediate a Ca²⁺-activated Cl⁻ conductance sensitive to DIDS when expressed in *Xenopus* oocytes or HEK293 cells (Fuller and Benos 2000b; Gruber et al. 2000). Since their discovery in the early 1990ies (Cunningham et al. 1995; Ran and Benos 1992, 1991; Zhu et al. 1991), 42 family members have been identified in altogether 11 mammalian species (Plog et al. 2009). Human and murine members are located on human chromosome 1p and murine chromosome 3, respectively (Ritzka et al. 2004).

In the nomenclature the species is indicated by h (human), m (murine), p (porcine), b (bovine), e (equine) and so forth. The homologs within a species are numbered in order of their discovery (Gruber et al. 2002).

A number of CLCA gene family members have been partially characterized including the four human homologs *hCLCA1* to *-4* (Agnel et al. 1999; Gruber et al. 1998a; Gruber and Pauli 1999) and the six murine homologs *mCLCA1* (Gandhi et al. 1998; Romio et al. 1999), *mCLCA2* (Lee et al. 1999), *mCLCA3* (Komiya et al. 1999; Leverkoehne and Gruber 2002), *mCLCA4* (Elble et al. 2002), *mCLCA5* (Braun et al. 2009; Evans et al. 2004) and *mCLCA6* (Bothe et al. 2008; Evans et al. 2004). Although they show high interspecies sequence homologies on mRNA (Figure 1) and

INTRODUCTION

protein levels they are characterized by a heterogeneous, species and cell-specific expression pattern for each family member (Gruber et al. 2000; Pauli et al. 2000).

Despite their name, biochemical and electrophysiological data suggest that some CLCA proteins may not represent channel proteins of their own but are more likely to function as extracellular signaling molecules and regulate other, yet unknown channels (Gibson et al. 2005; Mundhenk et al. 2006; Plog et al. 2009). Human hCLCA1 for example influences conductance of Ca^{2+} -dependent Cl^- channels (Hamann et al. 2009). Based on biochemical characterizations CLCA proteins can be grouped into two existing models. One group, including hCLCA1, its murine ortholog mCLCA3 and its porcine ortholog pCLCA1 (Gibson et al. 2005; Mundhenk et al. 2006; Plog et al. 2009), form heterodimeric glycoproteins that are fully secreted by the cell. The other group, including mCLCA6 (Bothe et al. 2008) and hCLCA2 (Eible et al. 2006) is anchored to the plasma membrane via a transmembrane domain.

Although CLCA proteins have electrophysiological similarities to CaCC, in contrast to CaCC they have time independent currents and are activated by high, non-physiological Ca^{2+} concentrations (Abdel-Ghany et al. 2003; Gandhi et al. 1998; Gruber et al. 1998a; Gruber and Pauli 1999).

Like CFTR, the majority of family members known to date are predominantly expressed in secretory epithelial cells (Gruber et al. 1998a). Of the murine family members the cellular localizations of mCLCA1 and mCLCA2 were previously narrowed down by *in situ* hybridization to intestinal epithelial cells of the crypts, respiratory epithelial cells, gall bladder epithelium, acinar glands of the pancreas, squamous epithelia and germinal centers of lymphoid tissues (Gruber et al. 1998a; Leverkoehne et al. 2002). Due to their high sequence identities, this expression pattern resembled an overlap between both family members. The cellular expression patterns were further determined in the Group of Frank Thevenod in cooperation with Gruber and coworkers (Roussa et al. 2010). They found mCLCA1 and -2 mRNA and protein in parotid and submandibular glands. Immunolabeling further revealed both to be present in small intestinal crypt enterocytes. The mCLCA3 protein was identified in goblet cells and other mucin-producing cells of various tissues including the respiratory and gastrointestinal tracts (Leverkoehne and Gruber 2002) using immunohistochemistry and immune electron microscopy. For mCLCA4, *in situ* hybridization revealed expression in smooth muscle cells of various organs including intestine and stomach as well as in the mucosa of the gastrointestinal tract (Eible et al. 2002). The cellular localization of the two most recently discovered murine CLCA family members mCLCA6 and mCLCA5 (Evans et al. 2004) are still unknown.

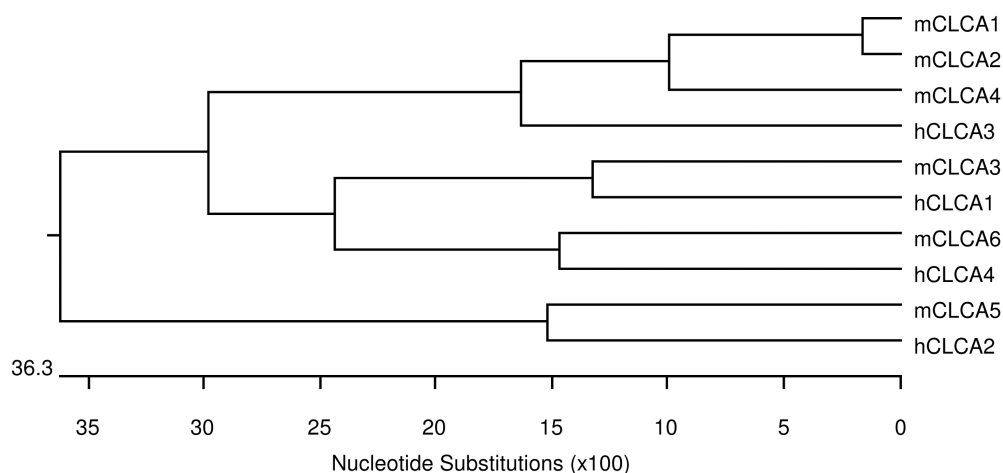
A number of functions including opposing results concerning their possible modulatory role in CF have been discussed between humans and mice (Abdel-Ghany et al. 2003; Fuller and Benos 2000a; Gruber et al. 2000; Pauli et al. 2000). Bronsveld and coworkers showed that in contrast to *CFTR*^{-/-} mouse models (Clarke et al. 1994; Rozmahel et al. 1996) the Ca²⁺-dependent Cl⁻ secretion in respiratory tissue showed no association with disease outcome in human patients (Bronsveld et al. 2001). Ritzka and coworkers found a strong correlation between allelic variants at the human CLCA gene locus *hCLCA1* or, less likely, *hCLCA4* and *CFTR*-independent Cl⁻ conductance in section biopsies of CF patients (Ritzka et al. 2004). Interestingly, the number of patients developing intestinal complications is less frequent than in mice (Cutting 2005).

Despite different hypotheses, their electrophysiological properties, which are similar to those of CaCC make all mCLCA potential candidate mediators for alternative Cl⁻ conductance. Additionally, their expression in CF relevant tissues supports relevance in CF. However, the cellular expression of the two most recently discovered murine family members mCLCA5 and mCLCA6 are still unknown.

Figure 1:

Phylogenetic Tree of Murine and Human CLCA mRNA Nucleotide Sequence Identities

Phylogenetic tree of the murine and human CLCA gene family members according to their mRNA nucleotide sequence identities. Sequence alignment and calculation of the phylogenetic tree were carried out with the ClustalV-Method (MegAlign, Lasergene, DNASTar Inc. Madison, WI, USA)



1.3 Mouse Models and Strains

To study the individual candidates for alternative Cl⁻ conductance the mouse was chosen as model species. Subsequently to the cloning of the *CFTR* gene in 1989, several mouse models of CF were generated by gene-targeting approaches. All 27 exons of the murine *cftr* are very similar to those of the human *CFTR* and the proteins show 78 % similarity (Ellsworth et al. 2000; Kukavica-Ibrulj and Levesque 2008). In 1992 the first mouse model was generated (Snouwaert et al. 1992). To date 11 mouse models have been developed which can be categorized into (1) CF mice with residual CF function; (2) *cftr* knockout mice with complete loss of function; and (3) mice with specific *cftr* mutations frequently observed in humans including Δ F508 (Scholte et al. 2004), G551D (Delaney et al. 1996) and G480C (Davidson and Rolfe 2001; Dickinson et al. 2000; Grubb and Gabriel 1997; Kukavica-Ibrulj and Levesque 2008).

Of the two CF mouse models used in this study one, *cftr*^{TgH(neoim)Hgu}, represents a mutation with residual *cftr* function (Dorin et al. 1992) and one, *cftr*^{tm1Cam}, represents a complete knockout (Ratcliff et al. 1993).

The following paragraphs give an outline about the important features concerning the two mouse models used in this study.

The *cftr*^{TgH(neoim)Hgu} mouse model was previously created by others by disrupting exon 10 of the *cftr* gene in embryonic stem cells using an insertional gene targeting vector (Dorin et al. 1992). Exon skipping and aberrant splicing allowed low levels of residual wild-type *cftr* mRNA to still be produced. 10% were still produced in the lungs and 20% in the intestine. Disease severity was thereby ameliorated in comparison to complete knockout mice (Dorin et al. 1994).

Similar to CF, especially to CF patients with Δ F508 mutation (Bronsveld et al. 2000), the mutant mice demonstrated a reduced cAMP-induced Cl⁻ secretion typically evoked by CFTR both in the intestinal and respiratory tracts *in vivo* and *in vitro* (Smith et al. 1995). The mice also showed an increased negative nasal potential difference which could be related to an increased Na⁺ absorption, again typical for CF (Smith et al. 1995). A Ca²⁺ related Cl⁻ secretion was reduced in the intestine and preserved in the respiratory tract.

Histopathological changes were also consistent with those seen in CF. The colon was only mildly dilated with increased mucus accumulation. Thus, *cftr*^{TgH(neoim)Hgu} mice resemble the human intestinal CF phenotype more strongly than other mouse

models (Dorin et al. 1994). Pancreas and lungs were predominantly without pathological finding (Dorin et al. 1992).

The *cftr*^{tm1Cam} mouse model mimics a complete functional knockout. A termination codon was previously introduced into the coding sequence of the murine *cftr* gene and caused a disruption of the *cftr* locus (Ratcliff et al. 1993). Homozygous pups failed to thrive and approximately 80% died in the first few days of life due to meconium ileus. Mice that survived the peripartal period suffered from intestinal obstructions. Interestingly, only about 15% of CF patients suffer meconium ileus.

Electrophysiologically, these mice lacked a cAMP-activated Cl⁻ conductance in tracheal and caecal measurements. In contrast to human airway epithelium, an increased Na⁺ absorption was not observed in the murine trachea. They did, however, have a Ca²⁺-dependent Cl⁻ secretion in airway epithelial cells.

In the pathohistological examinations the intestine showed dilation of the small intestinal crypts and accumulation of mucus. The pancreas showed dilation and blockage of the small pancreatic ducts in half of the examined animals older than 17 days. The lungs were without pathological finding (Ratcliff et al. 1993).

Both mouse models therefore mimic several important electrophysiological and histological aspects of the human CF disorder making them suitable models routinely used to study the pathology of CF.

Despite striking similarities between human CF and the effect of the *cftr* mutation on mice, the dissimilarities between both species need to be considered when projecting back to the human. Important differences between CF models and human CF patients are observed in pancreatic function and the pulmonary phenotype (Davidson and Rolfe 2001). Pancreatic insufficiency is a hallmark of most CF patients but mouse models fail to develop this manifestation. Similarly, pulmonary disease is cause of most morbidity and mortality in CF patients but mouse models do not share this phenotype. This may indicate alternative fluid secretory pathways, possibly CaCC, present in both organ systems (Clarke et al. 1994; Gray et al. 1995; Grubb and Gabriel 1997).

Anatomical differences between the lungs of mice and humans has led to doubt whether a *cftr*-mutant mouse could mimic the human CF lung disease (Collins 1992). In humans, lung disease gradually develops over years following repeated exacerbations of pulmonary infections with typical bacterial pathogens. Pulmonary disease is only poorly correlated with the *CFTR* genotype (Cutting 2005) and the predominantly neutrophilic inflammatory response is only poorly correlated to the

INTRODUCTION

amount of infectious stimuli (Muhlebach and Noah 2002). In contrast to humans, however, mouse models for research purposes are generally kept in SPF facilities and killed at the age of 10 weeks. Furthermore, despite lack of spontaneous lung disease, when challenged with bacterial pathogens, CF mice lack physiological clearance and develop marked inflammation (Davidson et al. 1995).

Differences in severity between the human CF and the mouse phenotype resembling CF may also be due to different levels of mRNA expression and genetic background of the mouse strain. The genetic background may correlate to varying activity of modifier genes (Kukavica-Ibrulj and Levesque 2008). Therefore we chose different genetic backgrounds for our studies. The following backgrounds were chosen, partially based on access and availability: C57BL/6, NMRI, BALB/c, DBA/2, the first three of which were used for publication.

C57BL/6 wild type mice were used for expression analyses for the purpose of better comparison with previous expression analyses on the same background done for other murine CLCA family members (Gruber et al. 1998b; Leverkoehne and Gruber 2002).

The *cfr*^{tm1Cam} mouse model had been generated, by others on a NMRI background to compensate for the high rate of early mortality. These mice have a longer survival rate when they additionally receive an oral laxative at weaning to prevent an intestinal ileus (Holle 2007). Again, the higher survival rate of the CF mutation in the NMRI strain suggests a strong influence of the genetic background on the development of the disease.

The *cfr*^{TgH(neoim)Hgu} mouse model had been generated by others on BALB/c, DBA/2 and C57BL/6 backgrounds each. In BALB/c mice a significantly higher DIDS-sensitive, Ca²⁺-activated Cl⁻ conductance was noticed previously compared to DBA/2 and C57BL/6 mice (Bleich et al. 2007). CF mice on a BALB/c background also exhibited the highest increase in Cl⁻ current in response to carbachol and forskolin stimulation of the proximal colon (Bleich et al. 2007). In addition, it had been speculated that mCLCA3 expression is induced in BALB/c mice with lost *CFTR* function but not in C57BL/6 mice and might compensate function in the airways (Leverkoehne and Gruber 2002).

1.4 Aims and Hypotheses

There were two primary aims and hypotheses:

First aim was to investigate the tissue expression pattern and cellular localization of the two new murine CLCA family members mCLCA5, ortholog to human hCLCA2, and mCLCA6, ortholog to human hCLCA4. Knowledge of their cellular localization is an important prerequisite for future functional studies and to pinpoint their potential relevance in the complex of CF. The hypothesis was that these two CLCA family members would occupy cell type specific niches relevant to CF, similar as was demonstrated for mCLCA3. The mRNA of both family members was quantified in a broad spectrum of murine tissues using real-time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). Next, laser capture microdissection (LCM) of relevant tissues expressing the candidates was employed to narrow down their cellular localization. For mCLCA5 additional immune electron microscopical investigations were carried out to localize the protein on ultrastructural level.

Second aim was to identify whether the CaCC candidates mCLCA1 to -6, mBEST1, mBEST2, mCLC-3B, mCLC-4, mTTYH3, mTMEM16A, mTMEM16F and mTMEM16K as well as the Cl⁻ channel SLC26A9 may play a role in CF. In particular, the aim was to test the hypothesis that these proposed candidates with modulatory roles for anion conductance in CF are up-regulated on transcriptional level in CF versus normal tissues. To do so the altogether 15 candidate genes were quantified in CF relevant tissues on mRNA level to identify a molecule or regulatory pathway involved.

Experiments were conducted in two CF mouse models: One with a mild intestinal phenotype (*cftr*^{TgH(neoim)Hgu}) on BALB/c, DBA/2 and C57BL/6 genetic backgrounds and one with a severe intestinal phenotype (*cftr*^{tm1Cam}) on a NMRI genetic background. Both animal models show classical phenotypical characteristics of CF including intestinal obstruction, severity dependent on genotype, goblet cell hyperplasia, mucus accumulation and crypt dilation, similar as seen in meconium ileus of human patients (Dorin et al. 1994; Ratcliff et al. 1993). They also have characteristic intestinal and airway electrophysiology including reduced or absent cAMP-induced Cl⁻ conductance in epithelial cells of intestine and airways dependent on genotype (Dorin et al. 1994; Ratcliff et al. 1993).

An up- or down-regulation of one or several of the candidate genes on transcriptional level could point towards a functional significance in the CF pathology and a potential target in CF therapy. Relative mRNA copy numbers were determined and compared

INTRODUCTION

between the CF and respective wild type tissues. Copy numbers were also compared between both CF mouse models to help identify modulating factors and between the different wild type background strains to account for variable other genetic influences.

Expression analyses carried out for mCLCA1, -2, -3, -4 and -6 on DBA/2 and C57BL/6 wild type strains and their CF-counterparts are included in the concluding discussion and depicted in the attachments. The CF mouse models and strains were used according to availability; we neither had the means to produce new mice of the same strains and models nor to breed new models.

2. Research Publications in Journals with Peer-Review

2.1 Murine mCLCA5 Is Expressed in Granular Layer Keratinocytes of Stratified Epithelia

With kind permission from Springer Science+Business Media: Histochemistry and Cell Biology, Murine mCLCA5 is expressed in granular layer keratinocytes of stratified epithelia, 133(3), 2009, 285-99, Josephine Braun, Melanie K. Bothe, Lars Mundhenk, Carol L. Beck, Achim D. Gruber, figure numbers 1 to 7, copyright Springer-Verlag 2009.

DOI <http://dx.doi.org/10.1007/s00418-009-0667-0>

2.2 Murine mCLCA6 Is an Integral Apical Membrane Protein of Non-goblet Cell Enterocytes and Co-localizes with the Cystic Fibrosis Transmembrane Conductance Regulator

Reproduced, with kind permission, from Melanie K. Bothe, Josephine Braun, Lars Mundhenk, Achim D. Gruber. "Murine mCLCA6 Is an Integral Apical Membrane Protein of Non-goblet Cell Enterocytes and Co-localizes With the Cystic Fibrosis Transmembrane Conductance Regulator." *Journal of Histochemistry and Cytochemistry*, 56(5):495-509, 2008.

DOI <http://dx.doi.org/10.1369/jhc.2008.950592>.

2.3 Quantitative Expression Analyses of Candidates for Alternative Anion Conductance in Cystic Fibrosis Mouse Models

Reproduced, with kind permission, from Josephine Braun, Lars Mundhenk, Friederike Range, Achim D. Gruber. "Quantitative expression analyses of candidates for alternative anion conductance in cystic fibrosis mouse models." *Journal of Cystic Fibrosis*, 9(5):351-64, 2010.

DOI <http://dx.doi.org/10.1016/j.jcf.2010.06.003>.

3. Declaration of Own Portion of Work in the Research Publications

3.1 Murine mCLCA5 Is Expressed in Granular Layer Keratinocytes of Stratified Epithelia

Authors: Braun J, Bothe MK, Mundhenk L, Beck CL, Gruber AD

Year: 2009

Journal: Histochem Cell Biol 133(3):285-99

My contributions: Independent design, preparation, completion and evaluation of all investigations involving laser capture microdissection and quantification of the target gene mRNA. Independent design, preparation and evaluation of all investigations involving immune electron microscopy. Independent subsequent creation of the entire manuscript with exception of only partial contribution to following parts:

In Materials and Methods: Sequence analyses and generation of antibodies; immunohistochemistry; biochemical protein analyses.

In the Results section: Sequence analyses and generation of antibodies; tissue expression pattern (by immunoblots, immunohistochemistry and confocal laser scanning microscopy); cellular processing and association with the plasma membrane.

In the Discussion: The paragraph concerning the biochemical data analysis; interpretation of the overall results.

Contributions of all authors: Design, preparation, completion and evaluation of investigations involving biochemistry and immunohistochemistry. Subsequent creation of parts of the manuscript relating to these analyses as mentioned in the exceptions above.

3.2 Murine mCLCA6 Is an Integral Apical Membrane Protein of Non-goblet Cell Enterocytes and Co-localizes with the Cystic Fibrosis Transmembrane Conductance Regulator

Authors: Bothe MK, Braun J, Mundhenk L, Gruber AD

Year: 2008

Journal: J Histochem Cytochem 56:495-509

My contributions: Independent design, preparation, completion and evaluation of all investigations involving laser capture microdissection and quantification of the target gene mRNA. Subsequent creation of all parts of the manuscript relating to these analyses.

Contributions of other authors: Design, preparation, completion and evaluation of investigations involving biochemistry, immunohistochemistry and immune electron microscopy. Subsequent creation of parts of the manuscript relating to these analyses.

3.3 Quantitative Expression Analyses of Candidates for Alternative Anion Conductance in Cystic Fibrosis Mouse Models

Authors: Braun J, Mundhenk L, Range F, Gruber AD

Year: 2010

Journal: J Cyst Fibros, doi: 10.1016/j.jcf.2010.06.003

My contributions: Independent design, preparation, completion and evaluation of all presented investigations and subsequent creation of the entire manuscript.

Contributions of all authors: Design and evaluation of investigations.

4. Concluding Discussion

This study focused on identifying the cellular localization of the two recently discovered murine CLCA family members mCLCA5 and mCLCA6. It also contributed to evaluating whether the candidate CaCC mediators mCLCA1 to -6, mBEST1, mBEST2, mCLC-3B, mCLC-4, mTTYH3, mTMEM16A, mTMEM16F and mTMEM16K as well as SLC26A9 may be involved in CF disease severity based on differences in quantitative mRNA expression.

4.1 Validation of Methods

Reliability of the results is based on the validity of the methods used. An important part of this study was therefore adequate sample acquisition and adequate preparation of RNA to ensure optimal mRNA quality. Tissue samples were processed immediately after killing the mice and were snap-frozen in liquid nitrogen. All steps involving handling of RNA were carried out without time delay and on ice unless stated otherwise according to the manufacturers' protocols. Roughly equal amounts of tissue samples were each sufficiently homogenized prior to nucleic acid extraction. Contamination with genomic DNA (deoxyribonucleic acid) was prevented by designing the primers to span an intron and treating the sample with DNase digestion. RNA templates were quality checked and quantified using the NanoDrop[®] ND-1000 Spectrophotometer.

The expression patterns of both mCLCA5 and mCLCA6 were analyzed on cellular level. In order to distinguish between the levels of mRNA expression within the different cell types, these specific cell types were isolated and collected individually by laser capture microdissection (LCM). Here, a pulsed UV-A (ultraviolet A) laser was focused through the objective lens of the microscope and used to separate the cells in a photochemical process in a non-contact manner without heat transfer. The process is therefore contamination-free and mRNA is ideally not affected. The isolated cells are then catapulted into the collecting device by the laser, again in a non-contact process (Burgemeister 2005).

Principle method used for evaluating the mRNA expression levels in various murine tissues and single cells both for expression analyses of mCLCA5 and mCLCA6 and for comparison of quantitative expression levels was a fluorescence-based real-time quantitative PCR. This method is the touchstone for nucleic acid quantification and

CONCLUDING DISCUSSION

has the capacity to detect minimal amounts of specific nucleic acid sequences with a high degree of sensitivity, specificity and quantitative precision.

In order to identify the mRNA expressing cell type specifically for mCLCA5 and mCLCA6 and in order to quantify the expression levels of the individual murine CLCA family members mCLCA1 to -6 the challenge in this study was to establish assays which could reliably distinguish between the closely related family members (Eible et al. 2002; Leverkushoehe et al. 2002) which share nucleic acid sequence identity of up to 96% between mCLCA1 and mCLCA2 (Table 1).

Table 1:

Murine and Human CLCA Nucleic Acid Sequence Pair Distances

Degree of nucleic acid sequence identities (top right) and divergences (bottom left) of the murine and human CLCA gene family members. Sequence alignment and calculation of pair distances was carried out with the ClustalV-Method (MegAlign, Lasergene, DNASTar Inc. Madison, WI, USA)

		% Nucleotide Identity									
		mCLCA1	mCLCA2	mCLCA3	mCLCA4	mCLCA5	mCLCA6	hCLCA1	hCLCA2	hCLCA3	hCLCA4
% Nucleotide Divergence	mCLCA1		96.0	47.3	75.9	42.3	45.8	45.6	42.5	68.4	48.5
	mCLCA2	3.3		47.7	71.4	38.8	46.1	43.7	40.3	62.8	46.4
	mCLCA3	59.1	60.2		43.9	35.0	50.4	71.0	37.4	43.1	53.6
	mCLCA4	18.2	21.5	58.5		39.1	42.8	38.5	41.0	61.6	44.4
	mCLCA5	69.1	73.0	75.3	71.8		34.3	36.8	65.6	36.5	33.7
	mCLCA6	59.3	60.8	54.4	62.2	78.9		53.5	38.5	45.2	69.0
	hCLCA1	55.9	58.3	26.5	56.1	76.5	50.5		36.8	44.3	57.9
	hCLCA2	62.6	67.6	75.8	68.5	30.5	76.3	74.0		40.0	38.8
	hCLCA3	29.2	32.2	64.2	34.6	72.6	61.9	58.0	66.0		46.4
	hCLCA4	56.8	59.1	49.6	60.7	78.2	29.4	40.6	71.5	57.4	

The primer and hydrolysis probe combinations used were either designed and purchased according to previous successful work of our team - for mCLCA1, mCLCA2, mCLCA4 (Leverkoeshoehe et al. 2006; Leverkushoehe et al. 2002); or newly designed and an assay established - for mCLCA3, mCLCA5, mCLCA6, mBEST1, mBEST2, mCLC-3B, mCLC-4, mTTYH3, mTMEM16A, mTMEM16F, mTMEM16K, SLC26A9. Assays were designed to run with an analytical sensitivity of measuring at least 10² mRNA copies of gene of interest accurately in the respective plasmid-cDNA (complementary deoxyribonucleic acid) or PCR-product cDNA. The analytical specificity was tested by several means. An *in silico* BLAST (basic local alignment search tool) search of the primers and probe combinations revealed 100% target specificity. Additionally, for the murine CLCA family members, cross-reactivity was

experimentally excluded for the mCLCA family members by following procedures: Amplification products were tested in conventional PCR showing specific sizes and only a single PCR product following agarose gel electrophoresis. Furthermore, DNA-sequencing of the respective PCR-products of all analyzed genes verified the specificity of the assays.

The PCR amplification efficiencies lay between 95% and 105% for all assays. Repeatability and reproducibility of the assays were further verified. Both intra-assay and inter-assay variance had a standard deviation of between 0 and 5% coinciding with the recommendations by Bustin (Bustin 2000). Based on these results a robust assay performance was assumed.

The reference gene used for normalization was elongation factor 1 α (EF1a). It is a universal cofactor in protein synthesis and is stably expressed in all living cells and tissue types (Gruber and Levine 1997) and was successfully used in several projects before (Leverkoehne et al. 2006; Leverkoehne et al. 2002). It was abundantly expressed in all tissue samples tested and correlated well with the total amount of RNA within each sample. Relative gene expressions were calculated as ratios representing the copy numbers of the gene of interest relative to the internal reference EF1a.

4.2 Cellular Expression of mCLCA5 and mCLCA6

The cellular localization of mCLCA3, the murine ortholog of hCLCA1, had been previously determined and those of mCLCA1, mCLCA2 and mCLCA4, the murine orthologs of the product of human pseudogene *hCLCA3*, were previously narrowed down: The mCLCA3 protein was identified in goblet cells and other mucin producing cells of various tissues including the respiratory and gastrointestinal tracts (Leverkoehne and Gruber 2002) using immunohistochemistry and immune electron microscopy. For mCLCA1 and mCLCA2 the quantitative mRNA tissue expression patterns were already established (Leverkoehne et al. 2002). Furthermore, their combined expression pattern was narrowed down by *in situ* hybridization to intestinal epithelial cells of the crypts, respiratory epithelial cells, gall bladder epithelium, acinar glands of the pancreas, squamous epithelia and germinal centers of lymphoid tissues (Gruber et al. 1998b). Due to their high sequence identities, this expression pattern resembled an overlap between both family members. The cellular expression patterns were further determined by the group of Frank Thevenod (Roussa et al. 2010). They found mCLCA1 and -2 mRNA and protein in parotid and submandibular

CONCLUDING DISCUSSION

glands. Immunolabeling further revealed both to be present in small intestinal crypt enterocytes. For mCLCA4, *in situ* hybridization with a mCLCA4 specific probe has revealed expression in smooth muscle cells of various organs including intestine and stomach as well as in the mucosa of the gastrointestinal tract (Elble et al. 2002).

In this study the cellular localization of the two most recently discovered murine CLCA family members were determined: mCLCA5, the murine ortholog of hCLCA2, and mCLCA6, the murine ortholog of hCLCA4. mCLCA5 and mCLCA6 were first discovered in 2004 (Beckley et al. 2004; Evans et al. 2004) and cloned in the same year by the group of Evans (Evans et al. 2004).

4.2.1 Cellular Expression of mCLCA5

mCLCA5 mRNA had a broad expression pattern in virtually all tissues analyzed but was predominantly detected in keratinocytes of all stratified squamous epithelia that undergo cornification including squamous epithelium of the skin and nonglandular mucosa of the stomach. Subsequent immune electron microscopy specifically localized the mCLCA5 protein to cytoplasmic granules of granular layer keratinocytes and, to a lesser extent, to spinous and cornified layer keratinocytes. In tissues physiologically lacking keratinocytes expression of mCLCA5 mRNA was close to the lower detection limit.

4.2.2 Cellular Expression of mCLCA6

mCLCA6 showed quite a different expression pattern: mCLCA6 mRNA was exclusively detected in the intestinal tract of all tissues analyzed with an expression peak in the ileum. Along the crypt-villous axis the epithelial cells of the villous tips of the jejunum and the luminal epithelial cells of the caecum (Attachments: Figure 2) showed highest mRNA expression. In contrast to its human ortholog hCLCA4, it was not expressed in the brain or trachea (Agnel et al. 1999).

Analyses defining the difference between the expression levels of both mCLCA6 and mCLCA3 between the lumenally located and basally located epithelial cells of the caecum were additionally carried out. The mean relative mRNA copy numbers of mCLCA6 and mCLCA3 were approximately 40-fold and 5-fold higher in the luminal enterocytes, respectively. mCLCA6 had an approximately 8-fold higher relative expression in the lumenally located enterocytes than mCLCA3. This is likely due to mCLCA6 being expressed in a more abundant type of epithelial cell than mCLCA3. In fact, these data can be backed up based on literature on protein level: Whereas

mCLCA3 is expressed in goblet cells (Leverkoehne and Gruber 2002) mCLCA6 is expressed in the more abundant non-goblet cell enterocytes (Bothe et al. 2008).

Immunohistochemical staining results carried out at the same time in our research group corroborated these results in the jejunum on the protein level. In the colon the crypts showed predominant staining (Bothe et al. 2008).

4.2.3 Do mCLCA5 and mCLCA6 Occupy Specific Functional Niches?

The expression patterns of both mCLCA5 and mCLCA6 could confirm the notion that different CLCA family members allocate to different cellular locations. CLCA are widely distributed in mammalian tissues with a species-specific cellular expression pattern for each family member described so far (Bothe et al. 2008). The cellular expression patterns of both mCLCA5 and mCLCA6 complement and broaden this picture.

This again could suggest that despite similar electrophysiological characteristics described in transfected cells, the protein products may have differing, cell specific functions or may even be multifunctional.

For example, all murine CLCA mRNA discovered and analyzed to date are expressed in different histological units of the gastro-intestinal tract: mCLCA1 and mCLCA2 were found in the stomach and the crypt epithelial cells of the intestine (Gruber et al. 1998b). Due to their high sequence homology and lack of differentiation in *in situ* hybridization in the intestine the expression pattern represented the sum of both. mCLCA3 was immunolocalized to secretory granules of goblet cells in the intestinal tract as well as the respiratory and reproductive tracts (Leverkoehne and Gruber 2002). Its human ortholog hCLCA1 and its orthologs in other species, equine eCLCA1 (Anton et al. 2005) and porcine pCLCA1 (Plog et al. 2009), all have an expression in goblet cells in common. mCLCA4 was located to smooth muscle cells (Eible et al. 2002) including those in the stomach and intestine. Here, mCLCA5 was predominantly expressed in the keratinocytes of the stomach. mCLCA6 on the other hand was found in the intestinal tract in the lumenally located epithelial cells, a niche where no other CLCA family member has been described.

Allocation of different murine CLCA family members to different cell types in the intestinal microenvironment supports the model of different evolutionary paths for each homolog and suggests the proteins filling different functional niches.

The mRNA localizations of mCLCA5 and mCLCA6 could lead to subsequent speculations about their protein function, provided that the proteins are functional.

CONCLUDING DISCUSSION

LCM and immune electron microscopy located mCLCA5 to keratinocytes of the stratified epithelium of the stomach. Therefore, a role in cornification of stratified squamous epithelia is plausible. In stratified squamous epithelia proliferation takes place only in the basal layer, whereas the stratum spinosum and stratum granulosum display layers with primarily differentiating and not proliferating keratinocytes (Yuspa et al. 1988). Keratinocyte differentiation in cornifying epithelium, which is largely Ca^{2+} -dependent (Yuspa et al. 1988) aims at developing the cornified envelope. In the pars nonglandularis of the stomach mCLCA5 protein was detected in keratinocytes in the granular layer, the layer that undergoes cornification. Again, the mRNA expression pattern and cellular localization could be backed by immunohistochemical staining results by our group (Braun et al. 2009).

A further aspect to consider is that the keratinocytes of the supra-basal layers are non-proliferative. A proliferation inhibiting function has already been described for mCLCA5 in association with growth suppression of metastatic breast cancer cells (Beckley et al. 2004). In that study HC11 and 4T1 cells (murine mammary epithelial cell lines) showed decreased proliferation when transiently transfected with mCLCA5, suggesting a proliferation-inhibiting role of mCLCA5.

Similar to mCLCA5, its human ortholog hCLCA2 was detected in the stratified epithelia of the cornea, skin, vagina, oesophagus and larynx with affinity-purified anti-hCLCA2 antibodies and real-time PCR and not in non-stratified epithelia of the small intestine and human stomach (Connon et al. 2005; Connon et al. 2004; Gruber et al. 1999). Unlike mCLCA5, hCLCA2 was localized to the basal cells adjacent to the basement membrane (Connon et al. 2004). The close apposition of hCLCA2 to hemidesmosomes led to the presumption of a role in epithelial stratification and particularly in adhesion mechanisms involving integrin- β_4 , a component of hemidesmosomes (Carter et al. 1990; Connon et al. 2004). A ligation between hCLCA2 and integrin- β_4 is a feature of both quiescent stratified epithelia and metastatic cancer cells (Connon et al. 2005). The mCLCA5 protein is also reported to have a β_4 binding domain (Abdel-Ghany et al. 2003) but was detected in the differentiated keratinocytes of the stratum granulosum of the stomach and not next to the basement membrane where hemidesmosomes are described (Braun et al. 2009). Surprisingly however, the mRNA expression pattern is broader than that described for the protein expression pattern which is limited to keratinocytes of all stratified epithelia (Braun et al. 2009). For example, only mCLCA5 mRNA could be detected in non-cornifying epithelia such as the cornea. This suggests that the mRNA may be transcribed into protein and suggests a role of the protein in the development of the

cornified envelope. Possibly mCLCA5 mRNA resides in all keratinocytes and the protein expression peaks during the process of cornification.

The heterogeneous distribution of mCLCA5 mRNA could further support the implications that it is expressed in tissues that are growth-arrested or apoptotic or being involved in anoikis (Beckley et al. 2004; Patel et al. 2006). Maybe the protein is expressed at specific times in the cell cycle. Alternatively, presuming the protein is involved in cornifying processes of stratified epithelia, the mRNA may be translated into a protein in tissues undergoing metaplasia into stratified squamous epithelium, a cell type which is not the prime target of CF disease severity.

Based on its location mCLCA5 is therefore unlikely to be a key player in the CF disease complex.

For mCLCA6, a role in transepithelial ion conduction is conceivable in enterocytes either as part of a more complex channel structure or as a modulator of other channel proteins (Bothe et al. 2008). Predominant expression of mCLCA6 at small intestinal villous tips may suggest a role in absorptive processes that typically take place in these locations. mRNA localization in the barrier cells of the intestine and taking into account that CLCA have electrophysiological characteristics of CaCC (Gandhi et al. 1998; Gruber et al. 1998b; Gruber and Pauli 1999) may suggest a role in the CF disease complex. Furthermore, mRNA expression could be corroborated on protein level by our group (Bothe et al. 2008).

Alternatively, a role as adhesion factor or in control of apoptosis and thus tumor growth inhibition as discussed previously cannot be entirely excluded (Abdel-Ghany et al. 2001; Elble and Pauli 2001).

4.3 Extrapolation from Mouse to Human

The diverging expression patterns of mCLCA5 to its human ortholog hCLCA2, and of mCLCA6 to its human ortholog hCLCA4 (Ritzka et al. 2003) could indicate the evolution of different functions of these proteins. For example, hCLCA2 was found to be the most abundant Cl⁻ channel in corneal epithelium of the eye possibly contributing to corneal transparency (Itoh et al. 2000) whereas mCLCA5 was only expressed on mRNA level in the cornea. As further example, in addition to its intestinal expression, hCLCA4 is also expressed in brain, trachea uterus, salivary gland and mammary gland (Agnel et al. 1999), whereas mCLCA6 mRNA was exclusively expressed in the intestine (Bothe et al. 2008).

CONCLUDING DISCUSSION

Although the coding sequences of the CLCA proteins are well conserved between the species, the respective non-coding regulatory sequences were subject to species-specific evolutionary change which may have led to functionally altered proteins (Ritzka et al. 2003). Moreover, different functional properties have been reported for direct orthologs of different species (Gruber et al. 1998a; Loewen et al. 2004). Therefore, in human tissues the orthologs may still succumb to different transcriptional regulation. None the less, considering the expression pattern of the two murine CLCA family members mCLCA5 and mCLCA6, mCLCA6 is more likely to play a role in CF than mCLCA5.

4.4 Quantitative mRNA Expression Comparison of Candidate CaCCs

To further investigate whether mCLCA5, mCLCA6, the remaining mCLCA family members mCLCA1 to -4, the potential alternative mediators of a CaCC (mBEST1, mBEST2, mCLC-3B, mCLC-4, mTTYH3, mTMEM16A, mTMEM16F, mTMEM16K) and SLC26A9 are differentially regulated between murine wild type and CF tissues, quantitative mRNA expression analyses were carried out. Differential expression could suggest a role in the complex of CF on transcriptional level.

The expression levels between the candidates themselves varied. mCLCA4, mTTYH3, mTMEM16A, mCLC-3B and mCLCA3 generally had highest mean value expression levels in the tissues tested. mCLCA5, mBEST1 and mCLCA1 had consistently minimal relative mRNA copy numbers.

4.4.1 Comparison of the Expression Levels between the Wild Type and *cftr* Mutant Mouse Strains

Of the six mCLCA family members, mCLCA6 had statistically significant higher expression levels in the caecum of the *cftr*^{TgH(neoim)Hgu} model on the DBA/2 background (Attachments: Figure 5) but not in any further intestinal segment tested nor in any other mouse model including the *cftr*^{tm1Cam} model exhibiting the severe intestinal phenotype. This supports the notion that mCLCA6 may be involved in the complex CF pathology in the caecum. An up-regulation in the mouse model with the mild intestinal phenotype but not in the mouse model with the severe intestinal phenotype may suggest a role in amelioration of disease severity or for a link to the *cftr*^{TgH(neoim)Hgu} genotype.

mCLCA5 was not differentially regulated between the wild type and the *cftr* mutant mouse strains. Thus, not only the cellular localization in keratinocytes but also

quantitative expression analyses in CF relevant tissues argue against a role in the CF pathology.

mCLCA3 had statistically significant higher expression levels in the stomach of the *cfr*^{TgH(neoim)Hgu} model on the DBA/2 background (Attachments: Figure 5).

Of all nine non-CLCA candidate genes tested, mTTYH3 mRNA had a strong and consistent differential expression on transcriptional level between the wild type NMRI and the CF mouse model *cfr*^{tm1Cam} throughout the intestinal tract. Both in the small and large intestinal segments mTTYH3 mRNA was down-regulated. Of note, no down-regulation was observed in the *cfr*^{TgH(neoim)Hgu} model exhibiting the milder intestinal disease phenotype. This suggests that mTTYH3 may be involved in the complex CF pathology. However, its down-regulation does not support the hypothesis of an alternative mediator of CaCC in the absence of *cfr*, at least not on transcriptional level.

All other candidate genes had only sporadically differential expression levels in select tissues especially when expressed in only minute copy numbers and without recognizable consistency (see also Attachments: Figures 5 and 6). This suggests that they are not differentially regulated on transcriptional level. CF disease modulation is therefore unlikely to be caused by an up- or down-regulation of mCLCA1, -2, -4, -5, mBEST1, mBEST2, mCLC-3B, mCLC-4, mTMEM16A, mTMEM16F or mTMEM16K on transcriptional level.

Thus, with the exception of mCLCA6 and mTTYH3 our results failed to identify consistently increased or decreased mRNA levels as evidence of transcriptional regulation or modulatory significance in CF tissues. Nevertheless, their proposed modulatory roles could still be based on posttranscriptional protein modification or functional regulation that have to be addressed in the future by other methodological approaches.

Quantitative mRNA expression levels are frequently insufficient to predict protein expression levels, both of which are of critical importance to assess functional significance in a biological system (Gygi et al. 1999). Furthermore, 20-30% of all human genes are regulated on post-transcriptional level by micro RNAs (miRNAs), which influence mRNA cleavage or protein synthesis (Landi et al. 2008).

Although mRNA of the candidate genes was detected in tissues relevant to CF, neither the presence nor the quantity of mRNA provide information of whether a translation will take place and if so whether a functional protein product will be generated at all (Bustin et al. 2009).

CONCLUDING DISCUSSION

For mCLCA6, the mRNA expression pattern was corroborated in wild type mice on protein level by work done in our group (Bothe et al. 2008) supporting its significance in the intestine, specifically in enterocytes. However, quantitative mRNA expression data frequently lack concordance with protein concentrations found in tissues (Gygi et al. 1999). Therefore, mCLCA6 may be differentially expressed between wild type and CF tissues on protein level. Semi-quantitative protein expression analyses for mCLCA3 for example revealed high protein expression levels compared to low mRNA expression levels in NMRI tissues (Leverkoehne et al. 2006), again revealing a discrepancy between both products. Therefore, it is not possible to conclude about the amount of protein based on copy numbers of mRNA.

4.4.2 Comparison of the Expression Levels between the Four Wild Type Mouse Strains of Different Genetic Background: NMRI, BALB/c, DBA/2 and C57BL/5

The expression levels of the candidate genes were compared between the four genetic backgrounds to identify potential strain influences. Comparison of the expression levels of all 15 candidates between the BALB/c and NMRI wild type strains revealed significant and consistent but reciprocal expressional differences in the intestinal tract and liver for mTMEM16A and mBEST1. mTMEM16A had an approximately 10-fold higher expression in the BALB/c strain whereas mBEST1 had an approximately 4-fold higher expression in the NMRI strain. There were significantly higher expression levels in the BALB/c strain compared the NMRI strain for mCLC-3B in the jejunum, 8-fold, and caecum, 16-fold, for mTMEM16F in the colon, 5-fold, and for mTMEM16K in the stomach, 4-fold, and caecum, 10-fold. Additional genetic effects therefore seem to have an effect on the transcriptional regulation of these two genes. All other candidates tested had no significant differences in expression between the NMRI and BALB/c wild type strains.

Data analyzing the comparison of the expression levels of mCLCA1, -2, -3, -4 and -6 between all four wild type strains are included as attachment.

They additionally revealed that mCLCA3 had overall strongest mean-value mRNA expression in the C57BL/6 strain. In the stomach this reached statistical significance to the DBA/2 and NMRI strains. mCLCA6 had overall weakest mean-value mRNA expression in the DBA/2 strain. In the caecum this reached statistical significance to the C57BL/6 and BALB/c strains (Attachments: Figure 3).

Overall, when viewing the strains in regard to their mCLCA expression, the DBA/2 strain had weakest expression of mCLCA1, -3 and -6 in almost all tissues tested, reaching statistical significance to the C57BL/6 or BALB/c strains in the stomach (mCLCA3), caecum (mCLCA6), colon (mCLCA1, mCLCA3) and lungs (mCLCA1). Other way around, individual mCLCA members were not predominantly expressed in one of the four mouse strains. Therefore, again additional genetic effects may be involved influencing the expression of the mCLCA gene products, however, only sporadically reaching statistical significance.

Taken together, these results suggest that the genetic background of the mice had an influence on the expression of select candidate genes, especially mTMEM16A and mBEST1. Strain specific differences in mRNA copy numbers have already been observed for CFTR (Ulatowski et al. 2004) and for members of the CLCA gene family (Leverkoehne et al. 2006). On the other hand, there is no evidence of the *cftr*^{TgH(neoim)Hgu} mutation itself having different effects on the mRNA expression dependent on the genetic background it was inbred on, at least not for the five mCLCA family members tested in the C57BL/6, DBA/2 and BALB/c strains in the tissues analyzed.

4.4.3 Comparison of the Expression Levels between the Two *cftr* Mutant Mouse Models: *cftr*^{TgH(neoim)Hgu} and *cftr*^{tm1Cam}

The expression levels between both mouse models were compared to identify a potential link between the type of *cftr* mutation and mRNA expression of the CaCC candidate. Statistically significant and consistent expressional differences were noted for mTTYH3 in the small and large intestine. The *cftr*^{tm1Cam} model had up to 125-fold reduced mean mRNA expression compared to the *cftr*^{TgH(neoim)Hgu} model. Although both models have different genetic backgrounds (BALB/c and NMRI for *cftr*^{TgH(neoim)Hgu} and *cftr*^{tm1Cam}, respectively) the expression of mTTYH3 mRNA in their wild type controls did not differ. This suggests that the down-regulation of mTTYH3 in the intestine of the *cftr*^{tm1Cam} model may be linked to the *cftr* genotype with the more severe phenotype.

4.4.4 Comparison of the Expression Levels between the Three *cftr*^{TgH(neoim)Hgu} Mutant Mouse Strains of Different Genetic Background: BALB/c, DBA/2 and C57BL/5

The three mouse strains BALB/c, DBA/2 and C57BL/6 were compared in order to analyze whether the strain specific expressional differences in the wild type mice were mirrored in their respective *cftr* mutant counterparts. The results are added as attachments (Attachments: Figure 4).

Only the higher expression of mCLCA1 mRNA in the lungs of the C57BL/6 CF mice compared to the DBA/2 CF strain matched the difference between the respective wild type strains in terms of statistical significance. Nevertheless, most mean value differences at least had the same tendency between the CF matched and wild type matched strains. For example, mCLCA3 had a strong expression in the stomach of the C57BL/6 CF strain and mCLCA6 had weak expression in the caecum of the DBA/2 CF strain similar as was reported in the wild type counterparts above, yet neither reached statistical significance to the other strains. Other way around, mCLCA1 had weakest expression in the caecum, colon and lung of the DBA/2 CF strain with a significant difference to the C57BL/6 strain of which only the same tendency was mirrored in the wild type strain comparison.

Otherwise, only sporadically different expression levels without recognizable consistency were noted between the four wild type strains, between the two CF models and between the three *cftr*^{TgH(neoim)Hgu} mutant strains. Therefore, the genetic background did not have an obvious different effect on the expression of the mCLCA family members dependent on the *cftr* genotype.

4.4.5 Comparison of Results with Previous Studies

Interestingly, in a previous study jejunum had an increased mRNA copy number of mCLCA3 in BALB/c *cftr*^{TgH(neoim)Hgu} and NMRI *cftr*^{tm1Cam} mouse models (Leverkoehne et al. 2006). Up-regulation of mCLCA3 was explained by an elevated number of mCLCA3 expressing goblet cells as observed in the intestine of most murine CF models (Grubb and Boucher 1999). In another study, mCLCA3 was significantly reduced in the intestine of *cftr*^{tm1Unc} mice on a C57BL/6 background although the number of goblet cells was elevated (Young et al. 2007). In this study, mCLCA3 had up-regulated expression in the stomach of the *cftr*^{TgH(neoim)Hgu} model on the DBA/2 background but not in any other tissues tested including the intestine and not in the

cftr^{tm1Cam} model. Differences in expression could be due to the different genetic backgrounds of the mice. In the case of contradictory expression levels between mice with the same genetic background factors including different housing conditions, environmental influences, nutrition and sample handling can not be excluded as having an effect on the expression levels of CLCA. Due to these contradictory results the significance of this finding and the evaluation of mCLCA3 as a modulator of the CF phenotype needs further investigations.

mCLCA2 on the other hand was hypothesized to be transcriptionally up-regulated in the jejunum of the *cftr*^{TgH(neoim)Hgu} mice on the BALB/c background (Leverkoehne et al. 2006). In this study mCLCA2 was not differentially expressed in the intestine.

However, both in this study and in the same sized groups of mice in the study by Leverkoehne (Leverkoehne et al. 2006) mRNA copy numbers of individual mice of the same group varied greatly. Most groups of five mice had at least one outlier or extreme value. These variations may conceal detection of statistically significant differences in small groups of mice. Small study populations have already led to conflicting results in the past (Buscher and Grasemann 2006).

For mCLCA5, a broader yet overlapping mRNA expression pattern was detected compared to immunohistochemical protein staining results carried out by our group. An arbitrary threshold of approximately 0.3 relative mCLCA5 copies separated tissues in which protein staining was detected (> 0.3 relative copies, except for the eye) from tissues with negative staining results (< 0.3 relative copies, except for the thymus). The observation that not all mRNA is translated into a protein could be due to differences in mRNA stability by post-transcriptional modifications or epigenetic effects on translation. Specific miRNA or small interfering RNA (siRNA) could also bind to the mRNA and increase or decrease their activity and prevent a protein product from being produced.

4.5 Do the Candidate Genes Play a Role in CF?

mCLCA6 and mCLCA3 were transcriptionally up-regulated in the caecum and the stomach of the *cftr*^{TgH(neoim)Hgu} DBA/2 mice, respectively, whereas mTTYH3 was transcriptionally down-regulated throughout the intestine of the *cftr*^{tm1Cam} NMRI mice. At least on transcriptional level both mCLCA6 and mCLCA3 may have a positive “alternative” modulatory effect on CaCC whereas mTTYH3 is more likely to have a negative modulatory effect, decreasing CaCC.

CONCLUDING DISCUSSION

The hypothesis that the CF mice lack CF like pulmonary disease and pancreas insufficiency as a consequence of transcriptional overexpression of the candidate genes tested here cannot be supported. However, the gene products may be regulated on post-transcriptional level evoking a modulatory function which is disguised when characterized by relative quantification of mRNA levels.

Based on their cellular localization and quantitative expression analyses, mCLCA6 is more likely to be involved in the pathophysiological mechanisms associated with CF than mCLCA5. Similar to several CLCA proteins, both mCLCA6 and mCLCA5 have been reported to evoke a Ca^{2+} -activated Cl^- conductance when heterologously transfected into HEK293 cells (Evans et al. 2004). Due to their different cellular localizations, different functional properties were discussed.

The localization of mCLCA6 mRNA in the intestine could point towards a potential role in modulating Cl^- secretion in the absence of functional CFTR in this localization. Differential expression on transcriptional level in the caecum supports enhanced functional properties of the gene product in the absence of CFTR and the potential to modulate other ion channel proteins similar as described for pCLCA1 (Hamann et al. 2009; Loewen et al. 2002) which subsequently could modulate disease severity. The colocalization of mCLCA6 with CFTR, as described in (Bothe et al. 2008), further supports the potential modulatory role of mCLCA6.

mCLCA5, on the other hand, was immune electron microscopically localized to stratified squamous epithelium which is not a prime target of CF disease and thus does not primarily point toward a potential role in modulating disease severity.

Based on differential mRNA expression, mTTYH3 may be involved in the complex CF pathology of the intestine. mTTYH3 mRNA was markedly down-regulated throughout the intestine of the *cfr*^{tm1Cam} mouse model with the complete functional CFTR knockout and the severe phenotype but not in the *cfr*^{TgH(neoim)Hgu} mouse model exhibiting residual CFTR Cl^- conductance and a milder intestinal phenotype. This suggests an association between the more severe intestinal phenotype and lack of mTTYH3 expression. The results argue against a direct compensatory action in the sense of an alternative anion conductance observed in CF epithelia (Clarke et al. 1994; Wilschanski et al. 1996). mTTYH3 is also unlikely to account for the CaCC conductance inhibited by CFTR or activated by defective CFTR (Kunzelmann et al. 1997b; Wei et al. 1999). On the contrary, the complete absence of functional CFTR may indirectly inhibit expression of mTTYH3. A lack or potential down-regulation of mTTYH3 expression may even exacerbate disease severity. On the other hand, it

would be interesting to investigate whether an up-regulation or enhancement of mTTYH3 expression changes intestinal phenotype, possibly reversing the process.

Taken together, the results of this project indicate that of all 15 candidates tested that possibly could modify disease severity based on their similar electrophysiological characteristics to CaCC and/or their tissue expression pattern, mCLCA6, mCLCA3 and mTTYH3 may play a role in CF as far as can be judged from the quantitative cellular RNA expression pattern analyses in CF mice. All candidates could, however, also be regulated on the functional or protein expressional level.

4.6 Conclusion

The two murine CLCA family members mCLCA5 and mCLCA6 have their own distinct cellular mRNA expression pattern. mCLCA5 mRNA had a broad expression pattern with elevated levels in tissues containing stratified squamous epithelia. mCLCA5 protein was immune electron microscopically localized to cytoplasmic granules of granular layer keratinocytes of stratified squamous epithelia. This suggests a role in cornification and thus barrier function. It does not support a potential role in CF. mCLCA6 mRNA was exclusively expressed in enterocytes suggesting a role in modulating transepithelial ion conductance and thus fluid secretion. Furthermore, it was up-regulated in the caecum of the *cfr*^{TgH(neoim)Hgu} DBA/2 mice with the mild disease phenotype further supporting a potential modulatory role in CF as part of an alternative CaCC pathway.

For mTTYH3 a significant and consistent down-regulation in the intestine of the *cfr*^{tm1Cam} mouse model compared to the wild type NMRI control was detected. This suggests a modulatory role of mTTYH3 in the pathogenesis of the severe intestinal CF phenotype most likely contributing to diminished CaCC. Only few sporadic and inconsistent significant differences were observed for the remaining candidates tested.

These investigations neither confirm nor reject the other candidate genes mCLCA1, -2, -4, -5, mBEST1, mBEST2, mCLC-3B, mCLC-4, mTMEM16A, mTMEM16F, mTMEM16K of SLC26A9 exerting modulatory functions. Thus, further investigations are necessary, especially on protein and functional levels in order to evaluate their roles in CF.

4.7 Outlook

In order to further assess the 15 candidates selected in this study as putative targets for CF disease intervention their functional niches and interacting molecules need to be clarified for further integration into the complexity of disease mechanisms.

To date, a number of genes modifying tissue specific CF disease severity have been identified (Cutting 2005). Some, for example TGFB1 (Transforming Growth Factor Beta 1), are associated with worse pulmonary outcome (Dorfman et al. 2008). Others, for example IFRD1 (Interferon-Related Developmental Regulator 1), modulate disease severity by regulating the inflammatory response in the lungs (Gu et al. 2009). In lungs, genes affecting inflammation play a key role in modulating disease severity (Collaco and Cutting 2008). Based on these observations, an interesting question to pursue is whether the candidates mCLC-3B, mCLC-4, mTTYH3, mTMEM16A, mTMEM16F and mTMEM16K expressed in the lung may stimulate increased Cl⁻ conductance after “activation” by inflammatory factors or by increased Ca²⁺ levels due to the inflammatory response secondary to the *cftr* gene-defect. For example, the cytokine IL-4 (interleukin-4), a mediator of inflammation, causes an increase in Ca²⁺-dependent Cl⁻ channel activity (Caputo et al. 2008). Thus, the inflammatory response, which is frequently a sequel to defective Cl⁻ conductance in CF, could modulate the candidate proteins expressed in the lung.

Dependent on the tissue, variability of the CF phenotype is more or less correlated with the *CFTR* genotype. For example, exocrine pancreas insufficiency is closely correlated with the *CFTR* genotype whereas lung disease is poorly correlated with the *CFTR* genotype (Cutting 2005). Intestinal disease severity is also strongly influenced by modifier genes (Cutting 2005) and mTTYH3 may be a negative modifier of the intestinal physiology in mice. A similar correlation is in fact demonstrated for ENaC (epithelial sodium channel). Absent ENaC inhibition impairs lung function in mice due to deficient *cftr* (Mall 2009).

Besides a Cl⁻ channel function of CFTR, its regulatory functions on other proteins are also important in regulating CF pathophysiology (Duran et al. 2009). CFTR is known to regulate channels and transporters including SLC26A3, a HCO₃⁻ transporter (Duran et al. 2009), ENaC (Mall et al. 2004), which is down-regulated by CFTR in respiratory epithelium (Grubb et al. 1994; Kunzelmann et al. 1997a), and volume-regulated anion channel VRAC (Vennekens et al. 1999). HCO₃⁻ secretion is important to CF pathology and is permeant through bestrophin channels (Qu and Hartzell 2008). Furthermore, Wei and coworkers concluded in their studies that CFTR and CaCC functionally interact and that CFTR reduces endogenous CaCC

currents (Wei et al. 1999). On the other hand, expression of defective CFTR could up-regulate CaCC channels (Kunzelmann et al. 1997b). The opposite seems to be true for mTTYH3 in the latter scenario. Reduced mTTYH3 expression in the *cftr*^{tm1Cam} model may be a direct consequence of lack of CFTR expression, possibly in terms of lack of activation by functional CFTR.

Electrophysiological interactions between CFTR and mTTYH3 could help clarify whether CFTR modulates the expression of mTTYH3 or whether other factors are involved. To investigate if the transcriptional down-regulation has a negative effect on the intestinal phenotype, histopathological examinations of *cftr*^{tm1Cam} mutant mice with over expression of mTTYH3 would be interesting.

The human orthologs of the two candidate gene products mBEST1 and mTMEM16A have been described to functionally interact. TMEM16A is activated by the Ca²⁺ signaling generated by BEST1 (Barro-Soria et al. 2009). It is therefore possible that in the mouse models used here mTMEM16A consistently had higher mean value relative copy numbers of mRNA compared to mBEST1 in all of the tissues tested due to enhanced specific Ca²⁺ signaling. It would therefore be interesting to test the difference in expression of mTMEM16A between wild type and mBEST1 knockout mice.

On transcriptional level, quantitative analyses of miRNAs for each candidate could reveal clues about whether these genes are regulated independently of the *cftr* genotype prior to translation (Cronin et al. 2004). miRNAs are a small class of noncoding RNA molecules, likely encoded by introns, which play a regulatory role in post-transcriptional gene regulation. They have for example been described to have an inhibitory effect on translation and cause mRNA destabilization, reviewed in (Bushati and Cohen 2007).

A comparative DNA chip analysis between wild type and CF tissues could then again confirm and/or uncover additional candidate modifier genes on the transcriptional regulatory level.

On post transcriptional level, quantitative protein and functional analyses of the candidates would be interesting in order to analyze in how far the mRNA expression levels and differences correlate with the protein expression levels.

Finally, similar expression analyses as carried out in the mouse models could be carried out in the recently developed pig models of CF, which resemble the human

CONCLUDING DISCUSSION

more closely in physiology, size, genetics and CF phenotype (Rogers et al. 2008a; Rogers et al. 2008b; Rogers et al. 2008c).

5. Summary

Candidates for Alternative Chloride Conductance in Cystic Fibrosis: Localization of mCLCA5 and mCLCA6 and Quantitative mRNA Expression Analyses of 15 Candidate Genes

Josephine Braun

Cystic Fibrosis (CF) is a fatal autosomal recessive inherited genetic disease in humans caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. *CFTR* encodes for a Cl⁻ channel in secretory epithelia. Its absence or malfunction lead to altered regulation of Cl⁻ secretion across epithelial membranes and a phenotype of severe pulmonary disease, pancreatic insufficiency, meconium ileus in newborns and other conditions.

Interestingly, the CF phenotype has large variations. Even dizygous twins sharing most environmental influences show clinical variations. This suggests that factors other than *CFTR* modulate disease severity. One of these factors is a *CFTR*-independent, Ca²⁺-activated Cl⁻ current (CaCC) which was demonstrated to ameliorate disease severity in the intestine, lungs and pancreas of CF mouse models and in part in humans. The molecules responsible for these CaCC may be potential therapeutical targets. However, its molecular identity is still unknown.

First aim of the study was to investigate the tissue specific and cellular mRNA expression pattern of two new murine CLCA family members (mCLCA5 and mCLCA6) as important prerequisite for protein and functional analyses. Second aim was to characterize the cellular mRNA expression levels of select CaCC candidates and modulators and possibly transcriptional regulatory changes under conditions of CF.

This study used laser capture microdissection (LCM) and immune electron microscopy to determine cellular localizations of mCLCA5 and mCLCA6. It used reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) to quantify and compare the mRNA expression of 15 candidate modulators of CaCC in tissues relevant to CF (stomach, duodenum, jejunum, caecum, colon, pancreas, liver, lung, kidney) between two different CF mouse models and their wild type controls. One mouse model (*cfr*^{TgH(neoim)Hgu} on BALB/c, DBA/2 and C57BL/6 backgrounds) exhibits residual *cfr* and a milder phenotype than the other mouse model (*cfr*^{tm1Cam} on NMRI background) with the complete *cfr* knockout.

SUMMARY

Candidates tested were the Ca^{2+} -activated Cl^- channels mBEST1, mBEST2, mTTYH3, mTMEM16A, mTMEM16F, mTMEM16K, the Ca^{2+} -activated Cl^- transporters mCLC-3B and mCLC-4, as well as six murine CLCA gene family members (mCLCA1 to -6), which encode proteins that evoke Ca^{2+} -activated Cl^- currents but do not form channels themselves. Additionally the Cl^- channel SLC26A9 was tested.

Similar to their homologs and orthologs within the CLCA gene family, mCLCA5 and mCLCA6 had their own distinct cellular localizations. mCLCA5 mRNA had a broad expression pattern whereas the protein was found exclusively in cytoplasmic granules of granular layer keratinocytes of stratified squamous epithelia suggesting a role in cornification and an inferior relevance in CF. mCLCA6 mRNA was exclusively expressed in enterocytes suggesting a role in transepithelial anion conductance and fluid secretion which may be of relevance in CF.

Of all investigated candidates, only mCLCA6 and mCLCA3 were up-regulated in the caecum and stomach of the *cftr*^{TgH(neoim)Hgu} DBA/2 mice respectively and only the tweety Cl^- channel mTTYH3 was markedly down-regulated throughout the intestinal tract of the *cftr*^{tm1Cam} NMRI mice. This supports mCLCA6 and mCLCA3 as potential mediators of “alternative” CaCC in CF but argues against a direct compensatory mechanism on transcriptional level in the lungs, pancreas or intestine. On the contrary, mTTYH3 may be linked to the more severe intestinal phenotype suggesting a negative modulatory effect. However, it cannot be excluded that any of the candidates tested are regulated on post-transcriptional level or by modulation of the protein activation status. Thus they may still play a role as modulators in the basic Cl^- secretory defect of CF and await further analyses.

6. Zusammenfassung

Kandidaten der alternativen Chloridleitfähigkeit bei Zystischer Fibrose: Lokalisierung von mCLCA5 und mCLCA6 und quantitative mRNA Expressionsanalysen von 15 Kandidatengenegen

Josephine Braun

Zystische Fibrose (englisch cystic fibrosis, CF) ist eine tödlich verlaufende, autosomal rezessiv vererbte Erkrankung, die durch Mutationen des *CFTR*-Gens verursacht wird. *CFTR* codiert für einen Chloridkanal in sekretorischen Epithelien, dessen Fehlen oder Defekt eine fehlregulierte Chloridströmung und schwerwiegende Lungenerkrankungen, Pankreasinsuffizienz sowie Mekoniumileus bei Neugeborenen hervorrufen kann.

Interessanterweise zeigen dizygote Zwillinge, die den identischen *CFTR*-Defekt tragen und unter gleichen Umweltbedingungen leben, eine hohe klinische und pathologische Variation des CF-Phänotyps. Über den *CFTR*-Gendefekt hinausgehende genetische Faktoren scheinen den Schweregrad der Erkrankung zu beeinflussen. Zu diesen sogenannten *modifier genes* gehört eine *CFTR*-unabhängige, Ca^{2+} -aktivierte Chloridleitfähigkeit (CaCC), die den Darm-, Lungen- und Pankreas-Phänotyp in CF-Mausmodellen mildert und sich möglicherweise als therapeutisches Ziel eignet. Leider ist deren molekulare Identität und Modulation bislang jedoch ungeklärt.

Erstes Ziel der Studie war es, das bislang unbekannte gewebsspezifische und zelluläre mRNA-Expressionsmuster von zwei murinen CLCA Vertretern (mCLCA5 und mCLCA6) zu bestimmen. Zweites Ziel war es die zellulären mRNA Expressionshöhen ausgewählter CaCC-Kandidaten und Modulatoren zu analysieren sowie mögliche regulatorische Veränderungen auf transkriptioneller Ebene unter CF Bedingungen festzustellen.

Diese Studie hat mittels Lasermikrodissektion und Immunelektronenmikroskopie die zelluläre Lokalisation von mCLCA5 und mCLCA6 bestimmt. Sie quantifiziert und vergleicht mithilfe der Reversen Transkriptase quantitativen Polymerasekettenreaktion (RT-qPCR) die mRNA-Expression von 14 Vertretern, die potenzielle CaCC-Kandidaten oder -Modulatoren darstellen, in CF-relevanten Geweben (Magen, Duodenum, Jejunum, Zäkum, Kolon, Pankreas, Leber, Lunge, Niere) zwischen zwei verschiedenen CF-Mausmodellen und deren Wildtyp-Kontrollen. Eines der hier untersuchten Mausmodelle (*cftr*^{TgH(neoim)Hgu} auf BALB/c, DBA/2 und C57BL/6 Hintergründen) zeigt eine residuelle *cftr*-Aktivität und einen milderen Phänotyp, während das andere

ZUSAMMENFASSUNG

Mausmodell (*cfr*^{tm1Cam} auf NMRI Hintergrund) einen kompletten *cfr*-Knockout darstellt und einen schwereren Phänotyp zeigt.

In den Mausmodellen wurden die Ca²⁺-aktivierten Chloridkanäle mBEST1, mBEST2, mTTYH3, mTMEM16A, mTMEM16F, mTMEM16K und die Ca²⁺-aktivierten Chloridtransporter mCLC-3B und mCLC-4 untersucht. Des Weiteren wurden sechs Mitglieder der CLCA Genfamilie (mCLCA1 bis -6) analysiert, die selbst keine Kanalproteine sind, jedoch Ca²⁺-aktivierte Chloridströme hervorrufen können. Zusätzlich wurde der Chloridkanal SLC26A9 untersucht.

mCLCA5 und mCLCA6 zeigten, ähnlich wie ihre Homologen und Orthologen, jeweils spezifische zelluläre Lokalisationen. mCLCA5 mRNA zeigte ein weites Expressionsmuster, wobei das Protein nur in zytoplasmatischen Granula der Keratinozyten des Stratum granulosum aller Plattenepithelien gefunden wurde, was auf eine Funktion in der Verhornung vermuten lässt, und für CF eine untergeordnete Rolle zu spielen scheint. mCLCA6-mRNA wurde ausschließlich in Enterozyten gefunden. Diese Lokalisation deutet auf eine Funktion bei der transepithelialen Anionenströmung hin, welche für CF von hoher Relevanz sein könnte.

Von allen untersuchten Kandidaten waren nur mCLCA6 und mCLCA3 im Zäkum bzw. Magen der *cfr*^{TgH(neoim)Hgu} DBA/2-Mäuse hochreguliert, wohingegen der Tweety-Chloridkanal mTTYH3 im gesamten Darmtrakt der *cfr*^{tm1Cam} NMRI-Mäuse herunterreguliert war.

Diese Ergebnisse sprechen dafür, dass mCLCA6 und mCLCA3 Mediatoren der „alternativen“ CaCC in CF sein könnten, sprechen jedoch gegen einen direkten kompensatorischen Mechanismus auf transkriptioneller Ebene in Lunge, Pankreas oder Darm, mTTYH3 könnte dagegen sogar mit einem schwerwiegenden intestinalen Phänotyp im Zusammenhang stehen. Es kann jedoch nicht ausgeschlossen werden, dass einer oder mehrere der anderen untersuchten Kandidaten auf post-transkriptioneller Ebene oder durch Modulation des Proteinaktivierungsstatus reguliert wird und daher trotzdem als Modulator im Erkrankungskomplex CF eine Rolle spielt.

7. References

- Abdel-Ghany M, Cheng HC, Elble RC, Lin H, DiBiasio J, Pauli BU (2003) The interacting binding domains of the beta(4) integrin and calcium-activated chloride channels (CLCAs) in metastasis. *J Biol Chem* 278:49406-49416
- Abdel-Ghany M, Cheng HC, Elble RC, Pauli BU (2001) The breast cancer beta 4 integrin and endothelial human CLCA2 mediate lung metastasis. *J Biol Chem* 276:25438-25446
- Agnel M, Vermat T, Culouscou JM (1999) Identification of three novel members of the calcium-dependent chloride channel (CaCC) family predominantly expressed in the digestive tract and trachea. *FEBS Lett* 455:295-301
- Anton F, Leverkoehne I, Mundhenk L, Thoreson WB, Gruber AD (2005) Overexpression of eCLCA1 in small airways of horses with recurrent airway obstruction. *J Histochem Cytochem* 53:1011-1021
- Barro Soria R, Spitzner M, Schreiber R, Kunzelmann K (2006) Bestrophin 1 enables Ca²⁺ activated Cl⁻ conductance in epithelia. *J Biol Chem* 284:29405-12.
- Barro-Soria R, Aldehni F, Almaca J, Witzgall R, Schreiber R, Kunzelmann K (2009) ER-localized bestrophin 1 activates Ca(2+)-dependent ion channels TMEM16A and SK4 possibly by acting as a counterion channel. *Pflugers Arch* 459:485-97.
- Barro-Soria R, Schreiber R, Kunzelmann K (2008) Bestrophin 1 and 2 are components of the Ca(2+) activated Cl(-) conductance in mouse airways. *Biochim Biophys Acta* 1783:1993-2000
- Beckley JR, Pauli BU, Elble RC (2004) Re-expression of detachment-inducible chloride channel mCLCA5 suppresses growth of metastatic breast cancer cells. *J Biol Chem* 279:41634-41641
- Bertrand CA, Zhang R, Pilewski JM, Frizzell RA (2009) SLC26A9 is a constitutively active, CFTR-regulated anion conductance in human bronchial epithelia. *J Gen Physiol* 133:421-438
- Blaisdell CJ, Howard TD, Stern A, Bamford P, Bleecker ER, Stine OC (2004) CLC-2 single nucleotide polymorphisms (SNPs) as potential modifiers of cystic fibrosis disease severity. *BMC Med Genet* 5:26
- Bleich EM, Leonhard-Marek S, Beyerbach M, Breves G (2007) Characterisation of chloride currents across the proximal colon in CfrTgH(neoim)1Hgu congenic mice. *J Comp Physiol B* 177:61-73

REFERENCES

- Bothe MK, Braun J, Mundhenk L, Gruber AD (2008) Murine mCLCA6 is an integral apical membrane protein of non-goblet cell enterocytes and co-localizes with the cystic fibrosis transmembrane conductance regulator. *J Histochem Cytochem* 56:495-509
- Braun J, Bothe MK, Mundhenk L, Beck CL, Gruber AD (2009) Murine mCLCA5 is expressed in granular layer keratinocytes of stratified epithelia. *Histochem Cell Biol* 133:285-99
- Bremer LA, Blackman SM, Vanscoy LL, McDougal KE, Bowers A, Naughton KM, Cutler DJ, Cutting GR (2008) Interaction between a novel TGFB1 haplotype and CFTR genotype is associated with improved lung function in cystic fibrosis. *Hum Mol Genet* 17:2228-2237
- Bronsveld I, Mekus F, Bijman J, Ballmann M, de Jonge HR, Laabs U, Halley DJ, Ellemunter H, Mastella G, Thomas S, Veeze HJ, Tummler B (2001) Chloride conductance and genetic background modulate the cystic fibrosis phenotype of Delta F508 homozygous twins and siblings. *J Clin Invest* 108:1705-1715
- Bronsveld I, Mekus F, Bijman J, Ballmann M, Greipel J, Hundrieser J, Halley DJ, Laabs U, Busche R, De Jonge HR, Tummler B, Veeze HJ (2000) Residual chloride secretion in intestinal tissue of deltaF508 homozygous twins and siblings with cystic fibrosis. The European CF Twin and Sibling Study Consortium. *Gastroenterology* 119:32-40
- Buchanan PJ, Ernst RK, Elborn JS, Schock B (2009) Role of CFTR, *Pseudomonas aeruginosa* and Toll-like receptors in cystic fibrosis lung inflammation. *Biochem Soc Trans* 37:863-867
- Burgemeister R (2005) New aspects of laser microdissection in research and routine. *J Histochem Cytochem* 53:409-412
- Buscher R, Grasemann H (2006) Disease modifying genes in cystic fibrosis: therapeutic option or one-way road? *Naunyn Schmiedebergs Arch Pharmacol* 374:65-77
- Bushati N, Cohen SM (2007) microRNA functions. *Annu Rev Cell Dev Biol* 23:175-205
- Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 25:169-193
- Bustin SA, Benes V, Garson JA, Hellems J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 55:611-622

- Caputo A, Caci E, Ferrera L, Pedemonte N, Barsanti C, Sondo E, Pfeffer U, Ravazzolo R, Zegarra-Moran O, Galletta LJ (2008) TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. *Science* 322:590-594
- Carter WG, Kaur P, Gil SG, Gahr PJ, Wayner EA (1990) Distinct functions for integrins alpha 3 beta 1 in focal adhesions and alpha 6 beta 4/bullous pemphigoid antigen in a new stable anchoring contact (SAC) of keratinocytes: relation to hemidesmosomes. *J Cell Biol* 111:3141-3154
- Clarke LL, Grubb BR, Yankaskas JR, Cotton CU, McKenzie A, Boucher RC (1994) Relationship of a non-cystic fibrosis transmembrane conductance regulator-mediated chloride conductance to organ-level disease in Cfr(-/-) mice. *Proc Natl Acad Sci U S A* 91:479-483
- Collaco JM, Cutting GR (2008) Update on gene modifiers in cystic fibrosis. *Curr Opin Pulm Med* 14:559-566
- Collins FS (1992) Cystic fibrosis: molecular biology and therapeutic implications. *Science* 256:774-779
- Connon CJ, Kawasaki S, Yamasaki K, Quantock AJ, Kinoshita S (2005) The quantification of hCLCA2 and colocalisation with integrin beta4 in stratified human epithelia. *Acta Histochem* 106:421-425
- Connon CJ, Yamasaki K, Kawasaki S, Quantock AJ, Koizumi N, Kinoshita S (2004) Calcium-activated chloride channel-2 in human epithelia. *J Histochem Cytochem* 52:415-418
- Cronin M, Ghosh K, Sistare F, Quackenbush J, Vilker V, O'Connell C (2004) Universal RNA reference materials for gene expression. *Clin Chem* 50:1464-1471
- Cunningham SA, Awayda MS, Bubien JK, Ismailov, II, Arrate MP, Berdiev BK, Benos DJ, Fuller CM (1995) Cloning of an epithelial chloride channel from bovine trachea. *J Biol Chem* 270:31016-31026
- Cutting GR (2005) Modifier genetics: cystic fibrosis. *Annu Rev Genomics Hum Genet* 6:237-260
- Davidson DJ, Dorin JR, McLachlan G, Ranaldi V, Lamb D, Doherty C, Govan J, Porteous DJ (1995) Lung disease in the cystic fibrosis mouse exposed to bacterial pathogens. *Nat Genet* 9:351-357
- Davidson DJ, Rolfe M (2001) Mouse models of cystic fibrosis. *Trends Genet* 17:29-37
- Delaney SJ, Alton EW, Smith SN, Lunn DP, Farley R, Lovelock PK, Thomson SA, Hume DA, Lamb D, Porteous DJ, Dorin JR, Wainwright BJ (1996) Cystic

REFERENCES

- fibrosis mice carrying the missense mutation G551D replicate human genotype-phenotype correlations. *EMBO J* 15:955-963
- Devuyst O, Guggino WB (2002) Chloride channels in the kidney: lessons learned from knockout animals. *Am J Physiol Renal Physiol* 283:1176-1191
- Dickinson P, Kimber WL, Kilanowski FM, Webb S, Stevenson BJ, Porteous DJ, Dorin JR (2000) Enhancing the efficiency of introducing precise mutations into the mouse genome by hit and run gene targeting. *Transgenic Res* 9:55-66
- Dorfman R, Sandford A, Taylor C, Huang B, Frangolias D, Wang Y, Sang R, Pereira L, Sun L, Berthiaume Y, Tsui LC, Pare PD, Durie P, Corey M, Zielenski J (2008) Complex two-gene modulation of lung disease severity in children with cystic fibrosis. *J Clin Invest* 118:1040-1049
- Dorin JR, Dickinson P, Alton EW, Smith SN, Geddes DM, Stevenson BJ, Kimber WL, Fleming S, Clarke AR, Hooper ML, et al. (1992) Cystic fibrosis in the mouse by targeted insertional mutagenesis. *Nature* 359:211-215
- Dorin JR, Stevenson BJ, Fleming S, Alton EW, Dickinson P, Porteous DJ (1994) Long-term survival of the exon 10 insertional cystic fibrosis mutant mouse is a consequence of low level residual wild-type *Cftr* gene expression. *Mamm Genome* 5:465-472
- Dorwart MR, Shcheynikov N, Yang D, Muallem S (2008) The solute carrier 26 family of proteins in epithelial ion transport. *Physiology (Bethesda)* 23:104-114
- Drumm ML (2001) Modifier genes and variation in cystic fibrosis. *Respir Res* 2:125-128
- Duran C, Thompson CH, Xiao Q, Hartzell HC (2009) Chloride Channels: Often Enigmatic, Rarely Predictable. *Annu Rev Physiol* 72:95-121
- Eggermont J (2004) Calcium-activated chloride channels: (un)known, (un)loved? *Proc Am Thorac Soc* 1:22-27
- Elble RC, Ji G, Nehrke K, DeBiasio J, Kingsley PD, Kotlikoff MI, Pauli BU (2002) Molecular and functional characterization of a murine calcium-activated chloride channel expressed in smooth muscle. *J Biol Chem* 277:18586-18591
- Elble RC, Pauli BU (2001) Tumor suppression by a proapoptotic calcium-activated chloride channel in mammary epithelium. *J Biol Chem* 276:40510-40517
- Elble RC, Walia V, Cheng HC, Connon CJ, Mundhenk L, Gruber AD, Pauli BU (2006) The putative chloride channel hCLCA2 has a single C-terminal transmembrane segment. *J Biol Chem* 281:29448-29454
- Ellsworth RE, Jamison DC, Touchman JW, Chissoe SL, Braden Maduro VV, Bouffard GG, Dietrich NL, Beckstrom-Sternberg SM, Iyer LM, Weintraub LA, Cotton M, Courtney L, Edwards J, Maupin R, Ozersky P, Rohlfing T,

- Wohldmann P, Miner T, Kemp K, Kramer J, Korf I, Pepin K, Antonacci-Fulton L, Fulton RS, Minx P, Hillier LW, Wilson RK, Waterston RH, Miller W, Green ED (2000) Comparative genomic sequence analysis of the human and mouse cystic fibrosis transmembrane conductance regulator genes. *Proc Natl Acad Sci U S A* 97:1172-1177
- Evans SR, Thoreson WB, Beck CL (2004) Molecular and functional analyses of two new calcium-activated chloride channel family members from mouse eye and intestine. *J Biol Chem* 279:41792-41800
- Fuller CM, Benos DJ (2000a) Ca(2+)-Activated Cl(-) Channels: A Newly Emerging Anion Transport Family. *News Physiol Sci* 15:165-171
- Fuller CM, Benos DJ (2000b) Electrophysiological characteristics of the Ca²⁺-activated Cl⁻ channel family of anion transport proteins. *Clin Exp Pharmacol Physiol* 27:906-910
- Gandhi R, Eible RC, Gruber AD, Schreur KD, Ji HL, Fuller CM, Pauli BU (1998) Molecular and functional characterization of a calcium-sensitive chloride channel from mouse lung. *J Biol Chem* 273:32096-32101
- Gentzsch M, Cui L, Mengos A, Chang XB, Chen JH, Riordan JR (2003) The PDZ-binding chloride channel ClC-3B localizes to the Golgi and associates with cystic fibrosis transmembrane conductance regulator-interacting PDZ proteins. *J Biol Chem* 278:6440-6449
- Gibson A, Lewis AP, Affleck K, Aitken AJ, Meldrum E, Thompson N (2005) hCLCA1 and mCLCA3 are secreted non-integral membrane proteins and therefore are not ion channels. *J Biol Chem* 280:27205-27212
- Gray MA, Winpenny JP, Porteous DJ, Dorin JR, Argent BE (1994) CFTR and calcium-activated chloride currents in pancreatic duct cells of a transgenic CF mouse. *Am J Physiol* 266:213-221
- Gray MA, Winpenny JP, Verdon B, McAlroy H, Argent BE (1995) Chloride channels and cystic fibrosis of the pancreas. *Biosci Rep* 15:531-541
- Grubb BR, Boucher RC (1999) Pathophysiology of gene-targeted mouse models for cystic fibrosis. *Physiol Rev* 79:193-214
- Grubb BR, Gabriel SE (1997) Intestinal physiology and pathology in gene-targeted mouse models of cystic fibrosis. *Am J Physiol* 273:258-266
- Grubb BR, Vick RN, Boucher RC (1994) Hyperabsorption of Na⁺ and raised Ca(2+)-mediated Cl⁻ secretion in nasal epithelia of CF mice. *Am J Physiol* 266:1478-1483
- Gruber AD, Eible RC, Ji HL, Schreur KD, Fuller CM, Pauli BU (1998a) Genomic cloning, molecular characterization, and functional analysis of human CLCA1,

REFERENCES

- the first human member of the family of Ca²⁺-activated Cl⁻ channel proteins. *Genomics* 54:200-214
- Gruber AD, Elble RC, Pauli BU (2002) Discovery and cloning of the CLCA gene family. *Curr Top Membr* 53:367-387
- Gruber AD, Fuller CM, Elble RC, Benos DJ, Pauli BU (2000) The CLCA gene family: a novel family of putative chloride channels. *Curr Genomics* 1:201-222
- Gruber AD, Gandhi R, Pauli BU (1998b) The murine calcium-sensitive chloride channel (mCaCC) is widely expressed in secretory epithelia and in other select tissues. *Histochem Cell Biol* 110:43-49
- Gruber AD, Levine RA (1997) In situ assessment of mRNA accessibility in heterogeneous tissue samples using elongation factor-1 alpha (EF-1 alpha). *Histochem Cell Biol* 107:411-416
- Gruber AD, Pauli BU (1999) Molecular cloning and biochemical characterization of a truncated, secreted member of the human family of Ca²⁺-activated Cl⁻ channels. *Biochim Biophys Acta* 1444:418-423
- Gruber AD, Schreur KD, Ji HL, Fuller CM, Pauli BU (1999) Molecular cloning and transmembrane structure of hCLCA2 from human lung, trachea, and mammary gland. *Am J Physiol* 276:1261-1270
- Gu Y, Harley IT, Henderson LB, Aronow BJ, Vietor I, Huber LA, Harley JB, Kilpatrick JR, Langefeld CD, Williams AH, Jegga AG, Chen J, Wills-Karp M, Arshad SH, Ewart SL, Thio CL, Flick LM, Filippi MD, Grimes HL, Drumm ML, Cutting GR, Knowles MR, Karp CL (2009) Identification of IFRD1 as a modifier gene for cystic fibrosis lung disease. *Nature* 458:1039-1042
- Gygi SP, Rochon Y, Franza BR, Aebersold R (1999) Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol* 19:1720-1730
- Gyomory K, Garami E, Galley K, Rommens JM, Bear CE (2001) Non-CFTR chloride channels likely contribute to secretion in the murine small intestine. *Pflugers Arch* 443:103-106
- Hamann M, Gibson A, Davies N, Jowett A, Walhin JP, Partington L, Affleck K, Trezise D, Main M (2009) Human clca1 modulates anionic conduction of calcium-dependent chloride currents. *J Physiol* 587:2255-74
- Hartzell C, Putzier I, Arreola J (2005) Calcium-activated chloride channels. *Annu Rev Physiol* 67:719-758
- Hartzell HC, Qu Z, Yu K, Xiao Q, Chien LT (2008) Molecular physiology of bestrophins: multifunctional membrane proteins linked to best disease and other retinopathies. *Physiol Rev* 88:639-672

- Hartzell HC, Yu K, Xiao Q, Chien LT, Qu Z (2009) Anoctamin/TMEM16 family members are Ca²⁺-activated Cl⁻ channels. *J Physiol* 587:2127-2139
- Holle H (2007) Quantitative Expressionsanalysen von CLCA-Homologen bei Mausmodellen der Zystischen Fibrose. Inaugural-Dissertation, Department of Veterinary Medicine Freie Universitaet Berlin, Berlin, Germany.
- Itoh R, Kawamoto S, Miyamoto Y, Kinoshita S, Okubo K (2000) Isolation and characterization of a Ca(2+)-activated chloride channel from human corneal epithelium. *Curr Eye Res* 21:918-925
- Jentsch TJ, Friedrich T, Schriever A, Yamada H (1999) The CLC chloride channel family. *Pflugers Arch* 437:783-795
- Jentsch TJ, Stein V, Weinreich F, Zdebik AA (2002) Molecular structure and physiological function of chloride channels. *Physiol Rev* 82:503-568
- Jonsdottir B, Bergsteinsson H, Baldursson O (2008) [Cystic fibrosis--review]. *Laeknabladid* 94:831-837
- Kerem E, Corey M, Kerem BS, Rommens J, Markiewicz D, Levison H, Tsui LC, Durie P (1990) The relation between genotype and phenotype in cystic fibrosis--analysis of the most common mutation (delta F508). *N Engl J Med* 323:1517-1522
- Komiya T, Tanigawa Y, Hirohashi S (1999) Cloning and identification of the gene gob-5, which is expressed in intestinal goblet cells in mice. *Biochem Biophys Res Commun* 255:347-351
- Kramer F, Stohr H, Weber BH (2004) Cloning and characterization of the murine Vmd2 RFP-TM gene family. *Cytogenet Genome Res* 105:107-114
- Kukavica-Ibrulj I, Levesque RC (2008) Animal models of chronic lung infection with *Pseudomonas aeruginosa*: useful tools for cystic fibrosis studies. *Lab Anim* 42:389-412
- Kunzelmann K, Kiser GL, Schreiber R, Riordan JR (1997a) Inhibition of epithelial Na⁺ currents by intracellular domains of the cystic fibrosis transmembrane conductance regulator. *FEBS Lett* 400:341-344
- Kunzelmann K, Mall M, Briel M, Hipper A, Nitschke R, Ricken S, Greger R (1997b) The cystic fibrosis transmembrane conductance regulator attenuates the endogenous Ca²⁺ activated Cl⁻ conductance of *Xenopus* oocytes. *Pflugers Arch* 435:178-181
- Kunzelmann K, Milenkovic VM, Spitzner M, Soria RB, Schreiber R (2007) Calcium-dependent chloride conductance in epithelia: is there a contribution by Bestrophin? *Pflugers Arch* 454:879-889

REFERENCES

- Kuruma A, Hartzell HC (2000) Bimodal control of a Ca(2+)-activated Cl(-) channel by different Ca(2+) signals. *J Gen Physiol* 115:59-80
- Landi D, Gemignani F, Naccarati A, Pardini B, Vodicka P, Vodickova L, Novotny J, Forsti A, Hemminki K, Canzian F, Landi S (2008) Polymorphisms within micro-RNA-binding sites and risk of sporadic colorectal cancer. *Carcinogenesis* 29:579-584
- Lee D, Ha S, Kho Y, Kim J, Cho K, Baik M, Choi Y (1999) Induction of mouse Ca(2+)-sensitive chloride channel 2 gene during involution of mammary gland. *Biochem Biophys Res Commun* 264:933-937
- Leverkoehne I, Gruber AD (2002) The murine mCLCA3 (alias gob-5) protein is located in the mucin granule membranes of intestinal, respiratory, and uterine goblet cells. *J Histochem Cytochem* 50:829-838
- Leverkoehne I, Holle H, Anton F, Gruber AD (2006) Differential expression of calcium-activated chloride channels (CLCA) gene family members in the small intestine of cystic fibrosis mouse models. *Histochem Cell Biol* 126:239-250
- Leverkoehne I, Horstmeier BA, von Samson-Himmelstjerna G, Scholte BJ, Gruber AD (2002) Real-time RT-PCR quantitation of mCLCA1 and mCLCA2 reveals differentially regulated expression in pre- and postnatal murine tissues. *Histochem Cell Biol* 118:11-17
- Loewen ME, Bekar LK, Gabriel SE, Walz W, Forsyth GW (2002) pCLCA1 becomes a cAMP-dependent chloride conductance mediator in Caco-2 cells. *Biochem Biophys Res Commun* 298:531-536
- Loewen ME, Bekar LK, Walz W, Forsyth GW, Gabriel SE (2004) pCLCA1 lacks inherent chloride channel activity in an epithelial colon carcinoma cell line. *Am J Physiol Gastrointest Liver Physiol* 287:33-41
- Loriol C, Dulong S, Avella M, Gabillat N, Boulukos K, Borgese F, Ehrenfeld J (2008) Characterization of SLC26A9, facilitation of Cl(-) transport by bicarbonate. *Cell Physiol Biochem* 22:15-30
- Mall M, Grubb BR, Harkema JR, O'Neal WK, Boucher RC (2004) Increased airway epithelial Na⁺ absorption produces cystic fibrosis-like lung disease in mice. *Nat Med* 10:487-493
- Mall MA (2009) Role of the amiloride-sensitive epithelial Na⁺ channel in the pathogenesis and as a therapeutic target for cystic fibrosis lung disease. *Exp Physiol* 94:171-174
- McIntosh I, Cutting GR (1992) Cystic fibrosis transmembrane conductance regulator and the etiology and pathogenesis of cystic fibrosis. *FASEB J* 6:2775-2782

- Milenkovic VM, Rivera A, Horling F, Weber BH (2007) Insertion and topology of normal and mutant bestrophin-1 in the endoplasmic reticulum membrane. *J Biol Chem* 282:1313-1321
- Mohammad-Panah R, Ackerley C, Rommens J, Choudhury M, Wang Y, Bear CE (2002) The chloride channel ClC-4 co-localizes with cystic fibrosis transmembrane conductance regulator and may mediate chloride flux across the apical membrane of intestinal epithelia. *J Biol Chem* 277:566-574
- Mount DB, Romero MF (2004) The SLC26 gene family of multifunctional anion exchangers. *Pflugers Arch* 447:710-721
- Muhlebach MS, Noah TL (2002) Endotoxin activity and inflammatory markers in the airways of young patients with cystic fibrosis. *Am J Respir Crit Care Med* 165:911-915
- Mundhenk L, Alfalah M, Elble RC, Pauli BU, Naim HY, Gruber AD (2006) Both cleavage products of the mCLCA3 protein are secreted soluble proteins. *J Biol Chem* 281:30072-30080
- Murray CB, Morales MM, Flotte TR, McGrath-Morrow SA, Guggino WB, Zeitlin PL (1995) ClC-2: a developmentally dependent chloride channel expressed in the fetal lung and downregulated after birth. *Am J Respir Cell Mol Biol* 12:597-604
- Nilius B, Droogmans G (2003) Amazing chloride channels: an overview. *Acta Physiol Scand* 177:119-147
- Patel AC, Morton JD, Kim EY, Alevy Y, Swanson S, Tucker J, Huang G, Agapov E, Phillips TE, Fuentes ME, Iglesias A, Aud D, Allard JD, Dabbagh K, Peltz G, Holtzman MJ (2006) Genetic segregation of airway disease traits despite redundancy of calcium-activated chloride channel family members. *Physiol Genomics* 25:502-513
- Pauli BU, Abdel-Ghany M, Cheng HC, Gruber AD, Archibald HA, Elble RC (2000) Molecular characteristics and functional diversity of CLCA family members. *Clin Exp Pharmacol Physiol* 27:901-905
- Pearson H (2009) Human genetics: One gene, twenty years. *Nature* 460:164-169
- Plog S, Mundhenk L, Klymiuk N, Gruber AD (2009) Genomic, Tissue Expression and Protein Characterization of pCLCA1, a Putative Modulator of Cystic Fibrosis in the Pig. *J Histochem Cytochem* 57:1169-81
- Pusch M (2004) Ca(2+)-activated chloride channels go molecular. *J Gen Physiol* 123:323-325
- Qu Z, Hartzell HC (2008) Bestrophin Cl⁻ channels are highly permeable to HCO₃⁻. *Am J Physiol Cell Physiol* 294:1371-1377

REFERENCES

- Rae FK, Hooper JD, Eyre HJ, Sutherland GR, Nicol DL, Clements JA (2001) TTYH2, a human homologue of the *Drosophila melanogaster* gene *tweety*, is located on 17q24 and upregulated in renal cell carcinoma. *Genomics* 77:200-207
- Ran S, Benos DJ (1992) Immunopurification and structural analysis of a putative epithelial Cl⁻ channel protein isolated from bovine trachea. *J Biol Chem* 267:3618-3625
- Ran S, Benos DJ (1991) Isolation and functional reconstitution of a 38-kDa chloride channel protein from bovine tracheal membranes. *J Biol Chem* 266:4782-4788
- Ratcliff R, Evans MJ, Cuthbert AW, MacVinish LJ, Foster D, Anderson JR, Colledge WH (1993) Production of a severe cystic fibrosis mutation in mice by gene targeting. *Nat Genet* 4:35-41
- Ratjen F, Doring G (2003) Cystic fibrosis. *Lancet* 361:681-689
- Ritzka M, Stanke F, Jansen S, Gruber AD, Pusch L, Woelfl S, Veeze HJ, Halley DJ, Tümmler B (2004) The CLCA gene locus as a modulator of the gastrointestinal basic defect in cystic fibrosis. *Hum Genet* 115:483-491
- Ritzka M, Weinel C, Stanke F, Tümmler B (2003) Sequence Comparison of the Whole Murine and Human CLCA Locus Reveals Conserved Synteny Between Both Species. *Genome Letters* 2:149-154
- Rock JR, O'Neal WK, Gabriel SE, Randell SH, Harfe BD, Boucher RC, Grubb BR (2009) Transmembrane protein 16A (TMEM16A) is a Ca²⁺-regulated Cl⁻ secretory channel in mouse airways. *J Biol Chem* 284:14875-14880
- Rogers CS, Abraham WM, Brogden KA, Engelhardt JF, Fisher JT, McCray PB, Jr., McLennan G, Meyerholz DK, Namati E, Ostedgaard LS, Prather RS, Sabater JR, Stoltz DA, Zabner J, Welsh MJ (2008a) The porcine lung as a potential model for cystic fibrosis. *Am J Physiol Lung Cell Mol Physiol* 295:240-263
- Rogers CS, Hao Y, Rokhlina T, Samuel M, Stoltz DA, Li Y, Petroff E, Vermeer DW, Kabel AC, Yan Z, Spate L, Wax D, Murphy CN, Rieke A, Whitworth K, Linville ML, Korte SW, Engelhardt JF, Welsh MJ, Prather RS (2008b) Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. *J Clin Invest* 118:1571-1577
- Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, Rogan MP, Pezzulo AA, Karp PH, Itani OA, Kabel AC, Wohlford-Lenane CL, Davis GJ, Hanfland RA, Smith TL, Samuel M, Wax D, Murphy CN, Rieke A, Whitworth K, Ue A, Starner TD, Brogden KA, Shilyansky J, McCray PB, Jr.,

- Zabner J, Prather RS, Welsh MJ (2008c) Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. *Science* 321:1837-1841
- Romio L, Musante L, Cinti R, Seri M, Moran O, Zegarra-Moran O, Galletta LJ (1999) Characterization of a murine gene homologous to the bovine CaCC chloride channel. *Gene* 228:181-188
- Roussa E, Wittschen P, Wolff NA, Torchalski B, Gruber AD, Thevenod F (2010) Cellular Distribution and Subcellular Localization of mCLCA1/2 in Murine Gastrointestinal Epithelia. *J Histochem Cytochem* 58:653-68
- Rowntree RK, Harris A (2003) The phenotypic consequences of CFTR mutations. *Ann Hum Genet* 67:471-485
- Rozmahel R, Wilschanski M, Matin A, Plyte S, Oliver M, Auerbach W, Moore A, Forstner J, Durie P, Nadeau J, Bear C, Tsui LC (1996) Modulation of disease severity in cystic fibrosis transmembrane conductance regulator deficient mice by a secondary genetic factor. *Nat Genet* 12:280-287
- Santis G, Osborne L, Knight RA, Hodson ME (1990) Independent genetic determinants of pancreatic and pulmonary status in cystic fibrosis. *Lancet* 336:1081-1084
- Scholte BJ, Davidson DJ, Wilke M, De Jonge HR (2004) Animal models of cystic fibrosis. *J Cyst Fibros* 3:183-90
- Schroeder BC, Cheng T, Jan YN, Jan LY (2008) Expression cloning of TMEM16A as a calcium-activated chloride channel subunit. *Cell* 134:1019-1029
- Schwiebert EM, Benos DJ, Fuller CM (1998) Cystic fibrosis: a multiple exocrinopathy caused by dysfunctions in a multifunctional transport protein. *Am J Med* 104:576-590
- Smith SN, Steel DM, Middleton PG, Munkonge FM, Geddes DM, Caplen NJ, Porteous DJ, Dorin JR, Alton EW (1995) Bioelectric characteristics of exon 10 insertional cystic fibrosis mouse: comparison with humans. *Am J Physiol* 268:297-307
- Snouwaert JN, Brigman KK, Latour AM, Malouf NN, Boucher RC, Smithies O, Koller BH (1992) An animal model for cystic fibrosis made by gene targeting. *Science* 257:1083-1088
- Suzuki M (2006) The Drosophila tweety family: molecular candidates for large-conductance Ca²⁺-activated Cl⁻ channels. *Exp Physiol* 91:141-147
- Suzuki M, Mizuno A (2004) A novel human Cl⁻ channel family related to Drosophila flightless locus. *J Biol Chem* 279:22461-22468

REFERENCES

- Tsunenari T, Sun H, Williams J, Cahill H, Smallwood P, Yau KW, Nathans J (2003) Structure-function analysis of the bestrophin family of anion channels. *J Biol Chem* 278:41114-41125
- Ulatowski LM, Whitmore KL, Romigh T, VanderWyden AS, Satinover SM, Drumm ML (2004) Strain-specific variants of the mouse *Cftr* promoter region reveal transcriptional regulatory elements. *Hum Mol Genet* 13:1933-1941
- Vennekens R, Trouet D, Vankeerberghen A, Voets T, Cuppens H, Eggermont J, Cassiman JJ, Droogmans G, Nilius B (1999) Inhibition of volume-regulated anion channels by expression of the cystic fibrosis transmembrane conductance regulator. *J Physiol* 515:75-85
- Wang T, Weinman SA (2004) Involvement of chloride channels in hepatic copper metabolism: *ClC-4* promotes copper incorporation into ceruloplasmin. *Gastroenterology* 126:1157-1166
- Wei L, Vankeerberghen A, Cuppens H, Eggermont J, Cassiman JJ, Droogmans G, Nilius B (1999) Interaction between calcium-activated chloride channels and the cystic fibrosis transmembrane conductance regulator. *Pflugers Arch* 438:635-641
- Wilschanski MA, Rozmahel R, Beharry S, Kent G, Li C, Tsui LC, Durie P, Bear CE (1996) In vivo measurements of ion transport in long-living CF mice. *Biochem Biophys Res Commun* 219:753-759
- Yang YD, Cho H, Koo JY, Tak MH, Cho Y, Shim WS, Park SP, Lee J, Lee B, Kim BM, Raouf R, Shin YK, Oh U (2008) *TMEM16A* confers receptor-activated calcium-dependent chloride conductance. *Nature* 455:1210-1215
- Young FD, Newbigging S, Choi C, Keet M, Kent G, Rozmahel RF (2007) Amelioration of cystic fibrosis intestinal mucous disease in mice by restoration of *mCLCA3*. *Gastroenterology* 133:1928-1937
- Yuspa SH, Hennings H, Tucker RW, Jaken S, Kilkenny AE, Roop DR (1988) Signal transduction for proliferation and differentiation in keratinocytes. *Ann N Y Acad Sci* 548:191-196
- Zdebik AA, Cuffe JE, Bertog M, Korbmacher C, Jentsch TJ (2004) Additional disruption of the *ClC-2* Cl^- channel does not exacerbate the cystic fibrosis phenotype of cystic fibrosis transmembrane conductance regulator mouse models. *J Biol Chem* 279:22276-22283
- Zhu DZ, Cheng CF, Pauli BU (1991) Mediation of lung metastasis of murine melanomas by a lung-specific endothelial cell adhesion molecule. *Proc Natl Acad Sci U S A* 88:9568-9572

Zielenski J, Corey M, Rozmahel R, Markiewicz D, Aznarez I, Casals T, Larriba S, Mercier B, Cutting GR, Krebsova A, Macek M, Jr., Langfelder-Schwind E, Marshall BC, DeCelle-Germana J, Claustres M, Palacio A, Bal J, Nowakowska A, Ferec C, Estivill X, Durie P, Tsui LC (1999) Detection of a cystic fibrosis modifier locus for meconium ileus on human chromosome 19q13. *Nat Genet* 22:128-129

8. Talks and Poster

Talks:

- 2nd European CF Young Investigator Meeting (EYIM) in Lille, France (2008)
Differential expression of murine CLCA-gene family members in cystic fibrosis mouse models.
- 7th Project Supervisor Meeting of the Mukoviszidose e.V. at Schloss Mickeln, Düsseldorf, Germany (2007) Differential expression of Clca genes in cystic fibrosis.

Poster presentations:

- 2nd European CF Young Investigator Meeting (EYIM) in Lille, France (2008)
Differential expression of murine CLCA-gene family members in cystic fibrosis mouse models.
- 10th German Mucoviscidosis Congress in Würzburg, Germany (2007)
Differentielle Expressionsanalysen von Clca-Genen bei zystischer Fibrose.

9. Attachments

9.1 Quantification of mCLCA6 and mCLCA3 in Luminal and Basal Crypt Epithelial Cells of the Caecum

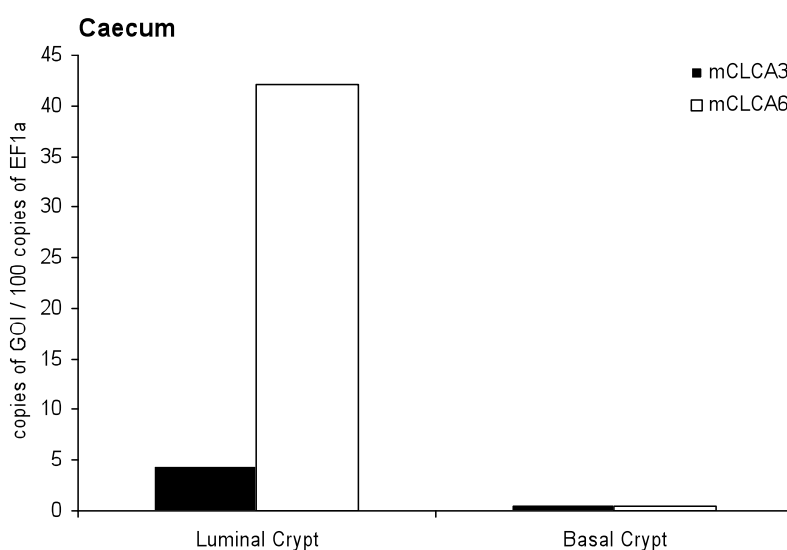
mCLCA6 and mCLCA3 mRNA were quantified in approximately 3 million μm^2 luminal and basal crypt epithelial cells of the caecum following laser capture microdissection. The caecum of one C57BL/6 10-week-old mouse was used. Killing of the mouse, tissue processing, laser capture microdissection, RNA isolation, reverse transcription, real-time quantitative polymerase chain reaction, quantification of target gene expression and statistical analyses were carried out according to the same procedures as described in the materials and methods section of the manuscript Bothe et al 2008.

The results of these data were presented and discussed in the discussion and are depicted in figure 2.

Figure 2:

Predominant expression of mCLCA6 and mCLCA3 in luminal epithelial cells of the caecum of the C57BL/6 strain

Laser capture microdissection was used to isolate approximately three million μm^2 of luminally located and basally located enterocytes of the caecum crypts prior to RT-qPCR. mCLCA6 had higher relative copy numbers in the luminal compared to the basal crypt epithelial cells and was more abundantly expressed in the luminal crypt epithelial cells than mCLCA3. n = 1 mouse.



9.2 Relative Quantification of mCLCA1, -2, -3, -4 and -6 in the *cftr*^{TgH(neoim)Hgu} Mouse Model Inbred on C57BL/6 and DBA/2, BALB/c and NMRI Genetic Backgrounds and Their Respective Wild Types

In addition to quantification in the BALB/c and NMRI strains, mCLCA1, mCLCA2, mCLCA3, mCLCA4 and mCLCA6 were also each quantified in the *cftr*^{TgH(neoim)Hgu} mouse model inbred on C57BL/6 and DBA/2 backgrounds.

Each group included five female 10-week-old mice. Killing of the mice, tissue processing, RNA isolation, reverse transcription, real-time quantitative polymerase chain reaction, quantification of target gene expression and statistical analyses were carried out according to the same procedures as described in the materials and methods section of the manuscript Braun et al 2009.

The results of these data were presented and discussed in the discussion and are depicted in figures 3 to 6.

Figure 3:

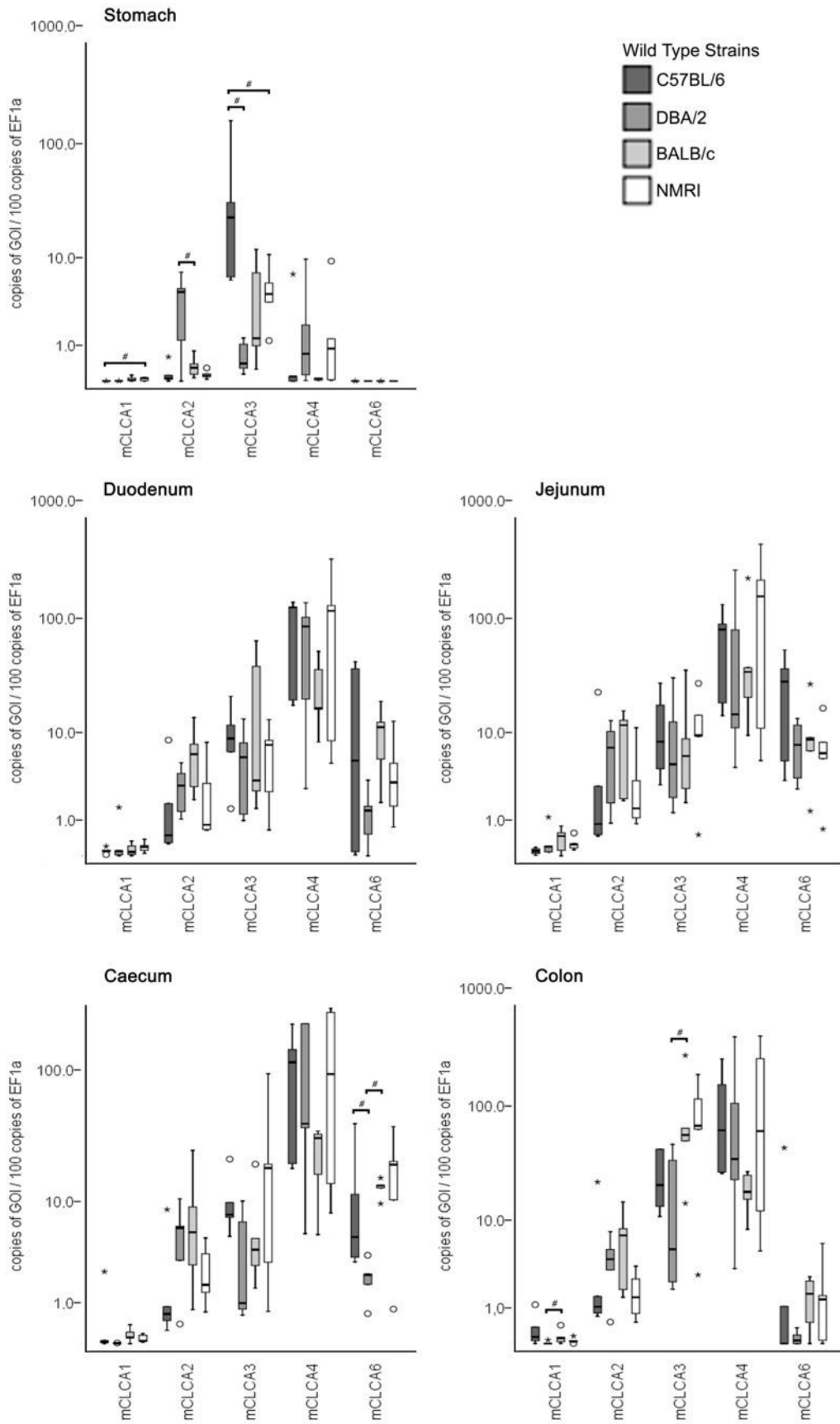
Comparison of relative expression levels of mCLCA members between the four wild type strains

For each organ analyzed the relative mRNA copy numbers of the genes of interest (GOI) for each of the four wild type strains are plotted: C57BL/6 (black columns), DBA/2 (dark grey columns), BALB/c (light grey columns) and NMRI (white columns).

mCLCA3 had overall strongest mean-value mRNA expression in the C57BL/6 strain. In the stomach this reached statistical significance to the DBA/2 and NMRI strains. mCLCA6 had overall weakest mean-value mRNA expression in the DBA/2 strain. In the caecum this reached statistical significance to the C57BL/6 and BALB/c strains.

Overall, when viewing the strains in regard to their mCLCA expression, the DBA/2 strain had weakest expression of mCLCA1, -3 and -6 in almost all tissues tested, reaching statistical significance to the C57BL/6 or BALB/c strains in the stomach (mCLCA3), caecum (mCLCA6), colon (mCLCA1, mCLCA3) and lungs (mCLCA1). Other way around, individual mCLCA members were not predominantly expressed in any of the four mouse strains.

In all box plots, outliers are marked by circles, extreme values by asterisks. An outlier has a value of more than 1.5 quartile spaces beyond the middle 50%. An extreme value is more than 3 quartile spaces beyond. A horizontal bracket with a hash marks a statistically significant difference between the wild type and CF groups. n = 5 mice per group. Please note that the y-scale is in log to the base of 10.



ATTACHMENTS

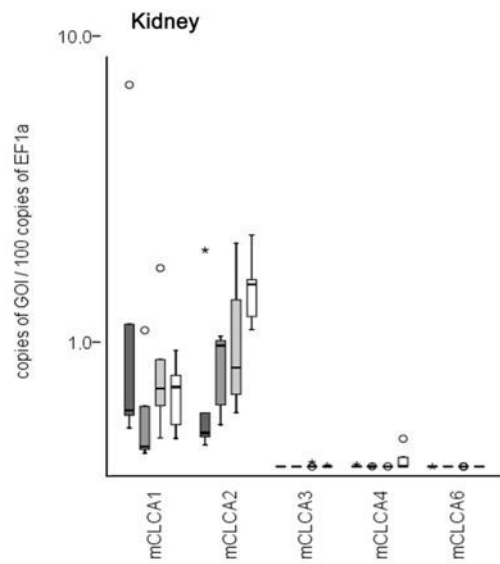
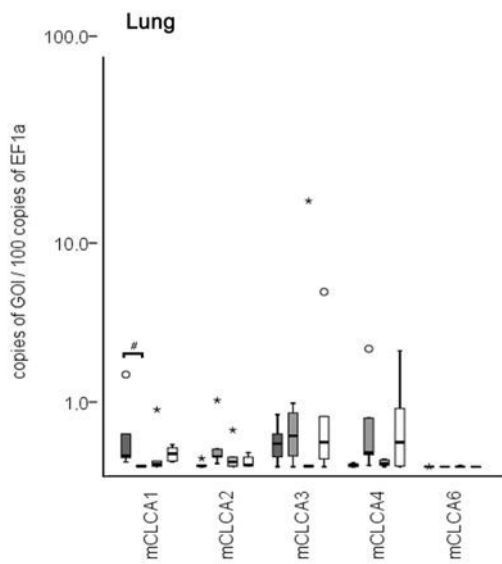
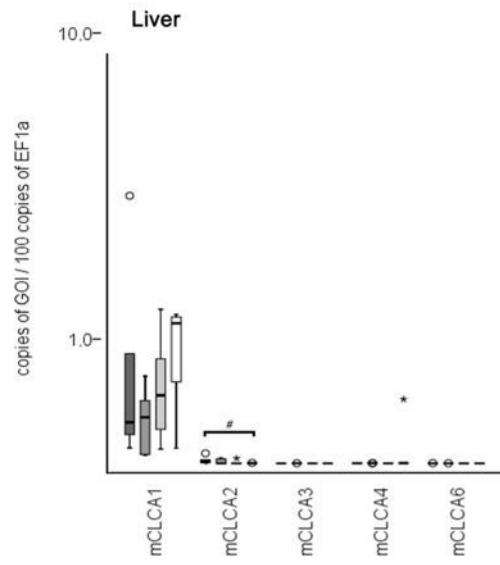
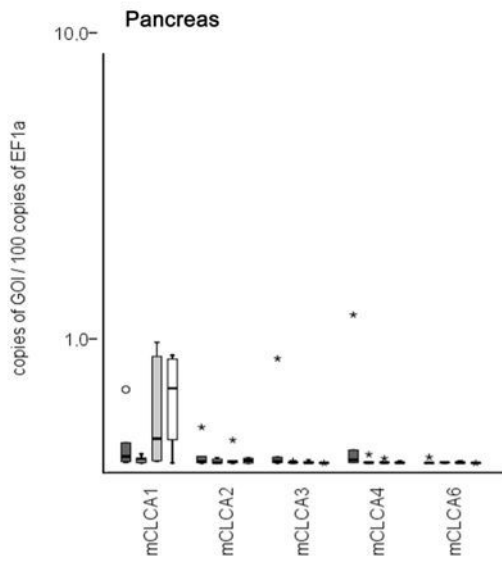


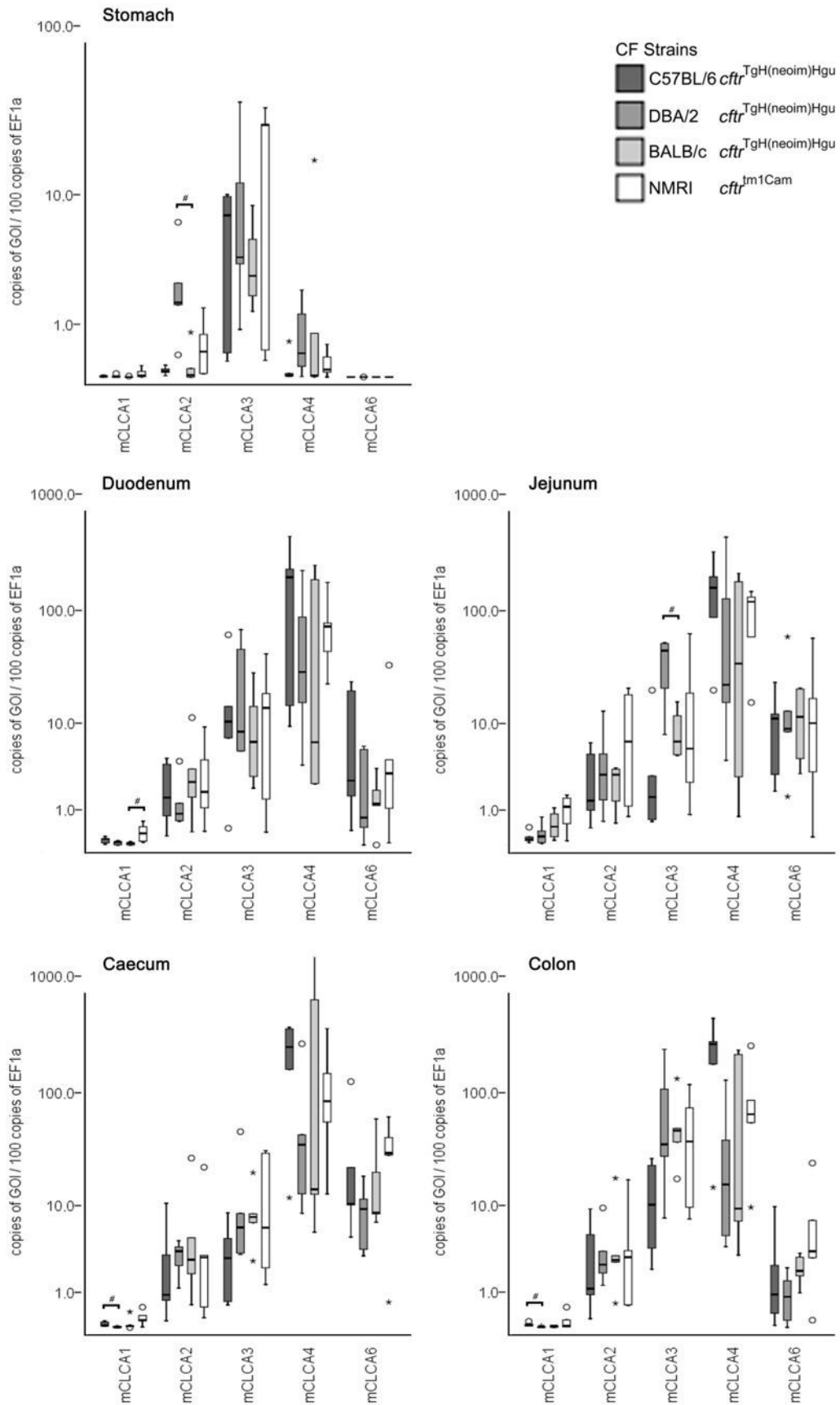
Figure 4:Comparison of relative expression levels of mCLCA members between the four CF strains

For each organ analyzed the relative mRNA copy numbers of the genes of interest (GOI) for each of the four CF strains are plotted: C57BL/6 *cftr*^{TgH(neoim)Hgu} (black columns), DBA/2 *cftr*^{TgH(neoim)Hgu} (dark grey columns), BALB/c *cftr*^{TgH(neoim)Hgu} (light grey columns) and NMRI *cftr*^{tm1Cam} (white columns).

Only the higher expression of mCLCA1 mRNA in the lungs of the C57BL/6 CF mice compared to the DBA/2 CF strain matched the difference between the respective wild type strains in terms of statistical significance. Nevertheless, most mean value differences at least had the same tendency between the CF matched and wild type matched strains. For example, mCLCA3 had a strong expression in the stomach of the C57BL/6 CF strain and mCLCA6 had weak expression in the caecum of the DBA/2 CF strain similar as was reported in the wild type counterparts, yet neither reached statistical significance to the other strains. Other way around, mCLCA1 had weakest expression in the caecum, colon and lung of the DBA/2 CF strain with a significant difference to the C57BL/6 strain of which only the same tendency was mirrored in the wild type strain comparison.

In all box plots, outliers are marked by circles, extreme values by asterisks. An outlier has a value of more than 1.5 quartile spaces beyond the middle 50%. An extreme value is more than 3 quartile spaces beyond. A horizontal bracket with a hash marks a statistically significant difference between the wild type and CF groups. n = 5 mice per group. Please note that the y-scale is in log to the base of 10.

ATTACHMENTS



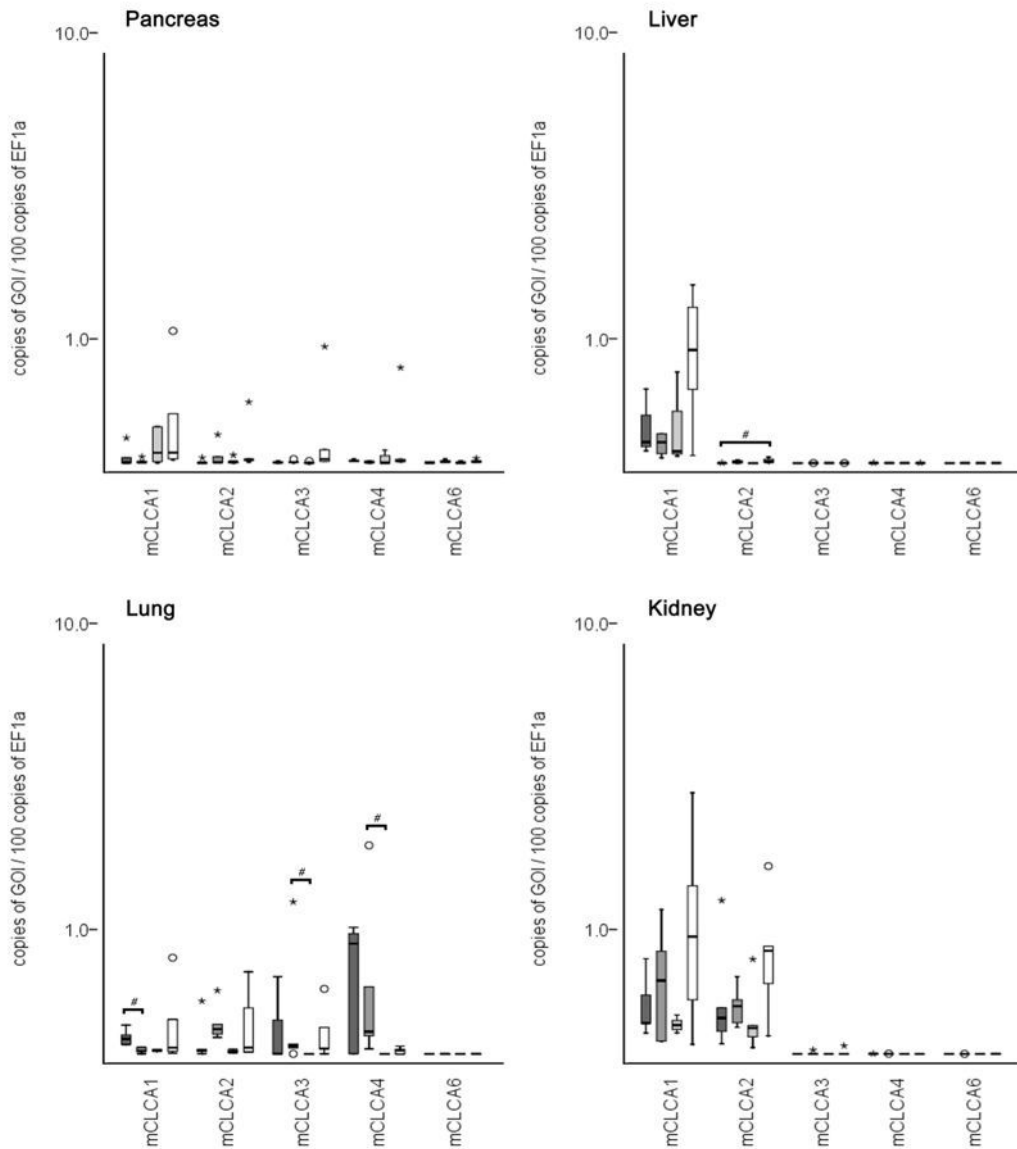


Figure 5:

Comparison of relative expression levels of mCLCA members between wild type and *ctfr*^{TgH(neoim)Hgu} mice on the DBA/2 background

The relative mRNA copy numbers of mCLCA1, mCLCA2, mCLCA3, mCLCA4 and mCLCA6 are plotted for both the wild type (black columns) and CF (grey columns) tissues of the DBA/2 mice.

mCLCA6 had statistically significant higher expression levels in the caecum and mCLCA3 had statistically significant higher expression levels in the stomach of the *ctfr*^{TgH(neoim)Hgu} model on the DBA/2 background.

In all box plots, outliers are marked by circles, extreme values by asterisks. An outlier has a value of more than 1.5 quartile spaces beyond the middle 50%. An extreme value is more than 3 quartile spaces beyond. A horizontal bracket with a hash marks a statistically significant difference between the wild type and CF groups. n = 5 mice per group. Please note that the y-scale is in log to the base of 10.

ATTACHMENTS

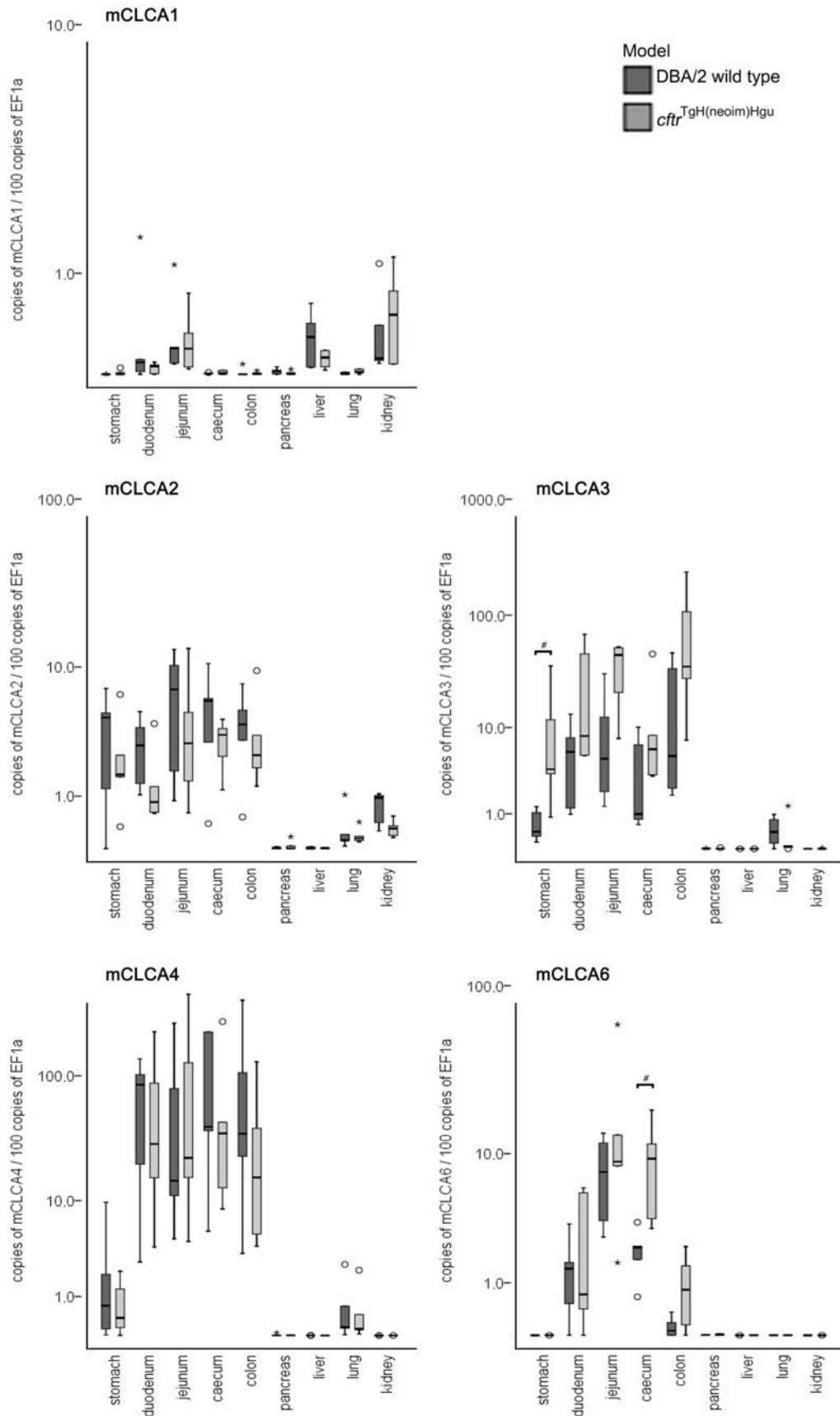


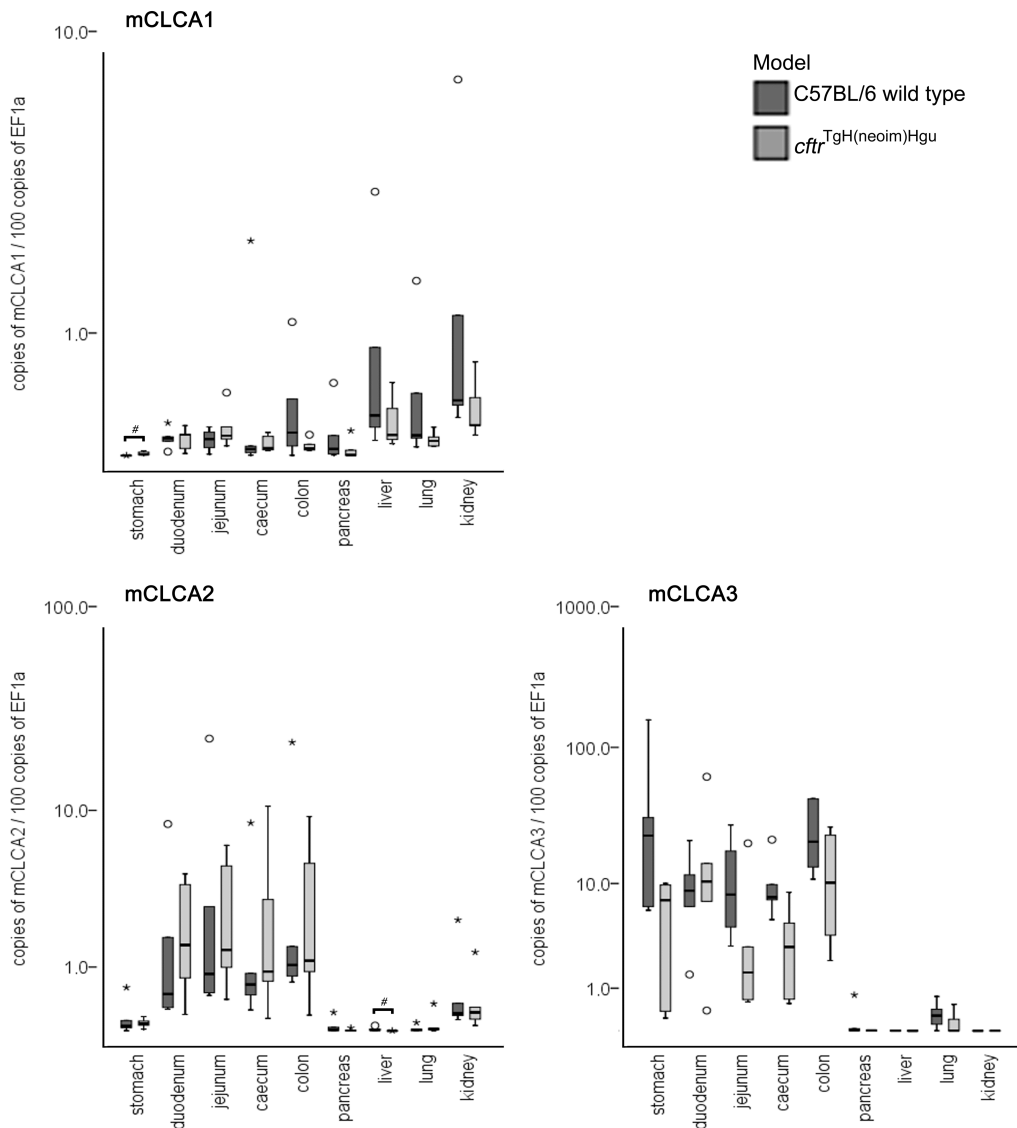
Figure 6:

Comparison of relative expression levels of mCLCA members between wild type and *cftr*^{TgH(neoim)Hgu} mice on the C57BL/6 background

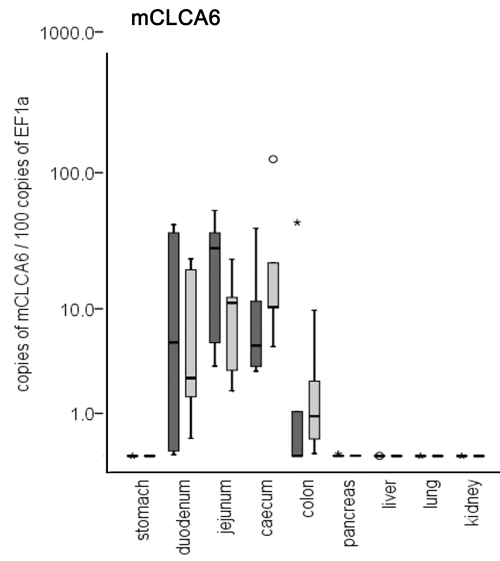
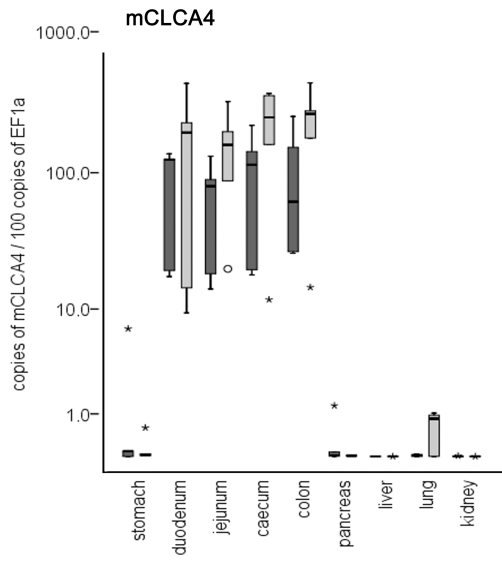
The relative mRNA copy numbers of mCLCA1, mCLCA2, mCLCA3, mCLCA4 and mCLCA6 are plotted for both the wild type (black columns) and CF (grey columns) tissues of the C57BL/6 mice.

Only sporadically differential expression levels in select tissues were detected especially when the GOI was expressed in only minute copy numbers without recognizable consistency.

In all box plots, outliers are marked by circles, extreme values by asterisks. An outlier has a value of more than 1.5 quartile spaces beyond the middle 50%. An extreme value is more than 3 quartile spaces beyond. A horizontal bracket with a hash marks a statistically significant difference between the wild type and CF groups. n = 5 mice per group. Please note that the y-scale is in log to the base of 10.



ATTACHMENTS



10. Acknowledgments / Danksagung

Herrn Prof. Dr. Achim D. Gruber, Ph.D., Geschäftsführender Direktor des Instituts für Tierpathologie der Freien Universität Berlin gilt mein ganz besonderer Dank. An diesem Institut ist die vorliegende Arbeit entstanden. Herzlich bedanke ich mich für die Aufnahme in seine Arbeitsgruppe, die kompetente Betreuung und seine jederzeit für ein Beratung offene Tür.

Der **Mukoviszidose e.V.** möchte ich besonderen Dank aussprechen für die finanzielle Unterstützung im Rahmen der Nachwuchsförderung und **Frau Dr. Jutta Bend, Frau Ingrid Frönbgen** und **Frau Dr. Sylvia Hafkemeyer** für ihre stets freundliche Betreuung.

Bei **Herrn Dr. Lars Mundhenk** bedanke ich mich ganz herzlich. Die Diskussionen mit ihm waren stets hilfreich. Mit viel Energie hat er die Arbeit mit voran gebracht. **Frau Dr. Nikola Heblinski** und **Frau Stephanie Plog** danke ich sehr herzlich für ihre unermüdliche Unterstützung. Es war eine Freude mit Euch zusammenzuarbeiten und Erfahrungen zu teilen. Bei **Frau Dr. Olivia Kershaw** und **Herrn Prof. Dr. Robert Klopffleisch** bedanke ich mich für ihre spontane Hilfsbereitschaft. Bei **Frau Dr. Friederike Range** bedanke ich mich für die Einführung in die Eigenarten des Labors, ihre Geduld und die vielen interessanten Gespräche. Bei **Frau Ursula Kobalz, Frau Anne Meyer** und **Frau Monika Schaerig** bedanke ich mich für ihre kräftige technische Unterstützung. Den weiteren **Mitarbeitern der Tierpathologie** danke ich für die andauernd freundliche Arbeitsatmosphäre, im besonderen **Frau Kathleen Lange** mit der ich die Freude hatte ein Büro zu teilen.

Frau Dr. Gisela Arndt aus dem Institut für Biometrie und Informationsverarbeitung der Freien Universität Berlin gilt mein Dank für ihre Beratung in der statistischen Auswertung und

Frau Verena Eckert-Funke aus dem Institut für Veterinär-Anatomie für ihre Unterstützung bei der Elektronenmikroskopie.

Bei **Frau Dr. Daniela Heinemann** möchte ich mich für ihre vielen aufmunternden Worte bedanken. Ein ganz besonderer Dank gilt meinen **Eltern, Riek, Pi, Bubi, Tante** und **Parvis**, die meine Nerven zusammen gehalten haben.

Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Escondido, 26.07.2010

Josephine Braun