2014
European Cystic Fibrosis Society

New Frontiers in Basic Science of Cystic Fibrosis

Conference Programme & Abstract Book

St.Julians, Malta

Chairpersons

Bob Ford, Marc Chanson and Ann Harris

26 - 29 March 2014

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CONFERENCE SPONSORSHIP & SUPPORT

The ECFS thanks the following for their support

Welcome from the ECFS President

Dear Friends and Colleagues,

It is a great pleasure to welcome you to the 11th European Cystic Fibrosis Conference entirely dedicated to Basic Science which, this year, takes place in Malta.

This year we are delighted to welcome Prof. Robert Ford as the conference Chairperson who will be supported by Dr. Marc Chanson and Prof. Ann Harris as co-chairpersons.

Basic scientific discovery is critical to our understanding of CF, and already we can see new therapies coming through from the hard work of this endeavour. The ECFS is committed to providing you with a platform to discuss your ideas with your colleagues during this important conference which will comprise a series of symposia with invited European and International guest speakers. It is a high priority for us to support the work of scientists in CF and we are proud that this conference is now established as a key annual event in the ECFS programme. Your active participation will contribute to a productive exchange of information and I hope productive collaborations.

I extend a very warm welcome to an exciting conference.

Stuart Elborn
President
European Cystic Fibrosis Society

Conference Chairpersons' Welcome

We are very happy to welcome you to the 11th Basic Science of CF conference 2014, in Malta.

Malta is an island strategically placed close to three major continents. Its history is a long and colourful one dating back to the dawn of civilisation.

The legacy of the Knights of St. John is hard to miss as they gave to Malta one of its best-known faces to the world, the eight-pointed Maltese Cross.
Across the Maltese Islands, a lot of military engineering and architectural feats are to be found: forts, bastions, fortifications, watch towers (one of them located on the premises of the conference venue), acquaducts, churches and cathedrals. We hope you will enjoy the location here in St Julians.

With this conference, you can expect to hear about recent unpublished results and engage in discussions of data and ideas in an informal and interactive environment. A key characteristic of the conference is the high attendance of young participants, both PhD students and post-docs, who contribute enormously to the success of this annual event with their enthusiasm and vivid discussions. Here you will have excellent opportunities to discuss your data and interact in a great atmosphere with the best of European and International experts in this field.
This year's programme includes a number of symposia with international experts covering topical aspects of basic research in cystic fibrosis together with invited talks from submitted abstracts. There are also two keynote lectures, one training workshop, one debate, as well as a Flash Paper session. On Thursday and Friday evening we will finish the day with poster sessions and a chance to win the best poster prize! This combination has been extremely successful in the past and the conference offers a forum for informal brain-storming-type discussions.
We welcome all scientists, not just from the field of CF research, but also from a diverse range of related topics, to an exciting conference of high scientific quality.

Bob Ford
University of Manchester
United Kingdom

Marc Chanson
University of Geneva
Switzerland

Ann Harris
Northwestern University
Chicago
United States

2014 ECFS Conference
New Frontiers in Basic Science of Cystic Fibrosis

26 March- 29 March 2014, St.Julians, Malta

Programme

Chairpersons: Bob Ford (Manchester, United Kingdom), Marc Chanson (Geneva, Switzerland) and Ann Harris (Chicago, United States)

Wednesday, 26 March 2014 (Day 1)	
11:30-17:00	**Pre-Conference Seminar - CFTR functional measurements in human models for diagnosis, prognosis and personalised therapy**
17:30-18:00	**Official Opening of the Meeting by the Conference Chairpersons** – Room Fortress
18:00-19:00	**Opening Keynote Lecture** A twenty-year scientific journey through CFTR pharmacology – Frédéric Becq (FR)
19:00-19:45	**Welcome Reception**
19:45-21:30	*Dinner*

Thursday, 27 March 2014 (Day 2)

07:30-08:45	*Breakfast*
08:45-10:30	**Symposium 1 – Structural defects of CFTR mutants**
	Chairs: David Dawson (US) - Lihua He (US)
08:45-09:10	Compounds that improve NBD1-CFTR folding efficiency synergize with compounds that promote domain assembly – Philip Thomas (US)
09:10-09:35	Major Cystic Fibrosis mutant exhibits defective phosphorylation in the amino terminal region of the regulatory "R" domain – Christine Bear (CA)
09:35-10:00	Models of the 3D structure of CFTR : from the understanding of the protein functions to the design of correctors – Isabelle Callebaut (FR)
10:00-10:10	Abstract 01: Rescue of NBD2 mutants N1303K and S1235R by small molecule correctors and transcomplementation - Liudmila Cebotaru (US)
10:10-10:20	Abstract 02: Some corrector compounds that directly modify the conformational stability of full-length F508del-CFTR stabilize and/or induce its active channel form after biosynthetic rescue - Paul Eckford (CA)
10:20-10:30	Abstract 04: Discovery of novel tailored F508del-CFTR binder correctors based on 3D structure models of entire CFTR protein for treating Cystic Fibrosis - Clément Boinot (FR)
10:30-11:00	*Coffee break* & **Poster viewing** – Bastions Rooms
11:00-12:45	**Symposium 2 – Regulation of CFTR and alternative channels**
	Chairs: Luis Galietta (IT) / Carlos Farinha (PT)
11:00-11:25	Functional regulation of ENaC by the bacterial serralysin proteases and inhibitors - Patrick Thibodeau (US)
11:25-11:50	CFTR is an external chloride-gated ion channel – Mike Gray (UK)
11:50-12:15	Purinergic regulation of mucociliary clearence and inflammation in CF - Eduardo Lazarowski (US)
12:15-12:25	Abstract 23**:** SLC6A14 elicits CFTR-mediated chloride and fluid secretion in CF airway - Tanja Gonska (CA)
12:25-12:35	Abstract 24: Regulation of the chloride channel ANO1 by microRNAs in Cystic Fibrosis patients - Florence Sonneville (FR)
12:35-12:45	Abstract 11**:** Impact of the F508del mutation on ovine CFTR, a Cl- channel with enhanced conductance and ATP-dependent gating - Zhiwei Cai (UK)
12:45-14:30	*Lunch*
14:30-15:30	**Flash Poster Session (even numbers)** **Chair: Bob Ford (UK)**
15:30-16:00	*Coffee break* & **Poster viewing**

16:00-17:45	**Symposium 3 – CFTR related disease**
	Chairs: Mike Gray (UK) / Peter Hegyi (HU)
16:00-16:25	Cystic fibrosis and bone disease: Defective human and murine osteoblast function with the F508del mutation in CFTR - Jacky Jacquot (FR)
16:25-16:50	Impairment of CFTR functional cooperation with SLC26A8, a sperm specific anion transporter, is associated with human asthenozoospermia - Aminata Touré (FR)
16:50-17:15	Modeling cystic fibrosis using primary intestinal CF organoids cultures - Jeffrey Beekman (NL)
17:15-17:25	Abstract 50: The FXR-FGF15 axis is impaired in mice with a deficiency for CFTR - Marcela Doktorova (NL)
17:25–17:35	Abstract 44: linking the CFTR to the Cystic Fibrosis Mucus Phenotype - Lauren Meiss (SE)
17:35-17:45	Abstract 71**:** Ethanol and fatty acids inhibit the activity of CFTR chloride channel and anion exchangers in pancreatic ductal epithelial cells - József Maléth (HU)
17:45-18:00	Break
18:00-19:15	**Flash Paper Session** **Chairs: William Skach US) / James Collawn (US)**
18:00-18:15	FP01: Correction of Chloride Transport and Mislocalization of CFTR Protein by Vardenafil in the Gastrointestinal Tract of Cystic Fibrosis Mice - Barbara Dhooghe (BE)
18:15-18:30	FP02: HGF stimulation of Rac1 signaling enhances pharmacological correction of the most prevalent Cystic Fibrosis mutant F508del-CFTR - Paulo Matos (PT)
18:30-18:45	FP03: Revertants, low temperature, and correctors reveal the mechanism of F508del-CFTR rescue by VX-809 and suggest multiple agents for full correction - Carlos Farinha (PT)
18:45-19:00	FP04: Airway hyperresponsiveness in FVB/N delta F508 cystic fibrosis transmembrane conductance regulator mice - Mark Bazett (CA)
19:00-19:15	FP05: Purification of the Cystic Fibrosis Transmembrane conductance Regulator protein expressed in Saccharomyces cerevisiae - Naomi Pollock (UK)
20:00-21:30	*Dinner*
21:30-23:00	**Evening Poster Session:** Posters with Even numbers - Bastions Rooms

Friday, 28 March 2014 (Day 3)	
07:30-08:45	*Breakfast*
08:45-10:30	**Symposium 4 – Host-pathogen interaction**
	Chairs: Marc Chanson (CH) / Maria Cristina Dechecchi (IT)
08:45-09:10	*P. aeruginosa* switch to mucoidity activates airway epithelial cells via the pattern recognition receptors TLR2 and TLR6 - Simon Rousseau (CA)
09:10-09:35	Interferon and Inflammasome signaling in response to *P. aeruginosa* - Alice Prince (US)
09:35-10:00	*P.aeruginosa* and modulation of IL-8 gene expression in bronchial epithelial cells - Giulio Cabrini (IT)
10:00-10:10	Abstract 34: Whole genome sequencing provides evidence of within-patient evolution and adaptation to the lung of *Mycobacterium abscessus* - Dorothy Grogono (UK)

10:10-10:20	Abstract 35: *Pseudomonas aeruginosa* reduces the expression of CFTR in airways via post translational modification of NHERF1 - Anna Tamanini (IT)
10:20-10:30	Abstract 38: Antiviral activity of azithromycin in cystic fibrosis airway epithelial cells – Aline Schögler (CH)
10:30-11:00	*Coffee break* & **Poster viewing** – Bastions Rooms
11:00-12:45	**Symposium 5 - Modifier Genes: identifying the mechanisms**
	Chairs: Mitch Drumm (US) / Patrick Harrison (IE)
11:00-11:25	Modifier genes in CF- Claude Férec (FR)
11:25-11:50	The 11p13 CF modifier region:investigating the chromatin landscape - Ann Harris (US)
11:50-12:15	Constituents of the Apical Plasma Membrane Contribute to Disease Severity Across Multiple CF-Affected Organs – Lisa Strug (CA)
12:15-12:25	Abstract 53: Genetic and functional correction of the human ΔF508 CFTR locus using designer nuclease technology – Christien Bednarski (DE)
12:25-12:35	Abstract 08**:** Study of long-range regulatory mechanisms of the CFTR gene - Stéphanie Moisan (FR)
12:35-12:45	Abstract 27: Role of microRNAs in human airway epithelium differentiation: Characterization of miR-449 as a central player in multiciliogenesis conserved in vertebrates – Benoit Chevalier (FR)
12:45-14:00	***Lunch***
14:00-17:30	***Free Afternoon***
17:30-18:30	**Flash Poster Session (odd numbers)** **Chair: David Sheppard (UK)**
18:30-20:15	**Symposium 6 – Cellular therapy for cystic fibrosis and airway epithelium repair** **Chairs: Christel Coraux (FR) / Bob Ford (UK)**
18:30-18:55	iPS cells for treatment of Cystic Fibrosis - Ulrich Martin (DE)
18:55-19:20	Cell Based Therapy: Cell Replacement and Niche Modification - Susan D. Reynolds (US)
19:20-19:45	New strategies for the improvement of CF airway epithelial repair - Emmanuelle Brochiero (CA)
19:45-19:55	Abstract 60: OligoG normalizes the CF mucus phenotype - Anna Ermund (SE)
19:55-20:05	Abstract 45: Primary human airway epithelial cells expanded with feeder cells and ROCK inhibitor for screening novel GSNO reductase inhibitors and CFTR correctors - Peter Bove (US)
20:05-20:15	Abstract 30: Cx26, Notch and PPARg-dependent pathways during human airway epithelial cell repair - Joanna Bou Saab (CH)
20:15-21:30	***Dinner***
21:30-23:00	**Evening Poster Session** - Posters with odd Numbers – Bastions Rooms

Saturday, 29 March 2013 (Day 4)	
07:30-08:45	Breakfast
08:45-10:30	**Symposium 7 – CFTR potentiators, correctors and treatments of cystic fibrosis** **Chairs: Frederic Becq (FR) / Jennifer Hollywood (IE)**
08:45-09:10	Strategies to correct the chloride transport defect in cystic fibrosis – Luis Galietta (IT)
09:10-09:35	Development and characterization of new correctors of F508del CFTR - John Hanrahan (CA)
09:35-10:00	407 compound, a new corrector for ΔF508CFTR: state of art - Aleksander Edelman (FR)
10:00-10:10	Abstract 58: Novel correctors of F508del-CFTR are additive to VX-809 in primary cultures of human lung cells homozygous for F508del or A561E - Nikhil Awatade (PT)
10:10-10:20	Abstract 61: The dual-acting small-molecule CFFT-004 rescues F508del-CFTR expression and function in recombinant cells and primary cultures - Jia Liu (UK)
10:20-10:30	Abstract 69: PTC124 is unable to significantly induce read-through of premature stop codons (PTC) in primary intestinal organoids of CF patients – Domenique van Ommen (NL)
10:30-11:00	*Coffee break & Poster viewing*
11:00-12:30	Debate – Correction at more than one domain is needed for F508delCFTR Chair Ineke Braakman (NL) Participants*:* Lihua He (US) – Philip Thomas (US) – Aleksander Edelman (FR)
11:00-12:30	Training workshop - What is the best method of repairing CFTR – gene or protein? Chair – Mitch Drumm (US)
12:30-14:00	*Lunch*
14:00-15:45	**Symposium 8 – Origin of chronic inflammation in CF** **Chairs: Alice Prince (US) / Mario Romano (IT)**
14:00-14:25	Inflammatory and fibrotic lung disease in CF: rational basis for cell-targeted therapy - Teresinha Leal (BE)
14:25-14:50	Worms and Amoebae as model systems to determine the developmental pre-sets driving chronic inflammation in post-natal CF - Anil Mehta (UK)
14:50-15:15	The Intracellular Calcium Hypothesis in Cystic Fibrosis (CF) - Clarisse Vandebrouck (FR)
15:15-15:25	Abstract 36 Macrophage Migration Inhibitory factor (MIF) accelerated biofilm formation, a potential novel therapeutic target - Aisling Tynan (IE)
15:25-15:35	Abstract 28 : Proteomic analyses of BALF from young CF children reveal altered lipid, cyclic nucleotide, and Iron signaling and metabolism during inflammation versus non-CF disease controls - Assem Ziady (US)
15:35-15:45	Abstract 29 : The mode of action of ADAM17 in resolution of inflammation in CF lung disease - Marta Stolarczyk (NL)
15:45-16:15	*Coffee Break & Poster Viewing* – Bastions Rooms

16:15-18:00	**Symposium 9 – CFTR trafficking** **Chairs: William Guggino (US) / Paul Eckford (CA)**
16:15-16:40	Identification of novel ERAD players controlling CFTR degradation - Agnès Delaunay-Moisan (FR)
16:40-17:05	CHIP Regulates ΔF508 CFTR Surface Stability at a Post-Endocytic Step - James Collawn (US)
17:05-17:30	A High-Content siRNA Screens Identify Novel CFTR Traffic Regulators in Human Airway Epithelial Cells - Margarida Amaral (PT)
17:30-17:40	Abstract 15: LMTK2 Mediated Phosphorylation Regulates CFTR Endocytosis in Human Airway Epithelial Cells - Carlos Farinha (PT)
17:40-17:50	Abstract 13: NBD1 Cotranslational Folding Intermediates as Targets for CF Drug Discovery – William Skach (US)
17:50-18:00	Abstract 55: New drug delivery system in treatment of Cystic Fibrosis using siRNA knock-down of the Aha-1 gene - Karen Schelde (DK)
18:00-18:15	*Break*
18:15-19:15	**Closing Keynote Lecture** - Room: Fortress CFTR: Structure, thermal stability and Paths to Drug Discovery - David Dawson (US)
20:30	*Dinner / Social Event*

POSTER TITLES & AUTHORS

P.01 **Rescue of NBD2 mutants N1303K and S1235R by small molecule correctors and transcomplementation.**
Daniele Rapino, Inna Sabirzhanova, Miqueias Lopes-Pacheco, William B. Guggino, Liudmila Cebotaru

P.02 **Some corrector compounds that directly modify the conformational stability of full-length F508del-CFTR stabilize and/or induce its active channel form after biosynthetic rescue**
Paul D.W Eckford, Mohabir Ramjeesingh, Christine E. Bear

P.03 **Restoration of NBD1 thermal stability is necessary and sufficient to correct ΔF508 CFTR folding and assembly**
Lihua He, Andrei A Aleksandrov, Jianli An, Zhengrong Yang, Christie G Brouillette, John R Riordan

P.04 **Discovery of novel tailored F508del-CFTR binder correctors based on 3D structure models of entire CFTR protein for treating Cystic Fibrosis**
Clément Boinot, Mathilde Jollivet Souchet, Brice Hoffmann, Jean-Paul Mornon, Benjamin Boucherle, Antoine Fortuné, Jean-Luc Decout, Pierre Lehn, Isabelle Callebaut, Frédéric Becq

P.05 **The membrane fluidity as well as the cytoskeleton modulate the activity of CFTR in response to stretch**
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P.06 **Glucocorticoids distinctively modulate the CFTR-channel with possible implications in lung development**
Mandy Laube, Ulrich H. Thome

P.08 **Study of long-range regulatory mechanisms of the *CFTR* gene**
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P.09 **Rattlesnake phospholipase A_2, a new potentiator for CFTR: structural and functional studies**
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P.19 **VX-809 stabilizes the early steps of F508del-CFTR biogenesis and folding**
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Jia Liu, Carlos Farinha, Nikhil Awatade, Ana M Romão, Hermann Bihler, Zhiwei Cai, Martin Mense, Margarida D Amaral, David N Sheppard

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Onofrio Laselva, Maria Favia, Anna Claudia Abbattiscianni, Lorenzo Guerra, Valentino Bezzerri, Stephan Joel Reshkin, Marianna Valentina Petruzzella, Roberto Gambari, Giulio Cabrini, Valeria Casavola

P.63 **SPLUNC1-Derived Peptides for the Treatment of Na^+ hyperabsorption in Cystic Fibrosis (CF) Lung Disease**
Robert Tarran, Alaina L Garland, Rui Cao, David Hill, Nicholas Moss, Mehmet Kesimer, William Arendshorst, Shawn Terryah

P.64 **Development of small molecules to correct the defective chloride transport in cystic fibrosis**
Marta Bellotti, Emanuela Pesce, Elvira Sondo, Paola Fossa, Luis J.V. Galietta, Enrico Millo

P.65 **Structure-activity relationships (SAR) of neoglycoconjugates derived from deoxynojirimycin modulating the metabolism of sphingolipids as possible anti-inflammatory agents for CF lung disease**
Silvia Munari, Susanna Khalil, Nicoletta Loberto, Valentina Lovato, Valentino Bezzerri, Anna Tamanini, Rosaria Bassi, Massimo Aureli, Maria Grazia Giri, Caroline Norez, Frederic Becq, Boris Vauzeilles, Sandro Sonnino, Giulio Cabrini, Maria Cristina Dechecchi

P.66 **Vessel associated progenitor cells in the cell based Therapy of the Cystic Fibrosis lung disease**
Chiara Vezzali, Stefania Antonini, Daniela De Stefano, Chiara Bonfanti, Renato Bacchetta, Valeria Villella, Luigi Maiuri, Graziella Messina

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Licia Totani, Concetta Amore, Antonio Piccoli, Giuseppe Dell'Elba, Angelomaria Di Santo, Nicola Martelli, Paolo Moretti, Mario Romano, Virgilio Evangelista

P.69 **PTC124 is unable to significantly induce read-through of premature stop codons (PTC) in primary intestinal organoids of CF patients**
Domenique D van Ommen, Lodewijk A W Vijftigschild, Hettie M Janssens, Karin M Winter-de Groot, Cornelis K van der Ent, Jeffrey M Beekman

P.70 **CFTR impairment in response to ethanol consumption and in alcoholic pancreatitis**
Péter Hegyi, Petra Pallagi, Lajos V Kemény, Zsolt Balla, Balázs Kui, Anita Balázs, Linda Judák, István Németh, Zoltán Rakonczay Jr., Viktória Venglovecz, Imre Földesi, Áron Somorácz, Katalin Borka, Doranda Perdomo, Gergely L Lukacs, Mike A Gray, Stefania Monterisi, Manuela Zaccolo, Markus M. Lerch, Miklós Sahin-Tóth, József Maléth

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József Maléth, Petra Pallagi, Lajos V Kemény, Linda Judák, Áron Somorácz, Katalin Borka, Zoltán Rakonczay Jr., Viktória Venglovecz, Mike A Gray, Péter Hegyi

P.72 **The buffer capacity of airway epithelial secretions**
Dusik Kim, Jie Liao, John W. Hanrahan

P.73 **Novel radiolabelled probes for in vivo detection of CFTR**
Vera FC Ferreira, Bruno L Oliveira, João DG Correia, Isabel Santos, Carlos M Farinha, Filipa F Mendes

P.74 **Radiolabelling of anti-CFTR antibodies for the detection of CFTR**
Vera FC Ferreira, Bruno L Oliveira, João DG Correia, Isabel Santos, Kathryn W Peters, Carlos M Farinha, Filipa F Mendes

AWARD WINNERS

ECFS Young Fellows Travel Award

Mark Bazett, CA
Christien Bednarski, DE
Sara Canato, PT
Marcella Doktorova, NL
Vera Ferreira, PT
Mandy Laube, DE
Joszef Maléth, HU

Student Helper Award

Clément Boinot, FR
Miriam Bossmann, DE
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Karen Schelde, DK
Florence Sonneville, FR
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Free Registration Young Researchers Supported by FFC

Nikhil Awatade, PT
Marta Bellotti, IT
Onofrio Laselva, IT
Jia Liu, UK

Vaincre La Mucoviscidose Travel Award

Jessica Bellec, FR
Benoït Chevalier, FR
Elma El Khouri, FR
Laura Vachel, FR

26 March – 18:00-19:00 **Room: Fortress**

Opening Keynote Lecture

A twenty-year scientific journey through CFTR pharmacology

Frédéric Becq

Laboratoire Signalisation & Transports Ioniques Membranaires, CNRS/Université de Poitiers, FRANCE

More than twenty years ago in the early '90s, the very first pharmacological agonists of CFTR chloride channels (IBMX, genistein…) were identified on the basis of the fresh knowledge that CFTR was a cAMP-regulated chloride channel. Then, tremendous efforts have rapidly been concentrated on the most common CF mutation F508del-CFTR. Indeed, it is remarkable to note that among the multiple advances, tricky research conducted step-by-step on dissecting the biosynthetic pathway and structural organization of CFTR begin to explain the molecular mechanism(s) of misfolding of the protein and its ER retention and offer now new opportunities to discover selective CFTR agonists.
The continuously rising discovery of novel drugs for CF, especially of molecules targeting directly the F508del-CFTR protein (named CFTR binders) or targeting proteostasis actors (named proteostasis modulators) is now beginning to shape the CF therapeutic landscape.
In two decades, the pharmacology of CFTR evolved from simple concepts based on the physiological regulation of CFTR to specific polypharmacology taking into account structural information and recent hypothesis on synergy to better address CFTR defects. These evolutions and concepts will be discussed and presented.

SYMPOSIUM 1
Structural defects of CFTR mutants
Chairs: David Dawson (US) / Lihua He (US)

S1.1 Compounds that improve NBD1-CFTR folding efficiency synergize with compounds that promote domain assembly

A Schmidt[1], G Miller[2], JF Dekkers[3], L Millen[1], J Mendoza[1], JM Richardson[1], Y Cheng[4], R Bridges[4], J Beekman[3], WC Wigley[2], PJ Thomas[1]

[1]UT Southwestern, Physiology, Dallas, United States, [2]Reata Pharmaceuticals, Irving, United States, [3]Utrecht University, Utrecht, Netherlands, [4]Rosalind Franklin Medical School, North Chicago, United States

Cystic Fibrosis (CF) is a monogenic, recessive disease, caused by dysfunction of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein. Loss of function of this Cl^--channel at the plasma membrane leads to multi-organ disease. The most common mutation, found in 90% of CF patients, is a deletion of phenylalanine at position 508, ΔF508-CFTR. This mutation interferes with the folding of CFTR, and its retention in the endoplasmic reticulum and subsequence degradation. Importantly, the mutant protein is appropriately translated in the correct cells. Thus, a possible strategy to treat the disease is to discover small molecules which correct the folding of the mutant protein and restore its normal cellular function. Recent work in our laboratory (Mendoza *et al.,* Cell 2012) and others (Rabeh *et al.,* Cell 2012), indicates that at least two defects are caused by the ΔF508 mutation, leading to misfolding and consequent degradation of the mutant protein: 1) decreased stability of the nucleotide binding domain 1 (NBD1), the domain containing the site of the deletion, and 2) poor assembly of the native multi-domain structure. A number of small molecules have been identified which partially restore ΔF508-CFTR Cl^--channel function, some of these are represented in the CFFT panel. To determine if CFFT panel small molecules act on different steps in CFTR folding, we utilized second-site mutations, which have been demonstrated to counteract each of the defects, NBD1 folding or domain assembly, and assays of isolated domain stability and folding. First, we assessed the isolated NBD1: To evaluate domain stability, *in vitro* thermal melts of the purified protein were performed. To evaluate NBD1 folding, an *in vivo* yield assay was employed. Second, in combination with step specific second site suppressor mutations, we assessed full-length trafficking using biochemical methods. Third, we performed functional assays on these combinations to evaluate the Cl^--channel function. These data suggest that some of the CFFT panel compounds target CFTR directly and act preferentially on one of the folding steps, but not the other, consistent with their limited efficacy. Importantly, combination of assembly step specific compounds identified from the CFFT panel, and a NBD1-folding step specific compound identified in a high throughput screen, act synergistically to robustly rescue ΔF508-CFTR maturation and function. Understanding the mechanism of action of these small molecules provides a rational path to effective combinations and to genotype specific treatment of other CFTR folding mutations.

S1.2 – Major Cystic Fibrosis mutant exhibits defective phosphorylation in the amino terminal region of the regulatory "R" domain

Stan Pasyk, Mohabir Ramjeesingh, Ling-Jun Huan, Stephanie Chin, Steven Molinski, Kai Du, Herman Yeger, Paul Taylor, Michael F. Moran, Christine E. Bear

The Research Institute, The Peter Gilgan Centre for Research and Learning, 686 Bay St, Toronto, Ontario, Canada, M5G 0A4

The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) is a multi-domain membrane protein. Deletion of F508 in the first nucleotide binding domain (NBD1), the major mutation, impairs the biosynthetic maturation and assembly of CFTR leading to loss of its functional expression as a channel on the cell surface. F508del disrupts the thermodynamic stability of NBD1 as well as its coupling to the second membrane spanning domain (MSD2) of CFTR. We asked if there are allosteric effects of F508del on the regulatory (R) domain, a region known to be important for phosphorylation regulated channel activity and implicated in inter-domain assembly. To identify defects in the R domain, we compared the phosphorylation status at PKA sites in the R domain of Wt and F508del-CFTR. Here we show that phosphorylation of Ser-660, quantified by selected reaction monitoring- mass spectrometry (SRM-MS), was significantly reduced in F508del-CFTR, as predicted if this region is engaged in aberrant intra-molecular interactions. This phosphorylation defect was not rescued by a “corrector” compound, structurally related to “correctors” in clinical trials, highlighting the need for novel structure-based interventions.

S1.3 – Models of the 3D structure of CFTR : from the understanding of the protein functions to the design of correctors.

JP Mornon[1], B. Hoffman[1], B. Boucherle[2], A. Fortune[2], R. Haudecoeur[2], C. Boinot[3], M. Jollivet[3], P. Lehn[4], F. Becq[3], JL Decout[2], I Callebaut[1]

1. IMPMC, Sorbonne Universités, Université Pierre et Marie Curie-Paris06, CNRS UMR7590, MNHN, IRD, Paris, France, 2. DPM, Université Jospeh Fourier, CNRS UMR5063, Grenoble, France, [4]. Université de Bretagne Occidentale, INSERM U1078, Brest, France, [4]. STIM, Université de Poitiers, CNRS ERL7368, Poitiers, France

In the absence of experimental 3D structures at atomic resolution for the entire CFTR protein, we and others have built homology models using as templates the experimental 3D structures of ABC exporters. These models provided significant insights into the structural and functional characteristics of CFTR, and were recently enriched by several modeling and dynamics studies by other groups, offering a description of the possible architecture of the anion channel.
We took into account an improved sequence alignment for assessing the accuracy of our Sav186-based model, considering the information provided by new experimental 3D structures and the internal symmetry linking the two halves of ABC exporters. We then ran short molecular dynamics simulations, without any constraint. This led to a relevant model of the full open form of the anion channel, with lateral funnels allowing ions and molecules access from the cytosol.
This model is particularly well consistent with the experimental data available today and provides insight into the molecular mechanisms ensuring the proper functioning of the CFTR protein, as well as into the impact of mutations in patients with cystic fibrosis (CFTR2 database). In addition, we also used models of the mutated forms of CFTR (F508del-CFTR in particular) to perform rational drug design. A series of molecules with potential corrective effects are currently synthesized and their biological activities evaluated by functional tests.

This work is supported by the French association "Vaincre La Mucoviscidose".

S1.4 Rescue of NBD2 mutants N1303K and S1235R by small molecule correctors and transcomplementation.

Daniele Rapino[1], Inna Sabirzhanova[2], Miqueias Lopes-Pacheco[2], William B. Guggino[2], Liudmila Cebotaru[3]

[1]Johns Hopkins U. School of Medicine, Physiology Department, Baltimore, United States, [2]Johns Hopkins U. School of Medicine, Physiology, Baltimore, United States, [3]Johns Hopkins U. School of Medicine, Ophthalmology and Physiology, Baltimore, United States

Although mutations in NBD 1 are most commonly associated with CF, disease causing mutations also occur in NBD2 of CFTR. Here we study two disease causing mutations in NBD2: N1303K, associated with severe disease and S1235R which causes mild or borderline symptoms. The goal is to gain more information on the impact of these mutations on CFTR processing and to devise ways to rescue them. Inhibition of proteasomes with MG132 or aggresomes with tubacin is able to rescue the immature band B and mature band C of N1303K and S1235R, indicating that degradation of both mutants is via proteasomes and aggresomes. VCP, the protein involved in translocation of mutant proteins to the proteasome and HDAC6, a protein involved in trafficking of mutant proteins to the aggresome, also bind to the NBD2 mutants indicative of their degradation in the proteasome and aggresome. There is no effect of the lysosome inhibitor (E64). We tested the effect of small molecule correctors in promoting maturation of NBD2 mutants. We found that several known correctors alone or in combination increased the maturation of B to C band of N1303K and S1235R. The best correction occurred with N1303K when a combination of the CFFT correctors, C4 plus C18 or C3 plus C4 was applied. In pull-down assays, we found that a number of chaperones, including HSP27, 40, 70 and 90 bind to the N1303K and S1235R mutants. Importantly, following treatment with the combination of correctors, chaperone binding was reduced especially in the N1303K mutant. This strengthens considerably our conclusion, that C3+C4 rescue both NBD2 mutants by reducing chaperone binding thereby promoting maturation. We next tested whether NBD2 mutants could be rescued by transcomplementation. Indeed transfection of the CFTR truncation, Δ27-264, into cell lines stably transfected with either of the NBD2 mutants was able to rescue them. This is the first time that the use truncated forms of CFTR proved to be effective in correcting NBD2 mutants. Our results suggest that the N1303K mutation has a profound effect on NBD2 processing compared to S1235R. Small molecule correctors are able to increase the maturation of band B to C in NBD2 mutants. In addition the truncation mutant, Δ27-264, is able transcomplement both NDB2 mutations. Our results suggest that patients bearing either the N1303K or S1235R mutations may be good candidates for both corrector and gene therapy. Funded by NACF Foundation.

S1.5 Some corrector compounds that directly modify the conformational stability of full-length F508del-CFTR stabilize and/or induce its active channel form after biosynthetic rescue

Paul D.W Eckford[1], Mohabir Ramjeesingh[1], Christine E. Bear[1,2,3]

[1]Hospital for Sick Children, Molecular Structure and Function, Toronto, Canada, [2]University of Toronto, Department of Biochemistry, Toronto, Canada, [3]University of Toronto, Department of Physiology, Toronto, Canada

F508del-CFTR, the most common CF-causing mutation in North America and Europe, impairs conformational maturation and stability of the CFTR protein, reducing its functional expression at the surface of cells in which it is expressed. “Corrector” compounds, such as the structurally related C18 (VRT-534) and VX-809 (lumicaftor) can rescue the biosynthetic maturation and forward trafficking of this mutant to the cell surface, to some extent. We propose that at least some corrector compounds will bind directly to the mutant CFTR protein to enhance its conformational stability and induce the channel-competent form of the protein once it has trafficked to the cell surface. We employed purified and reconstituted full-length Wt, F508del- and G551D-CFTR to test our hypothesis.

Purified full length F508del-CFTR protein in detergent solution aggregates rapidly whereas Wt-CFTR is significantly more stable in this *in vitro* system. Direct interaction of purified F508del-CFTR with small molecules including C18, VX-809 and the corrector VRT-325, but not an inactive analog of VRT-325, result in reduced aggregation which we interpret as a stabilizing effect on the conformation of the protein.

Acute treatment of purified full length Wt-, F508del and G551D-CFTR reconstituted into proteoliposomes with C18 but not vehicle enhanced the channel activity of the protein. Both the ATP-dependent and ATP-independent channel activities were enhanced by the treatment of C18, and the ATP-independent channel activity was inhibited by the CFTR-specific inhibitor, CFTRinh-172.

Together, these studies show that full length CFTR protein is a direct target of at least some corrector compounds and their binding can modulate regions involved in gating. These compounds may bind directly to a meta-stable F508del-CFTR structure post-translationally, to stabilize and/or induce a channel active form of the mutant protein. These findings enhance our understanding of the mechanism of action of this clinically relevant class of pharmacological corrector compounds.

This work was supported by Cystic Fibrosis Canada and Operating Grants to C.E.B from the CIHR.

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S1.6 Discovery of novel tailored F508del-CFTR binder correctors based on 3D structure models of entire CFTR protein for treating Cystic Fibrosis

Clément Boinot[1], Mathilde Jollivet Souchet[1], Brice Hoffmann[2], Jean-Paul Mornon[2], Benjamin Boucherle[3], Antoine Fortuné[3], Jean-Luc Decout[3], Pierre Lehn[4], Isabelle Callebaut[2], Frédéric Becq[1]

[1]Laboratoire de signalisation et transports ioniques membranaires (STIM), ERL7368, CNRS, Université de Poitiers, Poitiers, France, [2]Institut de Minéralogie et de Physique des Milieux Condensés, UMR7590, CNRS Université Pierre et Marie Curie-Paris 6, Paris, France, [3]Département de Pharmacochimie Moléculaire, UMR5063 UJF/CNRS, Université Joseph Fourier, Grenoble, France, [4]INSERM U1078, Université de Bretagne Occidentale, Brest, France

Cystic fibrosis (CF) is an autosomal and recessive disease due to mutations in the gene encoding a chloride ion channel CFTR (cystic fibrosis transmembrane conductance regulator). Deletion of phenylalamine at position 508 leads to the most common mutation, resulting from a mistrafficking of the CFTR protein and its retention in the Endoplasmic Reticulum (ER), abnormal gating of CFTR channel and endocytosis. Numerous small chemicals called CFTR correctors (VX809, SAHA, Corr-4a, iminosugars...) have been shown individually - albeit partially - to restore F508del-CFTR defective trafficking and functionally rescue chloride transport default. Although the mechanism of action of these correctors is still largely unknown, recent evidence suggests direct interaction of VX809 on the first transmembrane domain TMD1 of CFTR. Here, based on models refinement of the open and closed forms of CFTR, we used molecular docking and virtual screening to identify new active molecules able to bind and interact with identified F508del-CFTR pockets. This strategy led to (1) design novel tailored correctors following molecular docking prediction, allow to bind specifically in F508del-CFTR pockets, (2) synthetize these putative idealized compounds and (3) test them on the functional rescue of F508del-CFTR. We used our Bioscreen platform using a simple flux test combined to patch-clamp experiments in whole cell configuration on F508del-CFTR cell lines and human airway epithelial CFBE cells to screen these candidates for their potential correction efficacy. Preliminary experiments showed that after 24h of incubation some of these molecules restored the F508del-CFTR response to a cocktail of activators (forskolin + genistein) in CF cells with submicromolar $_{EC50}$ values. In a second set of experiments, using patch-clamp technique in whole-cell configuration on Hela F508del-CFTR, we tested 24h incubation of these novel correctors in combination with VX809 to investigate putative synergistic effects of two binder correctors on F508del-CFTR rescue. We demonstrated with this combination that the amplitude of the current densities recorded in response to activation was not significantly different compared to incubation with VX809 alone. Our next step will be to test second site suppressor mutations in combination with these novel molecules to investigate putative synergistic effects on F508del-CFTR folding and stability. Results will be presented opening perspectives on the development of new tailored correctors based on 3D structure model of entire CFTR protein to target the root cause of CF.

This work is supported by “Vaincre La Mucoviscidose”, “Mucovie” and FEDER funds.

Thursday 27 March – 11:00-12:45

SYMPOSIUM 2
Symposium 2 – Regulation of CFTR and alternative channels
Chairs: Luis Galietta (IT) / Carlos Farinha (PT)

S2.1 – Functional regulation of ENaC by the bacterial serralysin proteases and inhibitors

Michael Butterworth, Liang Zhang, Patrick Thibodeau

Department of Cell Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA

The epithelial sodium channel (ENaC) is a major regulator of sodium and water absorption across multiple epithelial tissues. Channel function is regulated by protein trafficking to the plasma membrane, as well as functional regulation associated with changes in open probability. Multiple studies have shown that proteolytic cleavage of the channel's extracellular domains result in increased channel open probability, leading to an increase in sodium flux. Though models for airway surface fluid homeostasis vary for CF, one hypothesis holds that ENaC plays a critical role in the maintenance of airway surface fluid. Activation of ENaC by either endogenous or exogenous proteases would putatively lead an increase in sodium absorption and is coupled to fluid absorption in the airway.

The serralysin family of metalloproteases is associated with the virulence of multiple gram-negative human pathogens, including *Pseudomonas aeruginosa* and *Serratia marcescens*. The secretion of the serralysin proteases has been connected to increased virulence for multiple pathogens. Increased protease secretion and activity have been associated with bacterial virulence in corneal keritits and in the CF lung. These proteases contain structurally conserved catalytic domains with similarity to multiple mammalian matrix metalloproteases.

To evaluate the potential that ENaC is a physiological target of the serralysin proteins, the proteases were cloned, expressed and purified for biochemical and electrophysiological studies. Protease addition to the apical baths in Ussing chambers resulted in an activation of ENaC, using multiple model cell lines and primary human airways cells. Both CF and non-CF primary cells showed similar responses with respect to channel activation. The activation of ENaC was slow relative to that induced by trypsin, but was consistent in all cell types tested. The serralysin-dependent activation was sensitive to mutations in the gamma-subunit of ENaC, suggesting the site of cleavage occurred within a previously identified regulatory region of the channel.

The serralysin proteases are often co-expressed with endogenous, intracellular or periplasmic inhibitors, which putatively protect the pathogen from unwanted or unregulated protease activities. To evaluate the potential use of these small protein inhibitors in regulating protease-induced activation of ENaC, protease inhibitors from Pseudomonas and Serratia marcescens were purified. The high-affinity inhibitor found in Pseudomonas was effective at blocking protease activity in biochemical and physiological experiments. Stoichiometric addition of the inhibitor resulted in a complete block of observable protease activity biochemically. Near stoichiometric addition of the inhibitors was sufficient to eliminate the protease-induced activation of ENaC in electrophysiological experiments.

These data provide evidence that a wide variety of proteases may regulate ENaC in a physiological setting and suggest that other metalloproteases could potentially serve to regulate channel function. Further, these data demonstrate that ENaC is a substrate for the serralysin proteases and that high affinity natural protein inhibitors can be utilized to block this protease-induced ENaC activation. Based on these observations, models for the potential mechanisms of serralysin-associated bacterial virulence will be discussed.

S2.2 - CFTR is an external chloride-gated ion channel.

Mike Gray

Institute for Cell & Molecular Biosciences, Newcastle University, UK

We have previously shown that an increase in luminal (extracellular) chloride concentration ([Cl]o) enhances CFTR activity (1,2) by increasing single channel open probability, and other work has reported that a reduction in [Cl]o alters the HCO3 /Cl permeability ratio and the conductance of the channel (3,4). Because most CFTR-expressing epithelia secrete substantial amounts of Cl and HCO3, and because the concentrations of these anions in the luminal fluid varies under different physiological conditions, such studies have led to the speculation that CFTR may be regulated by the Cl content of its own secretions (2,4). However, how [Cl]o is sensed by epithelial cells and how changes in [Cl]o are communicated to CFTR in order to modulate its gating, permeability and conductance are fundamentally important questions that have not been answered.

To investigate if CFTR itself could act as a 'Cl sensor', we first focussed on the nine positively charged (arginine and lysine) residues located in the extracellular domains and/or outer pore of CFTR. Using patch clamp recordings we found that neutralising just one of these residues in the third extracellular loop of CFTR abolished Cl sensing, providing strong evidence that CFTR is a sensor of external [Cl]. To begin to understand the mechanism that underlies the Cl-dependent changes in CFTR gating, we investigated the role of PKA phosphorylation and/or ATP binding and hydrolysis at the nucleotide binding domains (NBDs). Our results show that although Cl-dependent regulation of WT CFTR required PKA phosphorylation, it persisted in channels lacking the R domain, placing the [Cl]o-dependent response downstream of phosphorylation in the gating cycle. Using NBD nucleotide binding mutants, and ATP dose response studies in WT CFTR channels, we determined that [Cl]o sensing was linked to changes in ATP binding energy at NBD1, which likely impacts NBD dimer stability. Furthermore, biochemical measurements on purified and reconstituted CFTR showed that increasing [Cl]o decreased the intrinsic ATPase activity of CFTR mainly through a reduction in maximal ATP turnover.

Together, our studies indicate that CFTR is a Cl-gated ion channel that senses external Cl concentration, revealing a novel mechanism for regulating CFTR activity. Furthermore, our findings also suggest that the luminal ionic environment is an important arbiter of CFTR function, which has significant implications for salt and fluid homeostasis in epithelial tissues.

Supported by the Wellcome Trust

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S2.3 - Purinergic regulation of mucociliary clearence and inflammation in CF.

Eduardo Lazarowski

Cystic Fibrosis/Pulmonary Research & Treatment Center. University of North Carolina at Chapel Hill. USA

Extracellular ATP and its metabolic product adenosine regulate ion channel/fluid secretion activities necessary for mucin hydration via activation of airway epithelial purinergic receptors, mainly the ATP-sensing P2Y2 receptor and the adenosine-sensing A2B receptor. The concentrations of adenosine, ATP, and other purinergic agonists within the airway surface liquid are finely balanced to maintain effective mucociliary clearance (MCC) without promoting airway inflammation. In spite of the pathophysiological relevance of the responses triggered by extracellular nucleotides in the lung, the mechanisms of airway epithelial nucleotide release in normal and inflamed airways has only recently begun to be addressed. Insights into these mechanisms emerged from the identification of (i) pannexin 1 as a plasma membrane ATP channel, and (ii) solute carrier (SLC) transporters that control the uptake and storage of nucleotides in secretory vesicles/granules. We discovered that the SLC17A9 vesicular nucleotide transporter (VNUT), controls nucleotide levels within mucin granules and, therefore, contributes to the release of ATP from mucin secreting cells. Notably, cultures of bronchial epithelial (HBE) cells isolated from CF patients, as well as normal HBE cells exposed to inflammatory factors from CF airways, exhibited enhanced ATP release in response to hypotonicity, which was sensitive to inhibitors of the secretory pathway and correlated with enhanced expression of VNUT. Pannexin 1 greatly contributes to nucleotide release in normal airways, but pannexin 1 inhibition did not affect the ATP release response in CF-inflamed HBE cells. Thus, vesicular mechanisms contribute to the enhanced release of ATP from CF airway epithelia.

Sputum samples from CF patients contained abnormally high levels of ATP, consistent with the notion that nucleotide release is up-regulated in inflamed CF lungs. Furthermore, UDP-glucose, a potent and selective agonist at the P2Y14 receptor (P2Y14R), was present in CF sputum at levels that were 5-10 folds greater than ATP levels. These findings, together with the fact that the P2Y14R is highly expressed in neutrophils, prompted us to examine the potential pro-inflammatory activity of UDP-glucose. We found that UDP-glucose is a potent and stable neutrophil chemoattractant in vitro, and instillation of UDP-glucose into mouse tracheas resulted in increased neutrophil recruitment in the lung. Based on these results, we hypothesize that the enhanced airway epithelial nucleotide release associated with CF contributes to lung inflammation. Current studies in our lab are addressing the contribution of VNUT and pannexin 1 to MCC activities and inflammation in animal models of obstructed lung diseases.

S2.4 – SLC6A14 elicits CFTR-mediated chloride and fluid secretion in CF airway

Tanja Gonska[1,2], Mingyuan Li[3], Wan Ip[3], Andrew Lloyd-Kuzik[4], Saumel Ahmadi[4], Christine Bear[4]

[1]The Hospital for Sick Children, Research Institute, Toronto, Canada, [2]University of Toronto, Pediatrics, Toronto, Canada, [3]The Hospital for Sick Children, Toronto, Canada, [4]University of Toronto, Toronto, Canada

Background: SLC6A14 is associated to the severity of CF disease (Sun 2012). This transporter mediates the uptake of cationic and neutral amino acids across the apical membrane of airway epithelial cells (Galietta 1998) and we previously demonstrated that L-arginine, a substrate for SLC6A14, enhanced constitutive CFTR-mediated Cl^-secretion in human non-CF and CF primary bronchial epithelial cultures. We now postulate that SLC6A14 activation with L-arginine also effects transepithelial fluid transport in human airway epithelial cells.

Method: Primary human bronchial epithelial cells from CF (CFBE) and non-CF subjects (NBE) were obtained from lung transplants (Drs. Keshavjee and Zabner, University of Toronto and Iowa). Transepithelial L-arginine transport was measured as appearance of ^{3}H-L-arginine at the basolateral side of human airway epithelial cells following addition of L-arginine and ^{3}H-L-arginine to the apical side in an Ussing chamber. Airway surface liquid (ASL) height was measured following application of a PBS fluid bolus with texas red dextran using confocal microscopy. ASL height was assessed in presence of aprotinine to inhibit ENaC activity. L-arginin (Arg) was used as substrates and α-methyltryptophan (αMT, Karunakaran 2008) as inhibitor for SLC6A14.

Results: In NBE and CFBE cells transepithelial Arg transport was reduced by 60% in absence of Na^+ ($p=0.02$) as well as in presence of αMT ($p=0.02$) indicating that SLC6A14 is a main transport system for Arg across the airway epithelium. Our preliminary results show that SLC6A14 not only enhance CFTR-mediated $Cl^-_{leq,}$ but also affect airway epithelial fluid secretion. In NBE cells addition of Arg had no effect on the ASL height measured at 24 hrs ($p=0.27$). However, in CFBE cells addition of Arg led to a significant increase in the ASL of CFBE cells (5.4 ± 0.57 µm versus 8.2 ± 0.52 µm, $p=0.003$). This increase in ASL was inhibited in the presence of αMT. Ongoing investigations are evaluating intracellular pathways to explain the effect of SLC6A14 on CFTR function.

Conclusion: SLC6A14 is an important membrane constituent in airway epithelial cells. Activation of SLC6A14 increases constitutive CFTR-mediated $_{leq}$ as well as epithelial fluid secretion, which in turn leads to an improvement of the ASL height in CF human airways. Thus, SLC6A14 may be useful as a future drug target supporting our attempts to overcome the airway epithelial defect in CF.

(Funded by New Investigator Grant, CIHR/Sickkids foundation)

S2.5 - **Regulation of the chloride channel ANO1 by microRNAs in Cystic Fibrosis patients**

Florence Sonneville[1], Manon Ruffin[1], Philippe Le Rouzic[1], Sabine Blouquit-Laye[2], Loïc Guillot[1], Annick Clement[1], Harriett Corvol[1], Olivier Tabary[1]

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Introduction: Impairment of the activity of the CFTR chloride channel observed in cystic fibrosis (CF) patients is the main cause of the deterioration of lung function. This lung disease is the leading cause of morbidity and mortality in CF patients. In 2008, three groups have identified a novel chloride channel: the channel ANO1 (TMEM16a), proposed as a potential therapeutic target to restore chloride efflux in the lung in CF. Previous works from our laboratory have also shown that the activity and expression of ANO1 were reduced in a CF context (Ruffin et al., BBA Molecular Basis of Disease, 2013). Today, the origins of these decreases remain unknown. Some studies showed that CFTR is regulated by miRNAs which are small non-coding RNAs regulating negatively gene expression post-transcriptionally. One hypothesis is that miRNAs could have a major role in the deregulation of ANO1.

Objective: The objective of this work is to understand the origin of the reduced expression of ANO1 in CF cells by studying the role of miRNAs.

Methods: To answer this objective, we performed a gene candidate approach using bioinformatics databases to study miRNAs that could target ANO1. To initiate this project, we evaluated the expression levels of miRNAs candidates by RT-qPCR in bronchial epithelial cells lines and in CF and non-CF human lung explants. To validate the link between ANO1 and miRNAs, we performed functionality experiments studying the miRNAs candidates binding to the 3´UTR of ANO1 in condition of over-or under-expression of miRNAs, and quantifying the expression of ANO1 in these conditions.

Results: The bioinformatic study allowed us to identify different miRNAs including miR-9 and miR-144 as potential regulators of ANO1. In one hand, the study of the expression of miR-9 in CF versus non-CF context did not show difference of expression of miR-9 and in condition of over- or under-expression of miR-9, we did not observe regulation of ANO1. On the other hand, we observed a significant decrease in the expression of ANO1 in condition of over-expression of miR-144 but no difference in luciferase activity in the same conditions.

Conclusion: Our results suggest that miR-9, alone, do not regulate ANO1 in our experimental conditions and that miR-144 could indirectly regulate ANO1. It would be interesting to study through whom miR-144 regulates ANO1. Moreover, to go further, we make a miRNOME and transcriptome approach on primary air-liquid interface cultures of human airway epithelia CF and non-CF. The results of this dual approach must be received soon and will allow us to explore all deregulated miRNAs in the CF context.

S2.6 – **Impact of the F508del mutation on ovine CFTR, a Cl^- channel with enhanced conductance and ATP-dependent gating**

Zhiwei Cai[1], Timea Palmai-Pallag[2], Pissared Khuituan[1,3], Michael J Mutolo[2], Clément Boinot[1], Toby S Scott-Ward[1], David N Sheppard[1], Ann Harris[2]

[1]University of Bristol, School of Physiology and Pharmacology, Bristol, United Kingdom, [2]Human Molecular Genetics Program, Lurie Children's Research Center, and Department of Pediatrics, Northwestern University Feinberg School of Medicine, Chicago, United States, [3]Center of Calcium and Bone Research, Department of Physiology, Faculty of Science, Mahidol University, Bangkok, Thailand

Cross-species comparative studies are a powerful approach to understand cystic fibrosis transmembrane conductance regulator (CFTR) processing and function (1, 2). In this study, we investigated the single-channel activity of ovine CFTR and the impact of the F508del mutation using excised inside-out membrane patches from CHO cells transiently expressing CFTR constructs at 37 ºC as previously described (2, 3). Like human CFTR (3), ovine CFTR formed weakly inwardly rectifying Cl^- channels regulated by PKA-dependent phosphorylation and intracellular ATP. However, for three reasons ovine CFTR was noticeably more active than human CFTR. First, the single-channel conductance was increased (human, gamma = 8.55 ± 0.12 pS; ovine, gamma = 10.01 ± 0.21 pS; n = 6 and 8; $p < 0.05$). Second, ovine CFTR had a higher open probability (P_o) (human, P_o = 0.45 ± 0.02; ovine, P_o = 0.59 ± 0.02; n = 6 and 19; $p < 0.05$) because the frequency and duration of channel openings was increased. Third, ATP more strongly stimulated ovine CFTR channel gating (human, K_d = 0.167 mM; $P_{o(max)}$ = 0.57; ovine, K_d = 0.074 mM; $P_{o(max)}$ = 0.70 from Michaelis-Menten fits to mean data; n = 5 - 11 at each ATP concentration except at ATP (0.03 mM), where n = 3). Previous work by Ostedgaard et al. (1) demonstrated that the F508del mutation has a less severe impact on the single-channel activity of murine and pig CFTR. This was strikingly evident with ovine CFTR. The F508del mutation reduced the P_o of ovine CFTR by only ~30% to be equivalent to that of wild-type human CFTR (human: wild-type, P_o = 0.45 ± 0.02, F508del-CFTR, P_o = 0.06 ± 0.01; $p < 0.05$; ovine: wild-type, P_o = 0.59 ± 0.02, F508del-CFTR, P_o = 0.40 ± 0.02; $p < 0.05$; n = 6 - 19; $p = 0.074$ for human wild-type CFTR vs. ovine F508del-CFTR). However, the F508del mutation had a severe effect on the thermal stability of ovine CFTR. Like human F508del-CFTR, ovine F508del-CFTR Cl^- channels deactivated completely within 10 ~ 15 minutes in excised inside-out membrane patches at 37 ºC (n = 8). We conclude that ovine CFTR forms a regulated Cl^- channel with enhanced conductance and ATP-dependent channel gating compared to human CFTR. The F508del mutation has minimal impact on ovine CFTR channel gating, but strongly destabilises the channel at physiological temperatures. Our data suggest that F508del's impacts on gating and thermal stability are distinct effects. Supported by the CF Foundation, CF Trust, National Institutes of Health and the Strategic Scholarships Fellowships Frontier Research Networks, Office of the Higher Education Commission of Thailand.

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Thursday 27 March – 16:00-17:45

SYMPOSIUM 3
CFTR related disease
Chairs: Mike Gray (UK) / Peter Hegyi (HU)

S3.1 – Cystic fibrosis and bone disease: Defective human and murine osteoblast function with the F508del mutation in CFTR

Frédéric Velard[1], Martial Delion[1], Carole Le Henaff[1], Olivier Tabary[2], Christine Guillaume[1], Françoise Barthes[3], Lhousseine Touqui[4]i, Sophie Gangloff[1], Isabelle Sermet-Gaudelus[5] and Jacky Jacquot[1]

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Low bone density is commonly seen in patients with cystic fibrosis (CF) and begins at a young age. Rib and thoracic vertebral fractures can have adverse effects on lung health as the resulting pain and debilitation can impair airway clearance. Clinical and CF model studies have reported that F508del mutation in CFTR impacts bone formation; however, the underlying molecular defect in CFTR caused by the F508del-CFTR mutation in osteoblastogenesis, i.e, on the bone formation is unknown.

First, we have investigated whether targeting F508del-CFTR may rescue the skeletal osteopenic phenotype in murine CF. We found that oral administration of low-dose miglustat (N-butyldeoxynojyrimicin, NB-DNJ, 120 mg/kg/day once a day for 28 days) improved bone mass and microarchitecture in the lumbar spine and femur in F508del mice. The increased bone density was associated with increased bone formation rate. Exposure of primary F508del osteoblasts to miglustat partially restored the deficient CFTR-dependent chloride transport in these bone forming cells (Le Henaff et al. Am J Pathol, April 2014).

Second, human F508del osteoblasts were obtained from trabecular bone explants prepared from rib fragments harvested during lung transplantation in three young patients (two 13 and 15-year-old females, a 14-year-old male) homozygous for the F508del-CFTR mutation and a young patient (a 14-year-old male) with the F508del/G542X mutation in CFTR. Normal osteoblasts used as control, were obtained from trabecular bone explants of healthy young adults who underwent trauma surgery. We investigated whether the F508del mutation could affect the expression level of osteoblast genes relative to differentiation (e.g. collagen type1, osteocalcin (OC) osteopontin (ON) alkaline phosphatase (ALPL), RUNX2, OSX) and maturation (BMP2, SMAD1/2, osteoprotegerin (OPG), receptor activator of NF-kB ligand (RANKL), cyclooxygenase-2 (COX-2)). Fluorescence data showed the evidence of CFTR-dependent functional chloride conductance in primary normal osteoblasts, which was reduced in F508del osteoblasts. Compared to healthy osteoblasts, real time PCR data showed no significant difference in osteoblast gene expression related to differentiation (collagen type1, OC, ON, ALPL, RUNX2, OSX) in F508del osteoblasts. By contrast, a severe defective maturation of F508del osteoblasts was found in bone explants of the four CF patients. F508del osteoblasts exhibited a diminished expression of SMAD2, COX-2 and at a lesser extend for OPG mRNA but elevated RANKL mRNA level. Furthermore, we found that the basal production of OPG protein and COX-2 metabolite prostaglandin E2 (PGE2), two potent immuno-modulatory mediators, was greatly reduced by F508del osteoblasts. COX-2 activity and PGE2 have been previously identified as key mediators in the initial step of osteogenesis and the latter step of osteoblast maturation, supporting the role for COX-2 and PGE2 in the regulation of skeletal growth.

Our findings suggest that early bone loss in patients with the F508del mutation may be caused by a reduction in bone formation arising from a diminished OPG and COX-2/PGE2 expression and production in osteoblasts. The effect of correctors and potentiators of chloride channels on maturation-related genes of F508del osteoblast and consequently their effects on the new bone formation in patients with cystic fibrosis needs to be investigated for treatment of cystic fibrosis bone disease.

S3.2 - **Impairment of CFTR functional cooperation with SLC26A8, a sperm specific anion transporter, is associated with human asthenozoospermia**

Thassadite Dirami[1,2,3], Baptiste Rode[1,2,3], Mathilde Jollivet[4], Nathalie Da Silva[1,2,3], Denise Escalier[5], Natacha Gaitsch[6], Caroline Norez[4], Pierre Tuffery[7], Jean-Philippe Wolf[1,2,3,8], Frédéric Becq[4], Pierre F Ray[9], Emmanuel Dulioust[8], Gérard Gacon[1,2,3], Thierry Bienvenu[1,2,3,6], and Aminata Touré[1,2,3]

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Ion fluxes play an essential role in the control of sperm motility and capacitation (i.e. the maturation of the sperm occurring during the transit in the female genital tract and required for fertilization). In particular, calcium, chloride and bicarbonate are essential for both processes by inducing intracellular alcalinization, membrane hyperpolarization and cAMP-PKA-dependent phosphorylation events in the sperm. Several ion channels and transporters are present in the plasma membrane of the sperm and involved in these processes; however their physiological substrates together with their cross-regulation and functional hierarchy are very often poorly defined.

We previously characterized the SLC26A8 protein, also known as Testis Anion Transporter 1 (TAT1), as a male germ cell-specific member of the SLC26 (Solute Linked Carrier 26) family. SLC26 members are transmembrane proteins mediating the transport of a broad range of anions, including chloride, bicarbonate, sulfate and oxalate. We have shown that inactivation of Slc26A8 in the mouse induces male sterility with asthenozoospermia (i.e. lack of sperm motility); in particular Slc26A8 was shown to be required for the activation of the PKA-dependant phosphorylation events.

During the last decade the existence of a physical and functional interaction between several members of the SLC26 family and the CFTR channel has been well documented. Moreover the CFTR channel was shown to localize at the equatorial segment of the head and at the flagellum of mature sperm, and to be required for sperm motility and capacitation. We recently demonstrated that SLC26A8 interacts with the CFTR channel in vitro and in vivo, and strongly stimulates CFTR anion transport activity in Xenopus laevis oocytes and in CHO-K1 cells. We will report on three heterozygous missense mutations identified in the coding region of SLC26A8 by screening a cohort of infertile patients presenting with asthenozoospermia. All three mutations were found to impact on CFTR-SLC26A8 protein stability and to abrogate CFTR stimulation, demonstrating that in vivo the CFTR/SLC26A8 complex is essential for the regulation of anions fluxes during the processes of sperm motility and capacitation.

Acknowledgments

This work was supported by the Ministère de l'Education Nationale et de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, Centre National de la Recherche Scientifique, Université Paris Descartes, Agence Nationale de la Recherche (ANR-07-JCJC-0099; ANR-12-BSV1-0011-01 MUCOFERTIL) and Association Vaincre la Mucoviscidose (RF20110600465).

S3.3 – Modeling cystic fibrosis using primary intestinal CF organoids cultures

Florijn Dekkers[1,2,] Lodewijk Vijftigschild[1,2], Evelien Kruisselbrink[1,2], Annelotte Vonk[1,2], Inez Bronsveld[3], Karin de Winter[1], Hettie Janssens[4], Gerald Schwank[5], Kors van der Ent[1], Hans Clevers[5], Jeffrey Beekman[1,2]

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Human primary cell cultures from patients can model disease in vitro in a patient-specific fashion. We have set up a novel functional CFTR assay using intestinal organoids from CF patients. Intestinal organoids can be grown from rectal biopsies after their use for intestinal current measurements. We currently have developed two functional assays that both involve fluid secretion into the lumen of the organoid. The first assay induces rapid luminal fluid secretion and organoid swelling by an agonist such as forskolin with a dynamic range that allows discrimination of various CFTR mutations. The second assay quantifies the luminal surface area of the total organoid structure one day after passaging without addition of an agonist, and discriminates between CF and non-CF samples. These assays were used to study variability between patients in forskolin and B2-agonist induced CFTR function with or without CFTR-targeting drugs. Results indicate that CFTR residual function and response to drugs is determined by CFTR genotype, but also variable between patients with similar CF-causing mutations. Moreover, by comparing forskolin and adrenergic receptor stimulated swelling, we observed that coupling of adrenergic receptor signaling to CFTR function is also highly variable independent of CF-causing mutation. Next to the use of this model to study patient variability to CFTR-restoring drugs, we found that genetic correction of F508del by Cas9-CRISPR gene-editing normalize the swelling of CF organoids into wild type organoids, demonstrating that human adult stem cells can be corrected at the diseased loci for the first time. Collectively, these studies indicate that patient-specific intestinal organoids can play an important role in defining patient variables that can contribute to residual CFTR function and response to drugs in vivo.

S3.4 - The FXR-FGF15 axis is impaired in mice with a deficiency for CFTR

Marcela Doktorova[1], Frank A.J.A. Bodewes[1], Henkjan J. Verkade[1], Johan W. Jonker[1]

[1]University Medical Center Groningren, Center for Liver, Digestive and Metabolic Diseases, Department of Pediatrics, Groningen, Netherlands

Background: With the improved treatment of the pulmonary complications of cystic fibrosis (CF), gastrointestinal problems are rapidly becoming the major cause of morbidity and even mortality in CF. The gastrointestinal disorders include: fat malabsorption, CF related liver disease (CFLD), impaired growth, and impaired reabsorption of bile acids.
The FXR-FGF15 axis is an important pathway in the regulation of bile acid homeostasis as well as for liver growth and regeneration. Liver regeneration after partial hepatectomy is impaired in mice in which either the nuclear receptor FXR (FXR KO mice) or Fibroblast growth factor 15 (FGF15 KO mice) is inactivated. Hydrophobic bile acids such as cholic acid (CA) are strong activators of the FXR-FGF15 axis. Previously we found that chronic CA administration induces a liver growth and proliferation response in controls that is absent in cystic fibrosis (CF) mice, suggesting that the FXR-FGF15 axis is impaired in these mice. (unpublished data)
Methods: Here we determined whether the effect of dietary CA supplementation on liver growth is dependent on the presence of FXR in the intestine. We determined liver growth upon dietary CA supplementation in tissue specific

intestinal FXR $^{-/-}$ and control mice. Intestinal (villin-CRE) specific FXR knockout (iFXR$^{-/-}$) mice and wild-type (WT) littermates (n=7, age 9-12 weeks) were administered a semisynthetic, low fat diet (AB 4063 02) supplemented with 0.5% of cholic acid (CA) for 15 days. After this period mice were sacrificed, body and liver weights were measured and hepatic lipid composition was determined.

Results: After 15 days of CA treatment there was no difference in body weight between WT and iFXR$^{-/-}$ mice (23.0 vs. 22.8 g respectively; NS). There was a significant increase in liver weight in WT compared to iFXR$^{-/-}$ mice (1,4 vs. 1,2 g respectively; P=0.03). Body to liver weight ratio in WT was significantly higher compared to iFXR$^{-/-}$ mice

(0.07 vs. 0.06 respectively; $p< 0.001$). The hepatic triglyceride content was equal between WT and iFXR$^{-/-}$ mice (5.3 vs. 5.4 µmol/g of liver respectively; NS).
Conclusion: Intestinal FXR is essential for liver growth in response to dietary CA exposure. The liver proliferation

upon CA administration was, likewise as in CF mice, missing in intestinal FXR $^{-/-}$ mice. CA induced liver growth is not due to changes in hepatic lipid composition. Current results underline the important role of intestinal FXR in the regulation of bile salt metabolism and the hepatic response to prolonged hydrophobic bile salt exposure. Our data suggests that intestinal FXR signaling is impaired in CF mice which may be critical in the development of gastrointestinal complications of CF patients. Our current studies are directed to understanding the mechanism by which CFTR modulates the FXR-FGF15 axis and ultimately in improving the gastrointestinal complications of CF patients.

S3.5 - Linking the CFTR to the Cystic Fibrosis Mucus Phenotype

Lauren Meiss[1], Anna Ermund[1], Andrea Bähr[2], Gunnar C Hansson[1]

[1]Univ. Gothenburg, Dept. Medical Biochemistry, Gothenburg, Sweden, [2]LMU Munich, Chair for Molecular Animal Breeding and Biotechnology, Munich, Germany

The basic defect in cystic fibrosis (CF) is well characterized, but the link between defects in the CF transmembrane conductance regulator (CFTR), the causative gene of CF disease, and the phenomenon of stagnant mucus is not well understood. It has been shown that the ileal mucus in CF mice adheres to the epithelium, is denser, and is less penetrable than that of wild-type mice and that apical addition of 115 mM NaHCO3 to mucus partially normalizes this mucus phenotype (1, 2). Using this knowledge of effects on ileal mucus, we develop an airway experimental set-up. In the airway, CFTR is localized apically not to MUC5AC-expressing goblet cells, but to the neighboring ciliated epithelial cells. This is similar to the small intestine where the MUC2 secreting goblet cells are localized adjacent to the CFTR expressing enterocytes. Using bead-tracking and mass spectrometry analysis, we study the effects of therapies on the mucociliary transport rate, Muc5ac and Muc5b secretion, and mucus clearance patterns in the bronchotracheal tree of both wild-type and cystic fibrosis porcine models as well as in rat airway explants. Scanning electron microscopy is used to visualize the properties of the mucus layer, and transmission electron microscopy is used to study mucin secretion in detail. Preliminary results suggest the importance of bicarbonate in the proper unpacking and secretion of mucins. We hypothesize that the restoration of bicarbonate to the apical surface of the epithelium in combination with osmolytes may induce proper mucin unpacking in CF epithelia, and therefore could relieve the mucus obstruction that causes clinical problems for cystic fibrosis patients.

Support from the Fulbright Commission, the Whitaker International Program, Swedish Research Council, Cystic Fibrosis Foundation, Erica Lederhausens Foundation, RfCF Research Fund, and Lederhausen's CF Center.

S3.6 - Ethanol and fatty acids inhibit the activity of CFTR chloride channel and anion exchangers in pancreatic ductal epithelial cells

József Maléth[1], Petra Pallagi[1], Lajos V Kemény[1], Linda Judák[2], Áron Somorácz[3], Katalin Borka[3], Zoltán Rakonczay Jr.[1], Viktória Venglovecz[2], Mike A Gray[4], Péter Hegyi[1]

[1]University of Szeged, First Department of Medicine, Szeged, Hungary, [2]University of Szeged, Department of Pharmacology and Pharmacotherapy, Szeged, Hungary, [3]Semmelweis University, 2nd Department of Pathology, Budapest, Hungary, [4]Newcastle University, Institute for Cell & Molecular Biosciences, Newcastle upon Tyne, United Kingdom

Background: Excessive ethanol (EtOH) consumption is one of the most common causes of acute pancreatitis, which has no specific treatment yet. Pancreatic ductal epithelial cells (PDEC) secrete HCO_3^- rich pancreatic fluid via

Cl^-/HCO_3^- exchangers (CBE) and CFTR, which prevents acinar damage. No information is available about the effects of EtOH and EtOH metabolites (fatty acid ethyl esters and fatty acids) on PDEC, therefore our **aim** was to characterize these effects.

Methods: In our experiments human pancreatic epithelial cell line (Capan-1), guinea pig PDEC and human pancreatic tissue were used. Changes of intracellular pH (pH_i), Ca^{2+} concentration ($[Ca^{2+}]_i$), ATP [$(ATP)_i$] and mitochondrial membrane potential ($\Delta\Psi$) of PDEC were measured by microfluorometry or confocal microscopy. We

measured the effects of ethanol metabolites on CFTR Cl^- current of PDEC with patch clamp. The expression and localization of CFTR were analysed in PDEC and in human pancreatic tissue samples (from patients with alcohol induced acute (AP) or chronic pancreatitis (CP) or without pancreatic disease (NP)) with immunohistochemistry and RT-PCR.

Results: The administration of low EtOH concentration (10mM) stimulated pancreatic epithelial HCO_3^- secretion *in vitro* via IP_3 mediated $[Ca^{2+}]_i$ elevation. In contrast, both high concentration of EtOH (100mM) and palmitoleic acid (POA) (200µM) inhibited the HCO_3^- secretion of PDEC. Both the activities of the apical CBE and CFTR were decreased by 100mM EtOH or 200µM POA. The administration of 200µM POA induced sustained $[Ca^{2+}]_i$ elevation

by releasing Ca^{2+} from the endoplasmic reticulum via IP_3 and ryanodin receptor activation and extracellular Ca^{2+} influx. Moreover, 100mM EtOH and 200µM POA depleted the $(ATP)_i$ and decreased $\Delta\Psi$. The inhibitory effects of

EtOH and POA were mediated by sustained $[Ca^{2+}]_i$ elevation. We also showed that EtOH, POAEE and POA significantly decreased the expression of CFTR after 48h in PDEC. The expression of CFTR was significantly decreased on the luminal surface of pancreatic ducts in AP and CP patients.

Conclusions: These results suggest that one of the main targets of EtOH and fatty acids is CFTR. Both toxic factors inhibit pancreatic ductal HCO_3^- secretion as well. Restoration of CFTR function and the HCO_3^- secretion may be potential therapeutic possibilities in alcohol induced AP and CP.

Thursday 27 March – 18:00-19:15

FLASH PAPER SESSION
Chairs: William Skach (US) / James Collawn (US)

FP01: Correction of Chloride Transport and Mislocalization of CFTR Protein by Vardenafil in the Gastrointestinal Tract of Cystic Fibrosis Mice.

Dhooghe B, Noël S, Bouzin C, Behets-Wydemans G, Leal T.
Université catholique de Louvain

Although lung disease is the major cause of mortality in cystic fibrosis (CF), gastrointestinal (GI) manifestations are the first hallmarks in 15-20% of affected newborns presenting with meconium ileus, and remain major causes of morbidity throughout life. We have previously shown that cGMP-dependent phosphodiesterase type 5 (PDE5) inhibitors rescue defective CF Transmembrane conductance Regulator (CFTR)-dependent chloride transport across the mouse CF nasal mucosa. Using F508del-CF mice, we examined the transrectal potential difference 1 hour after intraperitoneal injection of the PDE5 inhibitor vardenafil or saline to assess the amiloride-sensitive sodium transport and the chloride gradient and forskolin-dependent chloride transport across the GI tract. In the same conditions, we performed immunohistostaining studies in distal colon to investigate CFTR expression and localization. F508del-CF mice displayed increased sodium transport and reduced chloride transport compared to their wild-type littermates. Vardenafil, applied at a human therapeutic dose (0.14 mg/kg) used to treat erectile dysfunction, increased chloride transport in F508del-CF mice. No effect on sodium transport was detected. In crypt colonocytes of wild-type mice, the immunofluorescence CFTR signal was mostly detected in the apical cell compartment. In F508del-CF mice, a 25% reduced signal was observed, located mostly in the subapical region. Vardenafil increased the peak of intensity of the fluorescence CFTR signal in F508del-CF mice and displaced it towards the apical cell compartment. Our findings point out the intestinal mucosa as a valuable tissue to study CFTR transport function and localization and to evaluate efficacy of therapeutic strategies in CF. From our data we conclude that vardenafil mediates potentiation of the CFTR chloride channel and corrects mislocalization of the mutant protein. The study provides compelling support for targeting the cGMP signaling pathway in CF pharmacotherapy.

Full Reference of the Paper:
PLoS One. 2013 Oct 24;8(10):e77314. doi: 10.1371/journal.pone.0077314.

FP02: HGF stimulation of Rac1 signaling enhances pharmacological correction of the most prevalent Cystic Fibrosis mutant F508del-CFTR

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Cystic fibrosis (CF), a major life-limiting genetic disease leading to severe respiratory symptoms, is caused by mutations in CF transmembrane conductance regulator (CFTR), a chloride (Cl-) channel expressed at the apical membrane of epithelial cells. Absence of functional CFTR from the surface of respiratory cells reduces mucociliary clearance, promoting airways obstruction, chronic infection and ultimately lung failure. The most frequent mutation, F508del, causes the channel to misfold, triggering its premature degradation and preventing it from reaching the cell surface. Recently, novel small-molecule correctors rescuing plasma membrane localization of F508del-CFTR underwent clinical trials but with limited success. Plausibly, this may be due to the mutant intrinsic plasma membrane (PM) instability. Herein, we show that restoration of F508del-CFTR PM localization by correctors can be dramatically improved through a novel pathway involving stimulation of signaling by the endogenous small GTPase Rac1 via hepatocyte growth factor (HGF). We first show that CFTR anchors to apical actin cytoskeleton (via Ezrin) upon activation of Rac1 signaling through PIP5K and Arp2/3. We then found that such anchoring retains pharmacologically rescued F508del-CFTR at the cell surface, boosting functional restoration by correctors up to 30% of wild-type channel levels in human airway epithelial cells. Our findings reveal that surface anchoring and retention is a major target pathway for CF pharmacotherapy, namely to achieve maximal restoration of F508del-CFTR in patients in combination with correctors. Moreover, this approach may also translate to other disorders caused by trafficking-deficient surface proteins.

Full Reference of the Paper:
Moniz S, Sousa M, Moraes BJ, Mendes AI, Palma M, Barreto C, Fragata JI, Amaral MD, Matos P. HGF stimulation of Rac1 signaling enhances pharmacological correction of the most prevalent cystic fibrosis mutant F508del-CFTR. ACS Chem Biol. 2013 Feb 15;8(2):432-42. doi: 10.1021/cb300484r. Epub 2012 Nov 20. PMID: 23148778.

FP03: Revertants, low temperature, and correctors reveal the mechanism of F508del-CFTR rescue by VX-809 and suggest multiple agents for full correction.

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Cystic Fibrosis is mostly due to the F508del-mutation, which impairs CFTR protein from exiting the endoplasmic reticulum due to misfolding. VX-809 is a small-molecule that rescues F508del-CFTR localization which recently went into clinical trial but with unknown mechanism of action (MoA). Herein, we assessed if VX-809 is additive or synergistic with genetic revertants of F508del-CFTR, other correctors and low temperature to determine its MoA.
We explored and integrated those various agents in combined treatments, showing how they add to each other to identify their complementary MoA and the full scope for F508del-CFTR correction.
Our experimental and modelling data, while compatible with putative binding of VX-809 to NBD1:ICL4 interface, also indicate scope for further synergistic F508del-CFTR correction by other compounds at distinct conformational sites/ cellular checkpoints, thus suggesting requirement of combined therapies to fully rescue F508del-CFTR.
Full Reference of the Paper:
Revertants, low temperature, and correctors reveal the mechanism of F508del-CFTR rescue by VX-809 and suggest multiple agents for full correction.
Farinha CM, King-Underwood J, Sousa M, Correia AR, Henriques BJ, Roxo-Rosa M, Da Paula AC, Williams J, Hirst S, Gomes CM, Amaral MD.
Chem Biol. 2013 Jul 25;20(7):943-55. doi: 10.1016/j.chembiol.2013.06.004.
PMID: 23890012

FP04: Airway hyperresponsiveness in FVB/N delta F508 cystic fibrosis transmembrane conductance regulator mice

Mark Bazett, Christina K. Haston

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Background
Airway hyperresponsiveness is a feature of clinical CF lung disease. In this study, we investigated whether the FVB/N ΔF508 CFTR mouse model has altered airway mechanics.
Methods
Mechanics were measured in 12–14 week old FVB/N Cftrtm1Eur (ΔF508) mice and wildtype littermates using the FlexiVent small animal ventilator. Lung disease was assayed by immunohistochemistry, histology and bronchoalveolar lavage analysis.
Results
Cftrtm1Eur mice presented with increased airway resistance, compared to wildtype littermates, in response to methacholine challenge. No differences in bronchoalveolar cell number or differential, or in tissue lymphocyte, goblet cell or smooth muscle actin levels were evident in mice grouped by CFTR genotype. The bronchoalveolar lavage of Cftrtm1Eur mice included significantly increased levels of interleukin 12(p40) and CXCL1 compared to controls.
Conclusion
We conclude that the pulmonary phenotype of Cftrtm1Eur mice includes airway hyperresponsiveness in the absence of overt lung inflammation or airway remodeling.
Full Reference of the Paper:
Mark Bazett, Christina K. Haston, Airway hyperresponsiveness in FVB/N delta F508 cystic fibrosis transmembrane conductance regulator mice, Journal of Cystic Fibrosis, Available online 24 December 2013, ISSN 1569-1993, http://dx.doi.org/10.1016/j.jcf.2013.11.010.

FP05: Purification of the Cystic Fibrosis Transmembrane conductance Regulator protein expressed in *Saccharomyces cerevisiae*.

Naomi Pollock [1], Natasha Cant [1], Tracy Rimington [1] and Robert C. Ford [1]

[1]. Faculty of Life Sciences, University of Manchester, UK

We describe two methods for the purification of the cystic fibrosis transmembrane conductance regulator (CFTR) from a eukaryotic heterologous expression system, *S. cerevisiae*. Like prokaryotic systems, S. cerevisiae can be rapidly grown in the lab at low cost, but in addition they can traffic and post-translationally modify large membrane proteins. The selection of detergents for solubilization and purification is a critical step in the purification of any membrane protein. Having screened for the solubility of CFTR in several detergents, we have chosen two contrasting detergents for use in the purification that allow the final CFTR preparation to be tailored to the subsequently planned experiments.

In this method, we provide a comparison of the purification of CFTR in dodecyl-β-D-maltoside (DDM) and 1-tetradecanoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (LPG-14). Protein purified in DDM by this method shows ATPase activity in functional assays. Protein purified in LPG-14 shows high purity and yield, can be employed to study post-translational modifications, and can be used for structural methods such as small-angle X-ray scattering and electron microscopy. However it displays significantly lower ATPase activity.
Full Reference of the Paper:
Journal of Visualised Experiments, 2014 (in press).

Friday 28 March – 08:45-10:30 **Room: Fortress**

SYMPOSIUM 4
CFTR Folding: Host-pathogen interaction
Chairs: Marc Chanson (CH) / Maria Cristina Dechecchi (IT)

S4.1 - *P. aeruginosa* switch to mucoidity activates airway epithelial cells via the pattern recognition receptors TLR2 and TLR6

Trevor Beaudoin [1], Shantelle Lafayette [2,] Dao Nguyen [1, 2] and Simon Rousseau [1, 2]

[1]Meakins-Christie Laboratories and 2Department of Medicine, McGill University, Montreal, Canada.

The presence of the mucoid phenotype of *P. aeruginosa* in the lungs of CF patients is a marker of poor survival. As CF lung disease results from chronic infection leading to airway inflammation, we determined whether the switch to a mucoid phenotype by *P. aeruginosa* has an impact on the inflammatory response of airway epithelial cells. Exposure of airway epithelial cells to non-mucoid and mucoid *P. aeruginosa*-derived material leads to p38α MAPK activation, a key protein kinase involved in transmitting inflammatory signals. However, while the non-mucoid strain PAO1 activates p38α MAPK pathway solely via TLR5, a clinical mucoid *P. aeruginosa* strain activates p38α MAPK via both TLR5 and TLR2. Inactivation of MucA (the gene responsible for the mucoid phenotype) in PAO1 leads to p38α MAPK activation by both TLR2 and TLR5, as observed in the clinical mucoid isolate. TLR2 heterodimerizes with TLR1 or TLR6 to recognize tri-acylated (TLR1) or di-acylated (TLR6) lipopeptides. The TLR1/TLR2 heterodimer is usually link with recognition of gram-negative bacteria. In order to determine, which receptor pair recognizes mucoid *P. aeruginosa*, we use bimolecular fluorescence complementation. Mucoid *P. aeruginosa* or the MucA PAO1 mutant activated much more strongly the TLR2/TLR6 pair than the TLR1/TLR2 pair. Dimerization of TLR2/TLR6 led to a strong NFkB and ATF2 reporter activity, showing that it was functional at driving inflammatory gene expression. Moreover, neutralization of TLR6 but not TLR1 decreased p38 MAPK activation in airway epithelial cells exposed mucoid *P. aeruginosa*. Therefore, the switch to mucoid phenotype contributes to heightened inflammation via TLR2/TLR6 activation in addition to TLR5 homodimerization. Our findings highlight an important and under recognized for TLR2 and TLR6 in the response of airway epithelial cells to gram-negative bacterial infections.

S4.2 - Interferon and Inflammasome signaling in response to *P. aeruginosa*

Taylor S. Cohen and Alice Prince

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The respiratory tract is normally well defended against inhaled pathogens through the actions of immune and epithelial cells. Given the redundancies in proinflammatory signaling, some of the responses evoked have detrimental effects, causing disruption of the epithelial barrier and airway damage. Using mouse models of acute *P.aeruginosa* infection, we demonstrated that activation of the NLRC4 inflammasome, predominantly in alveolar macrophages, was associated with lung damage and interfered with efficient bacterial clearance. Experimental strategies to block inflammasome activation (capsase-1 inhibition, infection with FliC null mutants, infection in mice lacking NLRC4 or the receptors for IL-1 or IL-18) all had significantly improved clearance. Cytokine production, particularly IL-1β correlated with pathology, not the accumulation of neutrophils in the airways.

CF patients are infected by motile *P. aeruginosa* that activate inflammasome signaling in the airways, producing IL-1β and IL-18. Normally, inflammasome transcription is regulated by the type I IFNs. We found that IFN-β is potently induced in the lung, in epithelial and predominantly in immune cells by *P. aeruginosa* LPS and DNA. Treatment of mice with polyI:C which stimulates a type I IFN response, significantly improved *P. aeruginosa* clearance. However, significantly less IFN-β and other type I IFN effectors are produced by cells with CFTR mutations. Thus, the CF cell is less capable of controlling the inflammatory responses generated by the inflammasome that is briskly stimulated by *P. aeruginosa* particularly in the early stages of infection.

The type III interferons (IFN-λ) are also activated by airway pathogens, both S. aureus and *P. aeruginosa*. IFN-λ signaling is mediated by the IL-28/IL-10 composite receptor that is expressed primarily in the mucosal epithelium, in contrast to the more generalized distribution of IFNAR, which responds to type I IFNs. IFN-λ signaling contributes significantly to proinflammatory cytokine production and lung pathology. There was significantly increased bacterial clearance in IL-28R-/- mice, lacking the IFN-λ receptor. The proinflammatory signaling activated by these IFN-λ-IL-28R is mediated by mIR-21 and its target PDCD4, which function to amplify epithelial inflammation and add to lung damage. Thus the IFN-λ cascade provides a potential target to block redundancies in proinflammatory gene expression in CF. In contrast, the type I IFN cascade provides regulation of the inflammasome and its production of toxic cytokines. These studies illustrate the complexity of innate immune signaling in the airway and offer multiple potential targets for immunomodulation.

S4.3 – *P.aeruginosa* and modulation of IL-8 gene expression in bronchial epithelial cells

Giulio Cabrini [1], Valentino Bezzerri [1], Enrica Fabbri [2], Valentina Lovato [1], Giulia Montagner [2], Nicoletta Bianchi [2], Silvia Munari [1], Susanna Khalil [1], Maria Cristina Dechecchi [1], Anna Tamanini 1, Francesca Salvatori [2], Alessia Finotti [2], Ilaria Lampronti [2], Monica Borgatti [2], Alessandro Rimessi [3], Simone Patergnani [3], Paolo Pinton [3], Roberto Gambari [2]

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CFTR mutations are known to affect the intracellular signaling activating the innate immune in CF bronchial epithelial cells. The potent chemokine IL-8, which drives the recruitment of polymorphonuclear neutrophils in CF bronchial mucosa, has been detected in the bronchoalveolar lavage fluid in CF infants even in the absence of bacterial infection. Different concomitant conditions, including the increased viscosity of the airway surface liquid due to the ion transport defect, favour bacterial infection in CF lungs, which massively amplifies the constitutive inflammatory response activated by mutant CFTR protein. In particular, *P.aeruginosa* infection exerts multiple direct and indirect effects on the mucosa of the conductive airways [1]. Here we report our results focused on how the interaction of *P.aeruginosa* with bronchial epithelial cells modulates the intracellular pro-inflammatory signaling regulating IL-8 gene expression [2-4].

Direct interaction of whole planktonic *P.aeruginosa* with Toll-like Receptors (TLR2, 5 and 4) strongly upregulates IL-8 gene expression. TLR2/5-dependent activation of MyD88 induces phosphorylation of different Mitogen Activated Protein kinases, such as ERK1/2, p38, JNK, and other downstream kinases as RSK1/2 and HSP27, leading to intranuclear translocation of transcription factors such as NF-kB, NF-IL6, AP-1, CREB and CHOP. MyD88-independent pathways can also contribute to regulate the expression of IL-8 gene. In this respect, the release of ATP in the extracellular milieu, which is associated with *P.aeruginosa* interaction with the epithelial cell, is able to sustain a P2Y2 purinergic receptors- and phospolipase C beta3-dependent intracellular calcium signaling, which potentiates the MyD88-dependent pathways on IL-8 gene transcription. In this specific model, post-transcriptional regulation of IL-8 mRNA in cells exposed to *P.aeruginosa* is mediated by MCM7-dependent expression of miR-93, which plays a relevant role as negative feedback mechanism. These issues will be discussed considering that insights on the intracellular signaling involved in IL-8 gene expression will be useful to develop novel anti-inflammatory drugs tailored to the specific pathophysiology of CF lung disease.

Supported by Italian Cystic Fibrosis Research Foundation to GC, RG and PP, grants FFC #18/2009, FFC #12/2010, FFC # 19/2011.

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S4.4 - Whole genome sequencing provides evidence of within-patient evolution and adaptation to the lung of *Mycobacterium abscessus*

Dorothy Grogono[1], Josephine Bryant[2], Daniel Greaves[3], Juliet Foweraker[4], Charles Haworth[1], Martin Curran[5], Simon Harris[2], Sharon Peacock[6], Julian Parkhill[2], Andres Floto[1,6,7]

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Aim

Mycobacterium abscessus is an emerging cause of infection in patients with cystic fibrosis. It can be difficult to treat and consequently many patients develop chronic infection. Our aim was to look for evidence of within-patient evolution of *Mycobacterium abscessus*, using whole genome sequencing.

Methods

Whole genome sequencing (average depth of coverage 111 fold) was performed on 155 isolates of *Mycobacterium abscessus* from 22 patients with cystic fibrosis. Genetic diversity across the whole genome was analysed. Colony morphology was documented for all isolates and antibiotic susceptibility testing performed on a subset. Clinical data was collected for all 22 patients - including treatment regimens, lung function (FEV1), and markers of systemic inflammation (C-reactive protein).

Results

The genetic diversity between isolates (measured by pairwise SNP distance) from the same individual increased with the length of infection (r^2=0.78, p-value = 0.0007). Genetic diversity within samples (assessed by the number of minority variants) also increased the longer the individual had been infected (r^2=0.72, p-value < 0.0001).

We found that genetic diversity correlated with bacterial load (using time to liquid culture positivity as a surrogate). Peaks in genetic diversity and bacterial load correlated with clinical exacerbations (evidenced by increases in CRP and/or drop in FEV1). Conversely effective treatment was associated with a drop in genetic diversity and bacterial burden.

Phenotypically we saw evidence for within-sample diversity of both rough-smooth morphotype and antibiotic susceptibility and, as expected, found heterozygosity within genes associated with antibiotic resistance and colony morphology.

We also detected convergent evolution of *Mycobacterium abscessus* isolated from different individuals, with repeated accumulation of non-synonymous SNPs in a number of genes thought to control virulence.

Conclusions

We have demonstrated for the first time that the genetic diversity of *Mycobacterium abscessus* increases over the course of infection and may play an important role in the development of antibiotic resistance and modulation of virulence. This genetic diversity may also explain the lack of correlation between laboratory antibiotic susceptibility testing and the clinical response of patients to specific treatments.

S4.5 *Pseudomonas aeruginosa* reduces the expression of CFTR in airways via post translational modification of NHERF1

Rosa Rubino[1], Valentino Bezzerri[2], Maria Favia[1], Marcella Facchini[3], Moira Paroni[3], Maria Cristina Dechecchi[2], Maela Tebon[2], Antonio Iannucci[4], Brigitte Riederer[5], Anurag Kumar Singh[5], Ursula Seidler[5], Alessandra Bragonzi[3], Giulio Cabrini[2,4], Stephan Reshkin[1], Anna Tamanini[2]

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P. aeruginosa infections of the airway cells decrease apical expression of both wild-type (wt) and F508del CFTR through the inhibition of apical endocytic recycling. CFTR endocytic recycling is known to be regulated by its interaction with PDZ domain containing proteins (Swiatecka-Urban A. *et al.* 2002, Guerra L. *et al*. 2005, Kwon SH. *et al*. 2007, Bomberger J.M. *et al*. 2011). Recent work has shown that the PDZ domain scaffolding protein NHERF1 finely regulates both wt and F508delCFTR membrane recycling (Favia M. *et al*. 2010, Monterisi S. *et al*., 2012). Here, we investigated the effect of *P. aeruginosa* infection on NHERF1 post-translational modifications and how this affects CFTR expression in bronchial epithelial cells and in murine lung.

Infection reduced expression CFTR and, in parallel, increased phosphorylation and ubiquitination of NHERF1, as a consequence of both bacterial pilin- and flagellin-mediated host-cell interaction, both in bronchial cells *in vitro* and in mice *in vivo*.

The ability of *P. aeruginosa* to down-regulate mature CFTR expression was partially reversed a) in NHERF1 knockout mice *in vivo* and b) in bronchial epithelial cells *in vitro* after silencing NHERF1 expression or by testing NHERF1 mutants impairing its phosphorylation at serines 279 and 301. These studies provide the first evidence that NHERF1 phosphorylation in response to infection may negatively regulate the assembly and function of multiprotein NHERF1 complexes which are relevant to stabilize CFTR on the plasma membrane. The identification of the transduction signals downstream Toll-like Receptors that could be involved in NHERF1 phosphorylation could highlight novel targets to block the potential interference of *P. aeruginosa* on the therapeutic efficacy of potentiator and/or corrector compounds.

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S4.6 - Antiviral activity of azithromycin in cystic fibrosis airway epithelial cells

Aline Schögler[1,2,3], Brigitte S. Kopf[1,2], Ricardo J. Muster[1,2], Elisabeth Kieninger[1,2], Carmen Casaulta[2], Andreas Jung[4], Alexander Moeller[4], Thomas Geiser[1,5], Marco P. Alves[1,2], Nicolas Regamey[1,2]

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Introduction. Virus-associated pulmonary exacerbations, most predominantly caused by rhinoviruses (RV), contribute to cystic fibrosis (CF) morbidity. However, there are only few therapeutic options to treat and/or prevent virus-induced CF pulmonary exacerbations. Recent evidence suggests that the macrolide antibiotic azithromycin (AZM) has antiviral properties in RV-infected primary human bronchial epithelial cells. We hypothesize that AZM induces antiviral mechanisms in primary CF bronchial epithelial cells.

Methods. Primary bronchial epithelial cells from 11 CF children (median [range] age: 8.21 [1.14-14.98] years) were pretreated with AZM and infected with the minor group virus RV1B. Viral RNA, interferon (IFN)λ2/3, IFNI3, IFN-stimulated genes (ISGs; oligoadenylate synthetase, MxA, viperin), RV1B surface receptor (low density lipoprotein) and pattern recognition receptors (PRRs; retinoic acid inducible gene I, melanoma differentiation associated gene 5, toll-like receptor (TLR)3, TLR2) expressions were measured by RT-qPCR. Pro-inflammatory cytokines and IFNI3 production were assessed by ELISA. Cell death was evaluated by Flow cytometry.

Results. After AZM pretreatment, RV1B load was 7-fold reduced in CF bronchial cells compared to untreated cells (median [interquartile range] 10^6 copies/reaction: 8.13 [5.28-34.4] vs. 56.8 [18.4-81.5]; p=0.001). The decreased RV1B load was not due to AZM-induced cytotoxicity in CF bronchial cells. Furthermore, AZM pretreatment significantly increased RV1B-induced IFNs and ISGs, and also RV1B surface receptor and PRRs mRNA expressions in CF bronchial cells. Interestingly, while stimulating antiviral responses, AZM pretreatment did not significantly prevent virus-induced inflammatory cytokines production.

Conclusion. AZM pretreatment reduces RV load in primary paediatric CF bronchial epithelial cells *in vitro*, possibly through inducing a robust antiviral response including the induction of IFNs and ISGs. This study points to the potential of AZM as a novel therapeutic approach to treat and/or prevent RV-induced CF pulmonary exacerbations.

Friday 28 March – 11:00-12:45

SYMPOSIUM 5
Modifier Genes: identifying the mechanisms
Chairs: Mitch Drumm (US) / Patrick Harrison (IE)

S5.1 - Modifier genes in CF

Claude Ferec[1,2]

[1] INSERM UMR1078 ,[2]University Hospital Brest

Twenty five years after the CFTR gene discovery, nearly two thousand mutations and many polymorphisms have now been reported in the gene responsible of CF. These mutated alleles could be classified into five classes according to their impact on the CFTR protein function . However the impact of the mutations on the severity of the phenotype has been a matter of debate and is still a matter of research.
There is a broad range of age of onset for lung disease, meconium ileus, diabetes or liver disease between CF patients, and this is true also for patients who are homozygous for the most common mutation, the F 508 del. Therefore other non CFTR genetic variations or other environmental or socio econnomic factors do contribute to the clinical variability. To determine which genes are involved, which gene/ environmental factors are involved in the disease evolution will provide insight into the mechanism of disease pathogenesis. Identification of modifiers gene will help us to understand why and how this mono genic disease is now really a complex disease. The lung disease severity was so far the most studied phenotype. The track for modifiers gene was first based on a candidate gene approach but rapidly genome wide association study (GWAS) on large cohorts of patients were performed. We will review the main results of these approaches obtained in the past years. Among the candidate genes approach cytokines as for example the TGFB1 1 has been largely studied and conflicting results have been reported but finally it appears that TGFB1 is a modifier of the CF lung phenotype.
Candidate genes involved in the infectious response like the mannose-binding lectine 2 have been reported to be associated with earlier colonization of *P Aeruginosa.*
Results from association studies identified 7 genomic regions that might be associated with CF lung disease. More recently an exome wide association study based on extreme phenotype selection (early versus late *P.aeruginosa* infection) identifies a DCTN4 gene encoding Dynactin 4, a protein involved in the autophagy process and degradation of microbes, is significantly associated with time to chronic *P Aeruginosa* infection.
The vast number of genetic modifiers contributing to the variability of the CF phenotype illustrates the complexity of the relationship between genotype and phenotype in CF. We can speculate that the whole genome association studies which are coming soon will certainly bring us new insights in this complex field.

S5.2 – The 11p13 CF modifier region: investigating the chromatin landscape

Sara Fossum, Rui Yang, Michael Mutolo, Jenny Kerschner, Lindsay Stolzenberg, Lisa Jones, Hong Dang, Wanda O'Neal, Michael Knowles, Ann Harris

Programme Note: Abstract details are not authorised for publication

S5.3 - Constituents of the Apical Plasma Membrane Contribute to Disease Severity Across Multiple CF-Affected Organs

Lisa J. Strug

[1]The Hospital foir Sick Children Research Institute
[2]Faculty of Public Health Sciences, The University of Toronto

CF affects the lungs, intestines and pancreas, among other organs. Individuals with the same CFTR genotype have variable morbidity across the affected organs that is heritable, suggesting modifier genes contribute to the complex CF phenotype. Given CFTR is a Cl- channel located in the apical membrane of epithelial cells, we hypothesized that with loss of CFTR other transporters that result in variation in any residual or adapted epithelial functions contributed by apical membrane constituents could modify CF phenotypes. Using a genome-wide association study (GWAS) framework, we tested this hypothesis with the meconium ileus (MI) phenotype. We began with MI since it is highly heritable, and occurs at birth with limited opportunity for environmental influence. Clinical and genome-wide genotype data were available on 3,763 individuals with severe CFTR genotypes associated with pancreatic insufficiency from the North American CF Gene Modifier Consortium (NACFGMC). We observed multiple constituents of the apical plasma membrane as a group, and independently, to be associated with MI, including proteins encoded by the solute carrier family genes, SLC26A9, SLC6A14, and SLC9A3. These findings were replicated in an independent set of CF patients from North America (n=1,140) and from France (n=1,232).

Motivated by the findings from a genome-wide association study that CF-related diabetes is associated with the same MI risk alleles in SLC26A9, we asked whether the MI gene modifiers, SLC26A9, SLC6A14 and SLC9A3, are associated with disease in other CF-affected organs such as exocrine pancreatic damage and early lung disease. Using the Canadian subset of 1,661 participants from the NACFGMC we assessed the evidence of pleiotropy for the three solute carriers. Early lung disease measures (n=1,661) were associated with markers in SLC9A3 (min p=1.5x10^-6) and in SLC6A14 (min p=0.0002); while the MI-associated markers in SLC26A9 were associated with newborn screened measures of immunoreactive trypsinogen (IRT), a biomarker of exocrine pancreatic damage at birth, in a subset of the NACFGMC (n=147) from Colorado and Wisconsin (min p=3.82 x 10-4 at rs7512462). Next we examined whether multiple constituents of the apical plasma membrane, as a group, contribute to early exocrine pancreatic damage and lung disease, as was the case for MI. In both the Canadian and French samples, lung disease was associated with the group of 155 genes that code for apical plasma membrane constituents (p=0.0112 and p=0.0125, respectively). Whereas, the single marker rs7512462 in SLC26A9 accounted for 11% of the variance in newborn screened IRT but no other gene or the apical gene set appeared to significantly contribute to early pancreatic disease.

S5.4 - Genetic and functional correction of the human ΔF508 *CFTR* locus using designer nuclease technology

Christien Bednarski[1,2], Katja Tomczak[3], Wolf Michael Weber[3], Toni Cathomen[1,2]

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Considerable effort has been invested to develop a sustainable gene therapy for Cystic Fibrosis. Most of these approaches have been based on transfer of a normal *CFTR* gene copy to therapeutically relevant cell types. Another promising, alternative strategy is the targeted *in situ* correction of the mutated *CFTR* locus using designer nucleases, such as zinc-finger nucleases (ZFN).

In this study, we aimed at correcting the ΔF508 mutation using the ZFN technology in combination with an appropriately designed donor DNA to correct the mutated *CFTR* locus via homologous recombination. Activity of the *CFTR* exon 10-specific ZFN was verified by employing cleavage assays *in vitro* and *in cellula*. Human bronchial epithelial cells (CFBE41o$^-$), which are homozygous for the ΔF508 mutation, were nucleofected with ZFN expression vectors and a donor DNA carrying a *CFTR* super-exon encoding exons 10-24 and a puromycin resistance cassette. PCR-based genotyping confirmed targeted integration of the super-exon into *CFTR* exon 10 in the selected cell population. Subsequent clonal analysis revealed a gene targeting frequency of 8%. All genetically corrected clones carried a monoallelic targeted integration of the super-exon, reflecting a heterozygous genotype. Furthermore, corrected clones showed donor specific *CFTR* mRNA expression, as evaluated by semi-quantitative RT-PCR.

Finally, one gene corrected CFBE41o$^-$ clone has been evaluated for restored CFTR function. Ion transport measurements in Ussing chamber measurements confirmed functional transepithelial characteristics when activated with cAMP and inhibited via CFTRInh172, indicative of restored CFTR channels.

In conclusion, our data demonstrate that designer nucleases are a powerful tool to genetically and functionally correct mutations in the CFTR model cell line CFBE41o$^-$. This proof of principle study will pave the way for subsequent genome editing strategies in patient-derived stem cells for future therapeutic applications.

S5.5 - Study of long-range regulatory mechanisms of the *CFTR* gene

Stéphanie Moisan[1,2], Claude Férec[1,2,3]

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The cystic fibrosis transmembrane conductance regulator *(CFTR)* gene was identified in 1989. The regulatory mechanisms controlling its complex expression are still not fully understood. Although, 1949 mutations have been identified, many cases of cystic fibrosis remain still of unknown origin.
The aim of our project is to study long-range regulatory mechanisms of the *CFTR* gene.
We first developped the Chromosome Conformation Captures (3C) approach to map these potentials regulatory elements which could interact specifically with the *CFTR* gene by tri-dimentional folding mechanism.
Subsequently, we enhanced our analyses with a high-throughput adaptation of 3C: the 3C-Carbon Copy (5C) technology. This approach allows the analysis of millions chromatin interactions.
A first region of 783kb, comprising the *CFTR* gene, was covered with 5C primers. Interactions between these regions and the *CFTR* promoter are analysed by next generation sequencing with the Ion PGMTM.
These analyses are performed with primary epithelial cells, which express the gene and primary skin fibroblasts, wich do not express the gene.
Results obtained from 8 epithelial's libraries and 6 fibroblastic's libraries, isolated from healthy controls, are reproducible. Some regions seem to specifically interact with the promoter.
Thanks to these studies, we want to describe an expression dependent conformation of the *CFTR* locus.
These analyses will be pursued on patients affected by cystic fibrosis in whom either a single mutation or none was found in the *CFTR* locus.

This work is supported by “Vaincre La Mucoviscidose”.

S5.6 - Role of microRNAs in human airway epithelium differentiation: Characterization of miR-449 as a central player in multiciliogenesis conserved in vertebrates.

Benoit Chevalier[1], Anna Adamiok[2], Olivier Mercey[1], Laure-Emmanuelle Zaragosi[1], Christelle Coraux[3], Andrea Pasini[2], Laurent Kodjabachian[2], Pascal Barbry[1], Brice Marcet[1]

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The airway epithelium is the first line of defense which protects the respiratory tract against frequent external aggressions (inhaled toxic particles, pathogens, allergens,...). This epithelium lining the surface of the airways is constituted by basal cells, goblet cells and multiciliated cells (MCCs) which exhibit hundreds of motile cilia. The coordinated beating of these motile cilia is crucial to the mucociliary clearance essential for airway cleansing. Cystic fibrosis (CF) is a genetic disease characterized by chronic pulmonary infections which lead to airway epithelium remodeling, a goblet cell hyperplasia and a loss of MCCs. As a result, the mucociliary clearance is impaired, ultimately provoking respiratory failure. The formation of motile cilia (multiciliogenesis) requires sequentially a cell cycle arrest, followed by a massive multiplication of centrioles which then migrate and dock into a dense apical actin network before axoneme elongation of each cilium. A better understanding of complex mechanisms governing multiciliogenesis could help to develop new regenerative therapeutic strategies in CF for restoring airway epithelium integrity and mucociliary clearance.

MicroRNAs (miR or miRNAs) are small non-coding regulatory RNAs implicated in numerous biological processes and more recently associated with several chronic airway diseases.

Using two distant models of mucociliary epithelium (*in vitro* with the human airway epithelium and *in vivo* with the the *Xenopus* embryonic epidermis) we have shown that miRNAs of the miR-449 family were specifically expressed in precursors and mature MCCs. Protector oligonucleotides, targeting miR-449 binding site on several targets (Notch pathway and small GTPase R-Ras), indicate that miR-449 promote the cell cycle arrest, control both the amplification of centrioles and the apical actin remodeling, and allow MCC precursors to differentiate. Our findings demonstrate that miR-449 miRNAs are key regulators of vertebrate multiciliogenesis which act as "chefs d'orchestre" by finely controlling several pathways to trigger MCC differentiation.

Friday 28 March – 18:30-20:15

SYMPOSIUM 6
Cellular therapy for cystic fibrosis and airway epithelium repair
Chairs: Christel Coraux (FR) / ()

S6.1 - iPS cells for treatment of Cystic Fibrosis

Ulrich Martin

Leibniz Research Laboratories for Biotechnology and Artificial Organs, Department for Cardiac, Thoracic and Transplantation and Vascular Surgery, REBIRTH - Cluster of Excellence, BREATH - German Center for Lung Research, Hannover Medical School
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The availability of diseases-specific human induced pluripotent stem cells (hiPSCs) with their almost unlimited potential for proliferation and differentiation now offers novel opportunities in personalized medicine. In case of hereditary diseases such as cystic fibrosis (CF), human iPSCs will be the basis for advanced in vitro systems for disease modelling including assessment of the impact of genetic background and epigenetic factors, and for set up of mutation- and organ-specific high throughput screens useful in primary drug screening and compound evaluation.
hiPSC generation has now become a routine approach and scalable production of large amounts of iPSCs is possible. Novel genome engineering technologies not only allow for efficient introduction of required reporter genes but also footprintless correction of CFTR mutations for generation of isogeneic WT control cells. Although not as advanced as for other organs such as the heart , remarkable advance is currently observed with regard to the development robust differentiation protocols that will allow production of iPSC-derived CFTR-expressing epithelia especially of lung and bile duct.
This presentation will provide an overview on recent developments in iPSC generation, controlled iPSC culture, novel genome engineering approaches and targeted differentiation into relevant cell lineages. Finally, opportunities for ex vivo gene repair and personalized cell therapy based on disease corrected respiratory iPSC derivatives including discussion of current risks and limitations are discussed.

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S6.2 - Cell Based Therapy: Cell Replacement and Niche Modification -

Susan D. Reynolds

National Jewish Health

Chronic lung diseases are the second most prevalent cause of death worldwide (1). Collectively, these diseases encompass airway-centered ailments (cystic fibrosis, asthma, chronic bronchitis) as well as a subset of more alveolar-centric illnesses (chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis). Symptomatic care has greatly improved quality of life for these patients; however, curative treatments await bold interventions that target causal mechanisms. I will focus on one such approach, Cell Based Therapy.

Abnormal epithelial repair is a central component of chronic lung disease and epithelial progenitor cell dysfunction is a known-instrument of the repair defect (2-11). Progenitor cell defects in non-respiratory tissues have been treated by cell transplantation (12-21). The success of these initiatives inspired an NHLBI Working Group to identify Cell Based Therapy as a core strategy to overcome chronic lung disease in our lifetime (http://www.nhlbi.nih.gov/meetings/stemcell_wg.htm). Cell Based Therapy depends on identification of a therapeutic cell that has extensive mitotic and differentiation potential. Thus, such initiatives often focus on a tissue stem cell (TSC). The TSC is defined as a cell that: 1) maintains itself through self-renewal, and 2) generates each of the differentiated cell types found in its home tissue (22, 23). Several groups showed that the tracheobronchial epithelium (TBE) is maintained by a TBE-TSC and that it is a basal cell subtype (24-38).

The unique TSC functions, self-renewal and multipotential differentiation, are governed by a specialized microenvironment termed the TSC niche. My group used a series of in vitro tests to purify the mouse TBE-TSC and showed that the TBE-TSC generated its own niche (28). Niche formation by the mouse TBE-TSC is initiated by direct cell contact between the TBE-TSC and fibroblasts (28). Our pilot analysis of human basal cells identifies a highly clonogenic subtype that also generates its own niche.

Cell Based Therapy employs two basic strategies, straightforward transplantation of TSC and more futuristic manipulation of the TSC niche. First, I will present preliminary data indicating that mouse and human TBE-TSC have therapeutic potential following transplantation, i.e., the *TSC Replacement Approach*. Second, I will present preliminary data indicating that TBE-TSC cell-division results in self-renewal and niche generation in vitro. Thus, manipulation of TBE-TSC cell division may be a useful intervention that will replace the niche or restore niche function i.e. *Niche Modification Therapy*.

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S6.3 New strategies for the improvement of CF airway epithelial repair

Nguyen Thu Ngan Trinh [1], Claudia Bilodeau [1], Alban Girault [1], Manon Ruffin [1], Émilie Maillé [1], Anik Privé [1], Damien Adam [2], Simon Rousseau [3], Christelle Coraux [2], Emmanuelle Brochiero [1]

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In cystic fibrosis (CF), mutations in the CFTR gene lead to dysfunctional Cl- secretion and reduced mucociliary clearance favoring bacterial colonization and chronic inflammation, which are responsible for the progressive lung damage. Our histological analysis indeed revealed extended areas of epithelial shedding and remodeling in CF human airways and alveoli. Epithelial injury is a deleterious component of the CF pathology, since it may further impair the defense against pathogens, thus creating a vicious circle of infections and injuries. Although repair processes are engaged after damage to restore the epithelial integrity, they are obviously insufficient to maintain lung function in CF patients. Our aim is thus to better understand the factors that could impact epithelial wound repair in CF and to develop new therapeutic strategies promoting airway epithelial repair.

We first demonstrated that alveolar and airway repair processes (including cell migration, proliferation and wound healing) are dependent on K+ channel function coupled to EGF/EGFR signaling as well as to integrin proteins. Indeed, the inhibition of KATP, KCa3.1 and KvLQT1 K+ channels reduced epithelial repair rates after EGF or integrin stimulation, whereas K+ channel activation promoted wound healing.

Then, our results indicated that Cl- transport, particularly through CFTR channels, is also crucial for airway epithelial repair. Indeed, we found that CFTR inhibition or silencing in non-CF primary human airway epithelial cell monolayers significantly slowed the repair processes. Furthermore, our data revealed a delay in CF bronchial repair compared to non-CF, even in pathogen-free condition. Thus, the basic defect of CFTR could be responsible, at least in part, of the inefficient epithelial repair in CF. Interestingly, we then discovered that CFTR correction, after wt-CFTR transduction or rescue with CFTR correctors, enhanced the repair capacity of airway monolayers, thus highlighting a new, unsuspected function of CFTR correctors.

Moreover, bacterial infection, particularly by *Pseudomonas aeruginosa*, is another deleterious component of the CF lung disease. In addition to its involvement in the development of airway damage, this pathogen also affected the repair capacity of airway epithelia. Furthermore, we recently discovered that the presence of *Pseudomonas aeruginosa* diffusible material (PsaDM) impaired CFTR maturation and functional rescue by CFTR correctors. We also showed that part of the beneficial effect of CFTR correctors on airway epithelial repair was prevented by PsaDM. Interestingly, our preliminary experiments revealed that K+ channel activation was still able to efficiently stimulate airway epithelial repair, even in infectious conditions.

Overall, our data thus provided evidence of the involvement of CFTR and K+ channels in epithelial repair. We believe that it will now be crucial to identify the best combination of CFTR correctors and/or K+ channel activators that will be able to efficiently promote lung epithelial repair, even in the presence of infection.

S6.4 - OligoG normalizes the CF mucus phenotype

Anna Ermund[1], Lauren Meiss[1], Edvar Onsøyen[2], Phil Rye[2], Arne Dessen[2], Yngvar Berg[2], Astrid Hilde Myrset[2], Gunnar C Hansson[1]

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Cystic fibrosis (CF) is a recessive genetic disease caused by nonfunctional chloride and bicarbonate ion transport via CFTR. In the lungs of these patients, dense, intractable mucus collects because it is insufficiently cleared by the mucociliary clearance system, something that leads to lung infections and lung damage.

We have previously shown that the gel-forming MUC2 mucin is packed in an orderly way that allows unpacking by calcium removal and raised pH (1). Using an explant system (2), we found that the mucus of the small intestine in mice without a functional CFTR channel (CftrΔF508) is, in contrast to normal mucus, attached to the epithelium. This phenotype could be reverted to a non-attached phenotype by apical solutions containing about 100 mM bicarbonate (3).

OligoG CF-5/20 (AlgiPharma, Sandvika, Norway) is a natural product derived from brown algae alginate comprised of mainly guluronate oligomers, with average length of 13 monomers. Previous studies have shown that this oligomer alters the rheology of mucin/alginate gels, mucin/DNA gels and CF sputum. Because of this, OligoG is being tested as an inhalation therapy on CF patients. We have now tested OligoG on mouse ileum CF mucus. Explants from the small intestine of CFTRΔ508 mutant mice were mounted in the horizontal Ussing-type chamber (2). OligoG (1%, 1.2%, 1.5%, 2%, 3% or 6%) was added to the apical buffer, pH of 7.4. The attachment of the already formed mucus was assessed by comparing the total mucus thickness before and after aspiration. OligoG at 1.5% or higher transformed the mucus to a non-adherent normal phenotype without increase in mucus thickness. At 1% OligoG the mucus remained attached and at 1.2% an intermediate phenotype was observed. The effects are likely due to OligoG's known ability to chelate calcium. These observations represent an indication of how OligoG could work in cystic fibrosis patients through a normalization of mucus layers in both the gut and potentially the lungs, at a concentration that could be achieved.

Support from the Fulbright Commission, the Whitaker International Program, Erica Lederhausens Foundation, RfCF Research Fund, and Lederhausen's CF Center.

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S6.5 - Primary human airway epithelial cells expanded with feeder cells and ROCK inhibitor for screening novel GSNO reductase inhibitors and CFTR correctors

Peter F Bove[1], Kirsten M Look[1], Michael A Suniga[1], Xicheng Sun[1], Charles H Scoggin[1], Sherif E Gabriel[1]

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The current cell culture technology limits our ability to indefinitely expand primary human airway epithelial (HAE) cells while retaining their cell-specific phenotype and function. Controlled experiments of primary normal and cystic fibrosis (CF) -derived HAE cells determined that they retain primary culture morphology and functional properties for only a few passages (up to passage 2), thereby limiting the number of critical *in vitro* experiments performed. Therefore, the ability to markedly increase the supply of functionally relevant primary HAE cells derived from patients with various CF mutations (i.e. ΔF508, G551D, R117H, etc.) will allow for a significant increase in additional future studies designed to evaluate essential therapeutics for mutation specific CFTR correction or potentiation. Recent studies demonstrated that primary normal and tumor cells could be isolated and cultured in combination with irradiated fibroblast/feeder cells (mouse 3T3 fibroblasts, J2 sub clone) and an inhibitor of the rho kinase (ROCK) signaling pathway (Y-27632, "Y"). Under these co-culture conditions, cells were shown to proliferate indefinitely with characteristics similar to that of adult stem cells. Cells were then grown onto membrane porous cell supports in the absence of feeder cells and ROCK inhibitor, and shown to revert back/differentiate to their original cell phenotype. Utilizing this co-culture system, we have successfully expanded normal as well as CF-derived HAE cells from patients expressing the ΔF508 mutation (up to passage 6). These cells were seeded onto membrane porous inserts where they maintained an air/liquid interface, retained their cell specific morphology, and expressed functional ion transport characteristics similar to well-differentiated HAE cultures. These newly expanded and well-differentiated HAE cultures were examined histologically by hematoxylin and eosin (H&E) staining as well as alcian blue-Periodic Acid Schiff (AB_PAS) staining. Ussing chamber analyses had been performed using these newly expanded primary HAE cells (up to passage 6) to confirm retention of characteristic HAE ion transport properties. Moreover, we utilized these newly expanded cells for screening of our lead novel GSNO reductase inhibitors and CFTR correctors to identify their capacity to increase CFTR-mediated Cl^- transport. In addition, we have initiated studies for identifying changes in airway surface liquid (ASL) height using these newly expanded normal and CF HAE cells by confocal microscopy, another *in vitro* based model for screening lead novel compounds. Collectively, we anticipate that by expanding our supply of normal and CF-derived HAE cells, we significantly increase our ability to perform functional *in vitro* cell based screening assays designed to target CFTR corrective/potentiative therapies.

S6.6 - Cx26, Notch and PPARg-dependent pathways during human airway epithelial cell repair

Joanna Bou Saab[1], Marc Bacchetta[1], Marc Chanson[1]

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Being subjected to continuous assaults, the pseudostratified airway epithelium undergoes a coordinated repair process in order to maintain its protective function. The mechanisms regulating this process, which may be deregulated in Cystic Fibrosis (CF) cells, are poorly known. Studies have shown the implication of Notch and peroxisome proliferator-activated receptor g (PPARg) pathways in the differentiation of epithelial cells. Furthermore, physical integrity of this tissue is essential to maintain its barrier functions. Since gap junctions, composed of connexin (Cx) proteins, contribute to homeostasis and barrier functions of the airway epithelium, we evaluated the possible relationship between Cx26, PPARg and Notch signaling pathways during repair of human airway epithelial cells (HAECs). HAECs from non-cystic fibrosis (NCF) and from CF patients were grown at the air-liquid interface to generate well polarized epithelia. The expression and/or localization of transcription factors and Cxs were monitored by qPCR and immunofluorescence (IF) in repairing HAECs, 24hrs after mechanical cell ablation (termed 'wounding') and at later times when the ablated area was covered (wound closure). Hes1 gene expression was used to monitor the activation of Notch signaling pathway during repair. PPARg activation was monitored by measuring the expression of HPGD; the enzyme providing its endogenous ligand 15-ketoprostaglandin E2 (15kPGE2) and the expression of paraoxonase2 (PON2). Wounding triggered proliferation and an increase in Cx26 expression, which returned to basal levels after wound closure, in both NCF and CF cells. Hes1 expression was shown to be unaltered during repair in NCF cells; however, in CF cells, its expression, and therefore Notch activation, was decreased 24h after wounding ($p < 0.05$). HPGD expression was also unaltered in NCF cells during repair; however, in CF cells, the basal levels were lower compared to NCF cells ($p < 0.05$). The size of Cx26 plaques revealed by IF was markedly decreased upon PPARg activation with 15-kPGE2 (7.25 ± 0.4 AU vs vehicle: 12.7 ± 1.1 AU; $p < 0.05$); however, PPARg inhibition with its pharmacological inhibitor GW9662 maintained Cx26 plaques (11 ± 0.5 AU). While Notch's pharmacological inhibition with DAPT had no effect on Cx26 expression or on its plaques formation, modulation of Cx plaques size by PPARg activator was more pronounced in the presence of DAPT (vehicle: 12.7 ± 1.1 AU; DAPT+15kPGE2: 0.03±0.02 AU, $p < 0.05$;). These results reveal that Notch and PPARg signaling pathways are deregulated in human airway CF cells. Moreover, they show that the expression of Cx26, which is a marker of repairing HAECs, is negatively regulated by PPARg activation. We suggest that Notch activation is crucial during the early phases of HAEC repair and that PPARg signaling pathway has a dominant effect on Notch to induce proper differentiation at later stages of the repair process. Altogether, our results show that Notch, PPARg and Cx26 are interrelated to finely tune the HAEC repair process. Since these pathways are deregulated in CF, future studies will evaluate if these defects alter the reestablishment of an integrated airway epithelium.

Saturday 29 March – 08:45-10:30

SYMPOSIUM 7
CFTR potentiators, correctors and treatments of cystic fibrosis
Chairs: Frederic Becq (FR) / Jennifer Hollywood (IE)

S7.1 - Strategies to correct the chloride transport defect in cystic fibrosis

Emanuela Pesce [1], Marta Bellotti [2], Emanuela Caci[1], Francesco Napolitano [3], Francesco Sirci [3], Elvira Sondo [1], Paolo Scudieri [1], Giulia Gorrieri [1], Loretta Ferrera [1], Valeria Tomati [1], Olga Zegarra-Moran [1], Enrico Millo [2], Nicoletta Pedemonte [1], Diego Di Bernardo [3], Luis J.V. Galietta [1]

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The chloride transport defect in cystic fibrosis may be corrected by rescuing the activity of the mutant CFTR protein or by stimulating the activity/expression of alternative chloride channels. To target the processing defect of F508del-CFTR, the most frequent mutant in cystic fibrosis patients, we are following two strategies: i) identification of small molecules that correct the processing defect; ii) investigation of CFTR interactome to identify new proteins involved in protein trafficking and degradation. For the pharmacological correction of F508del, we are investigating the properties of aminoarylthiazoles (AATs). These compounds have a dual effect: they improve the targeting of mutant CFTR to plasma membrane and also potentiate channel activity (although less effectively compared to “classical” potentiators). Interestingly, combination of most effective AATs with VX-809 generate synergic effects. However, the potency of AATs is still in the micromolar range. Therefore, novel AATs are being synthesized and tested to find correctors with improved potency and reduced cell toxicity. The synthesis of AATs is also guided by the hypothesis that these molecules bind to CFTR NBD1.

To explore the CFTR interactome, we previously screened a large siRNA library in order to identify proteins whose silencing leads to improved F508del-CFTR expression and function. This approach generated a list of possible targets that are now studied to find drugs able to mimic siRNA effects.

An alternative approach to stimulate chloride transport in cystic fibrosis epithelial cells is the pharmacological modulation of an alternative channel. For this purpose, we are studying the physiological role, interactome, and pharmacological sensitivity of TMEM16A, a chloride channel activated by cytosolic calcium. In particular, the screening of two libraries containing kinase inhibitors and other biologically-active molecules identified two small molecules able to potentiate the calcium-dependent activity of TMEM16A.

Supported by Italian Cystic Fibrosis Foundation (FFC #2/2012) and Telethon Foundation (GGP10026)

S7.2 – Development and characterization of new correctors of F508del CFTR

John W. Hanrahan[1], Graeme Carlile[2], Renaud Robert[1], Elizabeth Matthes[3], Julie Goepp[3], Veronique Birault[4], Roberto Solari[4], David Y. Thomas[2]

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CFTR correctors identified in cell-based screens are surprisingly diverse, suggesting they may have different targets and/or modes of action. This is encouraging because it implies there are multiple distinct ways to correct F508del-CFTR trafficking. The majority of hits in our screens have no clear similarity to compounds of known function, however searching adjacent chemical space has revealed structurally related molecules with known cellular targets including phosphodiesterases, histone deacetylases, kinases, E3 ligases, polyADP-ribose polymerases (PARP also termed ARTD), cyclooxygenase 2 and Na/K ATPase. Many of these compounds inhibit enzymes involved in the post-translational modification of proteins. While serine and tyrosine phosphorylation of CFTR has been implicated in biogenesis and trafficking and protein ubiquitination plays a central role in quality control, other post translational modifications such as ADP ribosylation remain to be explored.

One objective when characterizing a new corrector is to determine if it acts as a chemical chaperone or proteostasis modulator. To identify the target of the sponge-derived compound latonduine we performed SAR and identified a position where a biotin moiety could be attached without loss of corrector activity. We then used this derivative as bait to bind target molecules in cell extracts, which were analyzed by mass spectrometry. One of the proteins we identified is a member of the poly(ADP-ribose) polymerase family, PARP3. This identification was confirmed by latonduine inhibition of the isolated PARP3 enzyme *in vitro*, and also by correlating siRNA knock downs of PARP3 mRNA in cells with alteration of the EC50 for correction by latonduine. In principle proteostasis modulators may also be able to correct the trafficking of other mutant membrane proteins. Using several assays we found this to be the case. Some correctors were effective against other mutant proteins including the sulfonylurea receptor, a related ABC protein. Since correctors are less likely to act as pharmacological chaperones for structurally unrelated proteins, assaying the correction of other ER-retained mutant proteins can help classify correctors as proteostasis modulators.

Using hits from the McGill HTS campaign as the starting point, computational methods were used to identify analogues in the GSK compound collection. One of these underwent extensive (>400 derivatives) medicinal chemistry development in collaboration with GSK. This McG339 series is interesting because some members of the family have significant levels of correction alone and are also additive with the Vertex compound VX-809, giving significantly greater correction than what is currently thought to be the threshold needed for clinical benefit. We have also identified other combinations of compounds which display significant corrector synergy. These are being pursued using a bicistronic adenovirus that delivers dual fluorophores into primary HBE cells for ratiometric halide influx assays.

S7.3 – 407 compound, a new corrector for ΔF508CFTR: state of art

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Cystic fibrosis is due to the loss of epithelial chloride transport caused by mutations in the CFTR gene, the most frequent mutation being F508del. One of the strategies developed to find new treatment for Cystic fibrosis (CF) is to discover compounds that correct the trafficking of F508delCFTR to the plasma membrane. Using hypothesis-driven approach and combining modeling of NBD1, molecular docking and functional assays, we identified 4 compounds that correct F508delCFTR function in cells (including human primary bronchial cells in culture) and in F508del mice. Those new correctors probably act by interrupting the interaction between F508delCFTR with keratin 8 (Odolczyk et al EMBO Mol Med 2013).

An overview of current state of art of novel correctors will be presented by focusing on one of the most potent molecules, Mol 407. This will include studies on mechanisms of action of the compound, and evaluation of the effect in F508del mice.

Our current data suggest that 407 corrector 407 does not seem to stabilize F508delNBD1. In cell models data show that it prevents F508delCFTR from degradation by the proteasome, allowing delivery of the mutated CFTR to the plasma membrane probably through the non-conventional secretory pathway. The corrector increases significantly chloride current in cell cultures, nasal and intestinal epithelium in mice after topical and systemic administration.

In conclusion, in silico based approach lead to the identification of potent F508delCFTR corrector.

S7.4 – Novel correctors of F508del-CFTR are additive to VX-809 in primary cultures of human lung cells homozygous for F508del or A561E

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Background:

The most advanced investigational drug to treat CF patients with F508del-CFTR is VX-809 (Vertex), a molecule that works by rescuing F508del-CFTR from its abnormal intracellular localization to the cell surface probably by binding to the NBD1:ICL4 interface, a critical contact site in CFTR structure[1]. A second pocket in F508del-CFTR vs wt-CFTR was nevertheless postulated[1]. Although VX-809 exhibited ~25% rescuing efficacy in CFTR activity *in vitro*, there was no significant effect on lung function in Phase II clinical studies. Therefore a compound correcting F508del-CFTR with an additive profile to VX-809 would be of significant impact on the majority of CF patients.

Aim:

To assess the effects of novel F508del-CFTR correctors (B9, E12, Sygnature Discovery) and their additivity to VX-809. Ultimately, these compounds may be explored in triple drug combination therapy (two correctors and one potentiator).

Methods:

The effects of 2 novel correctors (B9, E12) and their additivity to VX-809 were investigated in F508del-CFTR-transduced CFBE cells and primary cultures of human bronchial epithelial cells F508del- or A561E-homozygous patients by measurements of genistein-inducible equivalent short-circuit current $_{(\Delta Ieq\text{-}SC\text{-}Gen)}$ in Ussing chamber. Monolayers transepithelial resistance was (±SD) 1550±40 0/cm^2.

Results:

Data for $_{\Delta Ieq\text{-}SC\text{-}Gen}$ in F508del-cells were (mean ±SD μA/cm^2): 0.836±0.173 (VX-809,n=3); 0.275±0.013 (B9,n=3); 0.461±0.095 (E12,n=3); 0.103±0.084 (DMSO,n=3). Additivity of novel compounds with VX-809 were (mean $\Delta I_{eq\text{-}SC\text{-}Gen}$ ±SD μA/cm^2): 1.47±0.233 (B9,n=3); 2.77±0.846 (E12,n=3), the latter is statistically significant vs VX-809 alone.

For A561E-cells, $_{\Delta Ieq\text{-}SC\text{-}Gen}$ were (mean ±SD μA/cm^2): $_{\Delta Ieq\text{-}SC\text{-}Gen}$ were (mean ±SD μA/cm^2): 1.28±0.205 (VX-809,n=4); 1.25±0.105(B9+VX-809,n=2); 1.137±0.13 (E12+VX-809, n=3); 0.174±0.025 (DMSO, n=3). Neither B9 nor E12 were significantly additive to VX-809.

Conclusions:

These data show that: 1) A561E is rescued by VX-809; 2) B9 and E12 rescue F508del-CFTR activity to a lesser extent than VX-809; 3) E12 significantly adds to VX-809 in rescuing F508del-CFTR. E12 seems to rescue F508del-CFTR by a different mechanism of action than VX-809. It thus has potential to be further developed into a corrector drug.

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1.Farinha CM et al (2013) Chem Biol 25: 943-68.

S7.5 – The dual-acting small-molecule CFFT-004 rescues F508del-CFTR expression and function in recombinant cells and primary cultures

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To overcome the processing and channel gating defects of F508del-CFTR requires small-molecules that traffick the mutant protein to the cell surface (CFTR correctors) and enhance channel gating (CFTR potentiators). Interestingly, some dual-acting small-molecules have been identified with both CFTR corrector and CFTR potentiator activities. Here, we investigate the mechanism of action of the dual-acting small-molecule CFFT-004. We tested its effects on the single-channel behaviour of F508del-CFTR using excised inside-out membrane patches from recombinant BHK cells. As controls, we studied low temperature-rescued F508del-CFTR and C18, an analogue of VX-809, the first CFTR corrector to be clinically evaluated. Low temperature-rescued F508del-CFTR has a severe gating defect with greatly reduced open probability (P_o) (wild-type: 0.42 ± 0.04; F508del-CFTR: 0.05 ± 0.01; n = 8 and 21; $P < 0.05$) because the duration of long closures separating channel openings is greatly increased. To investigate corrector activity, cells were incubated with CFFT-004 (5 μM) at 37 ºC for 24 h, then thoroughly washed before membrane patches were excised and CFTR channels studied at 37 ºC. The P_o of CFFT-004-rescued F508del-CFTR channels was 4-fold higher than low temperature-rescued F508del-CFTR (CFFT-004: 0.20 ± 0.02; n = 12; $P < 0.05$) because channel openings were more frequent and prolonged. To test for dual-activity, CFFT-004 (5 μM) was acutely added to the intracellular solution following the activation of CFFT-004-rescued F508del-CFTR channels by PKA and ATP. Acute addition of CFFT-004 (5 μM) further enhanced the P_o of CFFT-004-rescued F508del-CFTR by augmenting the frequency of channel openings (correction: 0.20 ± 0.02; correction+potentiation: 0.27 ± 0.04; n = 9; $P < 0.05$). In contrast to CFFT-004, C18 exhibited corrector, but not corrector-potentiator activity. To begin to explore the specificity of CFFT-004, we studied A561E-CFTR, the second most common CF mutation in Portugal, possessing a similar mechanism of CFTR dysfunction as F508del-CFTR. The activity of CFFT-004 (5 μM)-rescued A561E-CFTR channels was equivalent to that of low temperature-rescued A561E- and F508del-CFTR channels, suggesting that drug action might be mutation-specific. Consistent with this idea, analysis of CFTR expression in recombinant BHK cells by Western blotting revealed that treatment with CFFT-004 (5 μM) at 37 ºC for 24 h failed to promote the maturation of A561E-CFTR. However, CFFT-004 (5 μM) achieved minimal maturation of F508del-CFTR (1% band C relative to wild-type CFTR; n = 7), suggesting differential effects of the small-molecule on F508del-CFTR trafficking and channel gating. Finally, we investigated the effects of CFFT-004 on primary cultures of human bronchial epithelial (HBE) cells from patients homozygous for F508del or A561E by Western blotting and equivalent short-circuit current measurement. Corrector treatment with CFFT-004 (5 μM) at 37 ºC for 24 h augmented F508del-CFTR, but not A561E-CFTR expression and function in HBE cells. Moreover, acute addition of CFFT-004 (5 μM), to test for dual-activity, further enhanced F508del-CFTR, but not A561E-CFTR current. We conclude that CFFT-004 is a dual-acting small-molecule that rescues F508del-CFTR expression and function in recombinant cells and primary cultures. Supported by the CF Trust and PTDC/SAU-GMG/122299/2010 and BioFig (PEst-OE/BIA /UI4046/2011) grants from FCT/POCTI.

7.6 - PTC124 is unable to significantly induce read-through of premature stop codons (PTC) in primary intestinal organoids of CF patients

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Approximately 10% of patients with a genetic disease have premature termination codons (PTC). It is known from literature that aminoglycosides can facilitate read-through of PTC leading to functional protein expression. PTC124 has been developed recently to facilitate read-through in a well-tolerated and oral bioavailable way. Only limited to no efficacy in a phase III clinical trial was observed in CF patients with a PTC in the CFTR gene. We used a highly sensitive functional CFTR assay in primary intestinal organoids from CF patients with PTC to assess CFTR-restoring capacity of PTC124 and aminoglycoside G418 in vitro. Efficacy of PTC124 was compared with aminoglycoside G418 (a published PTC read-through inducing agent) and analyzed after o/n incubation, followed by forskolin-induced swelling of organoids that is fully CFTR-dependent. Organoid swelling was measured and quantified by confocal microscopy and imaging software.

Single treatment with PTC124 was not able to induce swelling in PTC-containing organoids, whereas G418 was able to restore CFTR function to some extent but not in organoids lacking PTC, demonstrating read-through. Moreover, VX-770 and VX-809 further enhanced G418-induced CFTR function in organoids expressing PTC and a CFTR frame shift mutation, suggesting functional interactions between these drugs. In organoids compound heterozygous for F508del and several different PTC, PTC124 activity was only detected in the presence of a W1282X mutation and co-treatment with VX-770 and VX-809. In other organoids with different PTC, no additive effects of PTC124 were measured on top of VX-770 and VX-809 treatment. The most optimal PTC read-through conditions with G418 and CFTR-targeting drugs VX-770 and VX-809 resulted in comparable swelling levels of VX-809 treated F508del homozygous organoids. Since the latter treatment showed no clinical efficacy in a phase II clinical trial, our data suggest that more potent PTC read-through drugs are required to induce clinically significant benefits in CF patients. Furthermore, read-trough agents can be combined with CFTR-targeting drugs to enhance their effect, and may show subject-specific or mutation-specific efficacy.

Saturday 29 March – 11:00-12:30 **Room: Fortress**

DEBATE

Correction at more than one domain is needed for F508delCFTR

Chair: Ineke Braakman (NL)

Participants: Philip Thomas (US) – Aleksander Edelman (FR)
Lihua He (US)

Saturday 29 March – 11:00-12:30 **Gardjola Room**

Training workshop

What is the best method of repairing CFTR – gene or protein?

Chair: Mitch Drumm (US)

Saturday 29 March – 14:00-15:45

SYMPOSIUM 8
Origin of chronic inflammation in CF
Chairs: Alice Prince (US) / Mario Romano (IT)

S8.1 - Inflammatory and fibrotic lung disease in CF: rational basis for cell-targeted therapy

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It has long been assumed that CFTR expression is limited to epithelial cells. However, other cells such as macrophages and fibroblasts also express CFTR, have altered inflammatory responses in CF and may act as target cells for CF therapeutic drug development.

Macrophages are ubiquitously accumulated and their phenotype is altered in CF. This feature has raised up the idea that macrophages may contribute to the cascade of infection/inflammation events characteristic in CF. The expression of different markers of both classically (M1) and alternatively (M2) activated macrophage polarization is increased in alveolar, lung resident and peritoneal macrophages isolated from F508del-CF mice compared to controls. The macrophage-related chemokine, CCL-2, is at least 10-fold more abundant in the alveolar space of mutant mice compared to controls. Azithromycin, a macrolide antibiotic with anti-inflammatory properties, downregulates proinflammatory cytokine expression (IL-1β, CCL-2, tumor necrosis factor-(TNF)-α) in M1-induced CF and wild-type alveolar macrophages. The PDE5 inhibitor vardenafil reduces the expression of proinflammatory markers, in particular of TNF-α, in lung resident macrophages expressing F508del-CFTR. We postulate that macrophages are the target cells of the anti-inflammatory effect of cGMP increasing agents.

Besides the exaggerated inflammatory responses, uncontrolled tissue rearrangements and excessive pulmonary fibrosis are common findings in end-stage CF lung disease. Responses of proinflammatory and profibrotic markers in lung and skin are enhanced in a murin model of bleomycin-induced fibrosis. Purified lung and skin fibroblasts from CF mice proliferate and differentiate into myofibroblasts more prominently and display higher sensitivity to TGF-β and PDGF growth factors. Under inflammatory stimulation (induced by LPS from *P aeruginosa* applied alone or with interferon-γ, or by IL-1β), mRNA and protein expression of proinflammatory mediators are higher in CF than in control fibroblasts, in which CFTR expression reached levels similar to those found in macrophages. Increased proinflammatory responses in CF fibroblasts are reduced by half with submicromolar concentrations of vardenafil.

These data indicate that inflammation and fibrosis in CF comprise systemic and complex processes involving multiple cell factors that can be targeted for CF drug therapy. As inflammation plays a major role in morbi-mortality in CF, understanding the contribution of each cell actor, either separately or in concert, in exaggerated inflammatory responses and in increased remodeling and fibrogenic processes in CF provides a rational basis for cell-targeted drug therapy.

S8.2 - **Worms and Amoebae as model systems to determine the developmental pre-sets driving chronic inflammation in post-natal CF**

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Hypothesis: from a fetal development perspective, a CF baby is pre-set to be pro-inflammatory.

Defective membrane-delimited GTP production occurs when CFTR is mutated/missing, involving dysfunctional, mal-interactive NDPK and AMPK, kinases mediating energy homeostasis (Muimo, Sheffield; Hallows,Pittsburgh; Kunzelmann,Regensberg & Brenman,Chapel Hill). We exploited the tractable social amoeba Dictyostelium (Dicty) and C. elegans (Ce) to model knockout/down of the homologue of the CF-defective GTP generator, nucleoside diphosphate kinase (NDPK/nm23; energy-deficient in CF). We chose NDPK because of its controlling role in tracheogenesis (FGF-NDPK signals; T.Hsu, Boston), its G-protein activatory role mediating ligand-independent cAMP synthesis (T.Wieland, Heidelberg), its binding to CFTR (Muimo/Mehta) and because fetal CFTR expression changes during fetal organogenesis (many data).

Results: Dicty-NDPK knockdown (1) restored both the disrupted vectorial homing/disturbed differentiation induced by constitutively over-activating AMPK (the latter creates a false low energy signal); (2) reduced Dicty micronutrient retention and (3) disturbed cell growth when the amoeba phagocytosed bacteria (Annesley, Fisher, Mehta;PloSOne). Genetic epistatic analysis is a tool whereby the site of action of proteins can be precisely localised. After Ce-NDPK deletion (Masoudi et al 2013, Development), the resulting genital tract malformation/sterility was epistatically localised downstream of the EGF-receptor, above MEK but below Raf, i.e. at KSR, the protein Kinase Scaffold controlling RAS/RAF/MEK assembly/signalling; KSR is an ERK-binding scaffold that times/restricts ERK activation.

Any developing organ has to balance proliferation against cell death. Ce-NDPK deletion induced defective uptake of apoptotic cell corpses, a 'macrophage-like' engulfment defect which is pro-inflammatory in post-natal life. In addition, we found developmental cell homing to be disrupted in Ce embryos (see Dicty equivalent above). We epistatically located the site of these defects to the small GTP-binding protein Rac, downstream of a well-established [and human-conserved pathway] at the head of which lies an ABC protein that belongs to the CFTR superfamily (ABC-A subfamily). Interestingly, Rac is a regulator of the inflammation-mediating DuOx system (see Mehta/Cohen, Dev. Dynamics, for links from DuOx to CFTR fragments devoid of F508).
Conclusions: Developmental data suggest that one of the origins of CF inflammation involves a 'pre-set' defective EGF receptor signalling pathway disrupting the KSR-NDPK interface, impeding Raf-MEK fidelity. We also find (Rac-dependent) defective clearance of dead cells during fetal organogenesis,that is predicted to disrupt final organ cell composition/number. Our data are consistent with: (1) KSR acting as a control point in the innate immune response to pseudomonas infection, as proposed by E. Gulbins) and a recent commentary on French data on EGFR's disrupted links to mutated CFTR, see Nadal in ERJ,2013).
Perspective: Disrupted energy supply to membrane signalosome complexes when CFTR is no longer correctly positioned in its microenvironment might explain why so many 'disconnected' pathways are disrupted by CFTR mutation; we propose a unifying theme to explain some of the 'R' in CFTR, noting that phospho-NDPK is a chloride-controlled protein in the membranes of epithelial cells (Muimo, Treharne Marshall Mehta, AJP,1994).

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S8.3 - The Intracellular Calcium Hypothesis in Cystic Fibrosis (CF)

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Inflammation is considered like a central subject in CF pathophysiology. Nevertheless, it constitutes an extremely complex ensemble of events and cellular actors. Among then, it has been shown that the intracellular Ca2+ increase is an intermediary step in the signal transduction events linking cell stimulation by inflammatory factors to NF-κB (Nuclear Factor KappaB) activation (Ribeiro et al., 2005; Tabary et al., 2006).
Here, we used human bronchial epithelial cells, devoid of pathogen infection, by targeting Ca2+ homeostasis and its relationship with CFTR function and expression. Especially, our group focuses its research on the identification of the Ca2+ channels implicated in the abnormal Ca2+ homeostasis observed in CF cells.
Previously, we demonstrated that in non-CF cells, there is no evidence in favor of the involvement of apical CFTR activity in the regulation of Ca2+ homeostasis. On the contrary, in CF cells, the different Ca2+ responses observed (versus non-CF cells) are dependent on the presence of CFTR to the plasma membrane (Antigny et al., 2088). One possible hypothesis is that TRPC channels constitute a missing link between the abnormal Ca2+ levels observed in CF cells and CFTR dysfunction. We have identified the TRPC6 channel as a major actor for supporting the abnormal Ca2+ entry in CF cells (Antigny et al., 2011), demonstrating a reciprocal functional coupling between TRPC6 dependent Ca2+ influx and CFTR that is related to both channel activities (Vachel et al., 2013). Then, we focused our investigations on other Ca2+ channels which could take part in the abnormal calcium influx in the CF cells. We identified additional new members of the TRP channels family which are deregulated in human bronchial epithelial CF cells. Indeed, our results now showed an abnormal activity of TRPV5 and V6 channels in CF cells. These two channels have a constitutively activated Ca2+ permeability at physiological membrane potentials and are highly selective for Ca2+ ions. In future experiments, we will explore the causes of this deregulation of the TRPV5/TRPV6 channels activity in CF airway epithelial cells.

(Our work has been supported by Vaincre la Mucoviscidose)

S8.4 - Macrophage Migration Inhibitory factor (MIF) accelerated biofilm formation, a potential novel therapeutic target

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Introduction: A major unmet clinical need in cystic fibrosis (CF) is defining novel mechanisms for accelerated bacterial biofilm formation which can then form the basis for focused innovative antibacterial therapies. In the context of *Pseudomonas aeruginosa* (*PA*), chronic infection leads to progressive lung destruction. Macrophage migration inhibitory factor (MIF) is a pro-inflammatory mediator with unique tautomerase enzymatic activity which has previously been shown to play a key role in driving an aggressive inflammatory phenotype in clinical disease.1,2,3

Aim: The aim of this project is to investigate the role MIF enzymatic activity plays in PA biofilm formation and the potential therapeutic efficacy of novel small molecular weight inhibitors targeting this enzymatic activity.

Method: Using the PA01 strain of *Pseudomonas aeruginosa*, biofilm formation with and without MIF (100ng/ml) was studied *in vitro* 96 well plate culture systems and flow cell system using a GFP tagged PA01. We also investigated the effect MIF had on antibiotic resistance of PA01 biofilms. qPCR was performed on RNA extracted from 24hr PA01 cultures with and without MIF (100ng/ml) to analyse genes involved in biofilm formation and cellular growth. We have established an animal model of bronchiectasis of PA01, to examine the effects that small molecular weight inhibitors, of MIF enzymatic activity, will have on infection and inflammation in the mouse lung.

Results: Results have shown that MIF significantly enhances biofilm formation of PA01 ($p< 0.01$) both in 96 well plates and pellicle formation. This MIF enhanced biofilm formation results in increased antibiotic resistance against Tobramycin (50µg/ml) ($p< 0.01$) in comparison to controls. In addition we have found a significant early induction of specific quorum sensing gene, AlgT, previously shown to be responsible for the conversion to a mucoid phenotype[4],($p< 0.05$). In the flow cell analysis of biofilms grown in the presence of MIF (100ng/ml) after 24hrs the structures were significantly larger in mass ($p< 0.05$) and more established than controls.

Conclusion:

We describe, for the first time, how the gram negative organism, *Pseudomonas aeruginosa,* hijacks a human cytokine, MIF, for its own survival advantage. Specifically, it accelerates biofilm formation contributing to enhanced antibiotic resistance. This work supports our contention that MIF inhibition represents a valid therapeutic target potentially as an adjunct therapy to maximize the effectiveness of antibiotics.

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S8.5 - Proteomic analyses of BALF from young CF children reveal altered lipid, cyclic nucleotide, and Iron signaling and metabolism during inflammation versus non-CF disease controls

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CF lung disease begins in infancy, and is characterized by mucus plugging, infection with inflammation, air trapping, and structural lung changes (eg: bronchiectasis) that are readily detectable by three years of age. Early markers of lung disease that are specific for CF, however, are lacking. This information is critical to our understanding of what initiates CF lung disease, and to identify proximal targets for intervention. This lack of specificity may in part represent examination that has been limited to candidate-based analysis of biofluids, coupled with comparisons with inadequate disease controls. We hypothesized that using an unbiased and blinded approach, proteomic analysis of BALF from young CF patients without chronic Pseudomonas infection compared with non-CF disease controls (ages birth-5 years) would identify disease pathways and biomarkers associated with early CF lung disease. Banked specimens from the CCHMC BALF Biorepository were selected based diagnosis (CF vs non-CF controls with chronic tracheostomy), age (< 4 yrs), microbiology (all CF patients were *Pseudomonas aeruginosa* culture negative) and matched for gender. All samples underwent common processing and were stored at -80C until analysis. Samples from each patient were assigned identification numbers, blinded, and randomized such that the researchers performing the proteomic analyses were prevented from having any information about the experimental subjects. Once proteomic analyses were concluded, the blind was partially broken so as to reveal which samples belonged in the same group, allowing for the comparison of data between cohorts, without revealing the identities of the groups. Protein was precipitated from BALF supernatants, resolubilized, and subjected to 1D and 2D gel SDS PAGE. Segments of the gels were excised and subjected to in-gel protein digestion, peptide fragments extracted and analyzed by LC-MS/MS running in data dependent mode. Quantitation was performed using gel densitometry (quantitative) and MS spectral counting (semi quantitative). In addition to identifying proteins that were significantly different in CF vs non-CF samples, signaling pathway analyses were conducted with the GeneGo software package. While a number of identified differential expressions validate findings by other groups, we identified a number of novel differences. These data support the notion that altered signaling specific to the CF condition occurs early in lung disease. Pathway analysis revealed that the most significant differences in CF vs non CF belong to pathways that reflect increased cellular metabolism, increased lipid and alcohol catabolism, and the increased catalysis of cyclic nucleotides such as cAMP. Transcriptional regulation analysis revealed significant differences in pathways regulated by CREB1, SP1, and androgen receptor in CF vs non CF. Our results indicate that CF BALF contains protein signatures that demonstrate increased metabolism, increased catabolism and an alteration in pathways that regulate lipid, alcohol, and iron metabolism during infection. These significant differences in CF vs non CF infected controls suggests that the CF BALF signature can be segregated from non CF disease controls, exhibiting different biomarkers that delineate pathways that may be informative about early disease pathogenesis and also serve as potential therapeutic targets.
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S8.6 - The mode of action of ADAM17 in resolution of inflammation in CF lung disease

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Background

We found in F508del CFTR mutant mice ($Cftr^{tm1eur}$) that lung injury causes induction of IL-6 and Amphiregulin (AREG) compared to wt. Recent studies link AREG with TGFß1-stimulated collagen accumulation in challenged murine lungs, leading to fibrosis. Further, both AREG/EGFR and IL6/IL6R/Gp130 pathways are involved in the resolution of lung inflammation and injury through the STAT3 transcription factor, which has recently been identified as a modulator of CF lung disease in humans.
Both AREG and IL6R are activated and released from the cell surface by the membrane bound protease ADAM17, which is regulated by extracellular and intracellular signaling.

Aim

We aimed to establish whether ADAM17 plays a pivotal role in CF lung disease and define the druggable targets in the molecular pathways up and downstream of this regulator.

Methods and Results

We addressed these issues in Primary Human Bronchial Epithelial cells in Air-Liquid Interface culture (HBEC-ALI) and the CF mutant immortalized cells CFBE41o-. In HBEC-ALI IL6R and AREG are shedded for 90% to the basolateral compartment. In both models AREG shedding is inhibited 80% ($P< 0.001$) by a specific inhibitor of ADAM17 (TMI-2, Wyeth). In HBEC-ALI IL6R is inhibited by 40% ($P< 0.01$), because a splice variant exists that is not a substrate of ADAM17.

We showed here that ADAM17 is a highly regulated enzyme in bronchial epithelial cells. Cigarette smoke treatment of normal HBEC-ALI, which is known to reduce CFTR activity, increases IL6R production by 50% and AREG shedding twofold. The ROS scavenger, N-acetylcysteine, reduced ADAM17 activity by 90% in CFBE cells ($P< 0.05$) and by 25% in HBEC-ALI ($P< 0.04$ tree different donors analysed in triplicates) suggesting a relationship between these two phenomena.
ADAM17 activity is tightly regulated by tyrosine phosphorylation. The tyrosine kinase inhibitor (genistein) reduced AREG shedding by ~80% ($P< 0.05$) in CFBE41o- cells, whereas an inhibitor of protein tyrosine phosphatases (orthovanadate) significantly increased AREG production. Furthermore, ADAM17 shedding activity depends on EGFR activity. AG 1478, a tyrosine kinase inhibitor that is specific for EGFR (ErbB1), reduced AREG production (~80%) to the same extent as an inhibitor of the kinase downstream of EGFR, MEK1/2 (U0126) and the ADAM17 inhibitor TMI-2 mentioned above.

Conclusions

In bronchial epithelial cells AREG and IL-6R released by ADAM17 act mainly in the basolateral compartment, what may affect epithelial (autocrine) and subepithelial receptors (transsignalling), involved in epithelial repair, inflammation and fibrosis. Our data further suggest that CFTR deficiency and stimulated ROS production affect ADAM17 activity. We show that phosphorylation is the crucial mechanism that regulates ADAM17 dependent shedding in this system. Since ROS is known to inactivate phosphatases, we suggest that ROS may affect ADAM17 activity by increasing its phosphorylation.
We propose that the druggable ADAM17/AREG/IL-6R/STAT3 pathway is a valid target for further study of experimental therapeutics in CF. We currently investigate changes in ADAM17 activity after induction of CFTR expression in the CF mutant CFBE41o- cells.

Astma fonds AF3.3.10.027

Saturday 29 March – 16:00-17:45

SYMPOSIUM 9
CFTR trafficking
Chairs: William Guggino (US) / Paul Eckford (CA)

S9.1 - Identification of novel ERAD players controlling CFTR degradation

El Khouri E., Tallet E., Le Pavec G., Toledano M.B., Delaunay-Moisan A.

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CFTRDeltaF508, the most frequent mutation responsible for Cystic fibrosis, hampers CFTR folding and results in its efficient recognition by the Endoplasmic Reticulum (ER) Quality Control (QC) machinery. Consequently, the mutant protein is retained in the ER where it is rapidly degraded by the Endoplasmic Reticulum Associated Degradation (ERAD) machinery. CFTR ERAD is considered as the initial step responsible for CFTR loss of function and it potentially competes with the production of correctable mutant proteins. Thereby excessive CFTR recognition by the ER quality control is considered as a relevant target to design therapeutic strategies.
Two strategies are currently undertaken to limit CFTRDeltaF508 recognition by ERQC. The first one involves the identification of molecules compensating the intrinsic conformational defects induced by the DeltaF508 mutation. The second relies on the identification of quality control activities whose modulation will tilt the balance toward less degradation and more folding.
In an effort to characterize the ERAD components targeting CFTRDeltaF508 to degradation and precise the mechanisms involved in this process, we have identified RNF185, as a new ER-bound E3 ligase targeting CFTR for ubiquitin/proteasome-dependent degradation in the ER (El Khouri et al., 2013). Two E3 ligases were previously involved in chaperone-mediated CFTR ERAD, RNF5 and CHIP. We found that RNF185 acts redundantly with RNF5. Strikingly, the simultaneous depletion of both RNF185 and RNF5 results in a profound stabilization of CFTRDeltaF508, much greater than that observed upon the depletion of each ligase. In these conditions, CFTRDeltaF508 stability is increased both during and after its synthesis has been completed. Thus, we identified the RNF5/RNF185 E3 ligase module (RNF module) as a major player of CFTR ERAD. We pursued with the identification of new partners of the RNF module. Among these, we focused on JAMP, a novel transmembrane protein interacting with the proteasome and different components of the ERAD machinery. We showed that JAMP is also required for CFTRDeltaF508 degradation through a still ill-defined mechanism. We now provide evidence that JAMP contains a redox-sensitive domain that modulates its ability to degrade CFTR. We hypothesize that the RNF-JAMP proteins constitute an original ERAD module regulating the targeting of CFTR to degradation in a potentially redox-dependant manner.

S9.2 - CHIP Regulates ΔF508 CFTR Surface Stability at a Post-Endocytic Step

Lianwu Fu, Andras Rab, Li Ping Tang, Steven M. Rowe, Zsuzsa Bebok, and James F. Collawn

Programme Note: Abstract details are not authorised for publication

S9.3 A High-Content siRNA Screens Identify Novel CFTR Traffic Regulators in Human Airway Epithelial Cells

Hugo M. Botelho[1,2], Shehrazade Dahimène[1,2], Inna Uliyakina[1,2], Beate Neumann[2], Christian Tischer[2], Rainer Pepperkok[2], Margarida D. Amaral[1]

[1]University of Lisboa, Faculty of Sciences (FCUL), BioFIG – Center for Biodiversity, Functional & Integrative Genomics, Lisboa, Portugal, [2]Cell Biology Biophysics Unit and Advanced Light Microscopy Facility (ALMF), European Molecular Biology Laboratory (EMBL), Heidelberg, Germany

Cystic Fibrosis (CF) is the most common genetic disease in Caucasians, caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a glycoprotein chloride channel expressed at the apical plasma membrane (APM) of epithelial cells. The F508del-CFTR mutation is responsible for about 85% of CF cases. This mutation yields a misfolded protein which is retained in the endoplasmic reticulum (ER) and prematurely targeted for degradation by the ER quality control (ERQC). This traffic defect originates an impaired transepithelial chloride transport which severely impacts on several organs, most notably the airways. Yet, even though the molecular basis for CF is known, no therapy exists for correcting the folding and traffic defects of F508del-CFTR. Therefore, we have set up to identify novel therapeutic targets for CF.

We hypothesize that cell proteostasis can be manipulated in order to alleviate the stringent ERQC on F508del-CFTR and allow it to reach the APM, where it is known to have residual function [1] which may rescue the disease phenotype [2]. Therefore, genes promoting wt-CFTR and/or rescuing F508del-CFTR to the cell surface upon knock-down are potential therapeutic targets for CF.

To identify novel CFTR traffic factors we developed a mCherry-CFTR construct encompassing an extracellular Flag-tag stably expressed in A549 alveolar epithelial cells or CFBE airway epithelial cells under the control of an inducible (Tet-ON) promotor [3]. This construct was used to perform a siRNA-based high-content screen where a fluorescence ratiometric measurement of immunodetected Flag versus mCherry informed on CFTR traffic efficiency. Screening one third of a druggable genome siRNA library revealed 178 siRNAs enhancing and 126 siRNAs inhibiting wt-CFTR traffic in A549 cells. A secondary (validation) screen confirmed hits including COPI coat components, G protein-coupled receptors, chaperones and proteins involved in sterol binding, β-catenin signalling, nuclear/chromatin structuring, cytoskeletal dynamics, cell differentiation and channel regulation. Several siRNAs rescuing F508del-CFTR to the APM have been identified in preliminary experiments. Overall, these results point to a complex involvement of several cellular functions in the regulation of CFTR traffic, whose relevance in the context of basic cell biology and CF therapeutics will be discussed.

Acknowledgements
Cystic Fibrosis Foundation grant 7207534, TargetScreen2 project EU-FP6-2005-LH-7-037365 (to MDA and RP), PTDC/SAU-GMG/122299/2010 from FCT/MCTES/PIDDAC (to MDA) and PEst-OE/BIA/UI4046/2011 centre grant (to BioFIG). CFF post-doc fellowship (to HMB).

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S9.4: LMTK2 Mediated Phosphorylation Regulates CFTR Endocytosis in Human Airway Epithelial Cells

Simão Luz[1], Kristine M Cihil[2], David L Brautigan[3], Margarida D Amaral[1], Carlos M Farinha[1], Agnieszka Swiatecka-Urban[2,4]

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Background: CFTR is a cAMP-activated, Cl^--selective ion channel expressed at the apical surface of fluid-transporting epithelia and CFTR-mediated Cl^- secretion is regulated by adjusting activity and abundance of CFTR at the plasma membrane (PM), mostly through clathrin-mediated endocytosis. Nonetheless, the protein interactions that control these processes in epithelial cells have only been partially explored. Previously, we showed that kinases have a key role in this process - as both SYK and WNK4 affect CFTR levels at the membrane [1,2].
Lemur Tyrosine Kinase 2 (LMTK2) is a transmembrane serine/threonine kinase playing a role in membrane trafficking. LMTK2 also directly binds to myosin VI, a motor protein known to facilitate CFTR endocytosis [3]. Although LMTK2 was previously shown *in vitro* to phosphorylate the S^{737}-residue in the regulatory domain (RD) of CFTR [4], it remains unknown whether also *in vivo* and if it impacts CFTR endocytic traffic.
Aim: To determine whether LMTK2 regulates CFTR endocytosis in human airway epithelial cells and if phopshorylation of S^{737} is involved in the process.
Methods: Western blot (WB), co-immunoprecipitation (co-IP), cell surface biotinylation and endocytosis assays. Human airway epithelial cells: CFBE41o- and Calu-3.
Results: Data demonstrate that endogenous LMTK2 localizes at the PM, including the apical membrane domain in both cell types. Endogenous LMTK2 and CFTR co-IP and LMTK2 phosphorylates the CFTR-S^{737}. Partial depletion of endogenous LMTK2 or overexpression of an LMTK2 fragment with impaired kinase activity increases CFTR abundance at the PM by attenuating its endocytosis. Compared to wt-CFTR, the CFTR mutant with the serine to alanine substitution in the LMTK2 phosphorylation site CFTR-S^{737A} also shows increased PM abundance and decreased endocytosis. Furthermore, depletion of endogenous LMTK2 in cells expressing F508del-CFTR also increases the short-circuit current when compared to mock-transfected cells.
Conclusion: Altogether, our results demonstrate that in human airway epithelial cells LMTK2 facilitates CFTR endocytosis and reduces its cell surface density by a mechanism that involves phosphorylation of CFTR-S^{737}, also contributing to the inhibitory effect of the S^{737} phosphorylation on CFTR mediated Cl^- secretion. These findings are particularly relevant as CFTR-S^{737} is also a substrate for PKA and AMPK. Additionally, modulation of LMTK2 activity may also be of therapeutic interest, contributing to the stabilization of F508del-CFTR at the plasma membrane.

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S9.5: NBD1 Cotranslational Folding Intermediates as Targets for CF Drug Discovery

Soo Jung Kim[1], Jae Seok Yoon[1], Hideki Shshido[1], William R Skach[2]

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The primary molecular defect in most CF patients is caused by CFTR misfolding Moreover, the efficiency of NBD1 folding itself limits CFTR trafficking, and is markedly compromised by the ΔF508 mutation. An important goal in CF therapeutics, therefore, is to understand how NBD1 acquires and maintains its folded state and to devise pharmacological strategies to improve its folding efficiency. Using fluorescence resonance energy transfer (FRET) we show that NBD1 folds cotranslationally as the nascent polypeptide emerges from the ribosome. Folding occurs sequentially via compaction of three distinct subdomains: the N-terminal, alpha-helical, and alpha-beta core. The timing of these folding events is critical as premature alpha-subdomain folding prevents subsequent formation of the beta-sheet core. To coordinate these events, the ribosome modulates intrinsic folding propensity of the nascent polypeptide by: first, delaying alpha-subdomain folding; second, facilitating intercalation of beta-strands within the parallel b-sheet core, and third balancing kinetics of synthesis and folding via selective codon usage. These studies pinpoint core formation as a critical step in de novo NBD1 folding. We next used the algorithm of Spencer et al, (J Mol Biol. 2012;422:328-35) to introduce synonymous codon substitutions into full length CFTR that are predicted to increase (or decrease) the alpha-subdomain and alpha/beta core translation rate (residues 525-593). When expressed in HEK cells, the “fast” coding sequence increased aggregation of immature (band B) CFTR resulting in delayed accumulation of immature CFTR (band B) in the insoluble cell lysate fraction. In addition, “fast” NBD1 CFTR that matured to band C, showed increased reactivity to the 7D12 monoclonal antibody epitope in the alpha-helical subdomain, indicating that the translation rate can co-translationally impact folding outcome.

The ability to access structural intermediates that exist only transiently during CFTR synthesis provides a potential means to pharmacologically manipulate the cotranslational folding pathway. To exploit these findings, we developed a high throughput screening assay, wherein immobilized ribosome nascent chain complexes (RNCs) can be imaged by high content microscopy in a 384 well format. This approach allows quantification of FRET efficiency on agarose beads containing as little as 1.8 attomole of NBD1 (1 million molecules). Solid-state FRET measurements recapitulate nascent chain folding observed in solution and can detect perturbations that both improve and inhibit NBD1 folding at specific stages of synthesis. This approach provides a novel tool to investigate normal and abnormal co-translational folding pathways and establishes a new CF drug discovery platform for screening small molecules that improve nascent NBD1 folding in CF patients. (Supported by CFFT and NIH DK51818).

S9.6: New drug delivery system in treatment of Cystic Fibrosis using siRNA knock-down of the Aha-1 gene

Karen K Schelde[1], Anne Poulsen[1], Chuanxy Yang[1], Eskild Petersen[2], Jørgen Kjems[1]

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Introduction: Cystic fibrosis is a genetic disease with mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. The most common mutation is the deletion of F508, which causes a misfolding of the CFTR protein and thereby a degradation in the ubiquitin-proteasome-system in the cell. Earlier research has shown that inhibition of the hsp90 co-chaperone, Aha-1, locates the misfolded CFTR to the plasma membrane, where the function of the channel is restored (Sun et al, 2008). Our aim is to use the RNA interference (RNAi) mediated by 22 nucleotides long small interfering RNAs (siRNAs) inside the cell to degrade mRNA with complementary sequences. In this way siRNA targeted against Aha-1 mRNA will interfere with Aha-1 protein expression and restore CFTR in the plasma membrane.

Methods: To achieve this goal both *in vitro*, *ex vivo* and *in vivo* experiments are carried out. The CuFi-1 cells (Cystic fibrosis cell line with del F508 mutation) are seeded out in 12-wells plates and transfected with particles composed of siRNA formulated in different types of chitosan. RNA isolated from the cells are analysed with qPCR and a knock down is registered. To set up an *ex vivo* experiment primary lung epithelial cells from healthy pigs are harvested and seeded out on semipermeable membranes and transfected with chitosan/siRNA particles.

To optimize delivery and lung distribution in a human-like anatomic setting we established a nanoparticle delivery protocol for pigs. A pig model (60kg) was established where particles are distributed into the lungs of an anaesthetised pig (20mL/h Fentanyl and 15mL/h Propofol i.v.) by the use of an ultrasonic nebuliser attached to the ventilator. 5mL 1:100 Infrared 715/755 Fluospheres (Life Technologies, #F8799) were injected as described above to investigate the distribution method of the ultrasonic nebuliser. After killing the pig (4mL/kg Pentobarbital), lungs are taken out and kept on ice prior to scanning in an In Vivo Imaging Scanner (IVIS).

Chitosan particles are known to promote mucus adhesion and efficient knockdown has been demonstrated in a mouse model (Howard et al. 2006; Nielsen et al. 2010). To move this delivery platform to the pig model we are currently performing a comparative in vivo study where biodistribution of chitosan formulated Cy7-labelled siRNA is studied over time in the pig.

Results: A knock down of Aha-1 is seen in the CuFi-1 cell line and validation of knockdown in primary lung epithelial cells is currently ongoing. Fluorescent nanoparticles are observed to be distributed throughout the lungs by the use of the ultrasonic nebulizer and to a better extent when using a catheter to inject intra tracheal. This distribution method works very well and is easy to use in the anaesthetised pig.

Conclusion: A highly efficient Aha-1 target for siRNA knockdown has been established *in vitro* and an air-liquid interphase cell culture system has been established for primary lung epithelial cells. An efficient treatment protocol for siRNA delivery to pig lung has been established and RNAi experiments are in progress.

Saturday 29 March – 18:15-19:15

Keynote Lecture

CFTR: Structure, Thermal stability and Paths to Drug Discovery

David C. Dawson

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Molecular models of the CFTR chloride channel have the potential to accelerate drug discovery by providing a platform for in silico screening of compound libraries. We have developed molecular homology models of CFTR based on the crystal structures of the prokaryotic transporters, Sav1866 and TM287/288. The models were refined using molecular dynamics simulation and validated by means of multiple comparisons of model predictions with experimental findings, including cysteine scanning of pore-lining residues, gating state-dependent, covalent labeling, docking of an open-channel blocker within the pore and successful prediction of a mutation-induced change in binding free energy. A major limitation of Sav-based homology models is their failure to demonstrate a fully open, continuous, anion-selective pore. We have used two approaches in an attempt to find open-pore conformations in our CFTR models without forcing them into an open conformation. One approach involved using dynamic importance sampling (DIMS) to interpolate between the outward-facing, Sav-based model and the inward-facing, TM287/288-based model. The second was based on extending the MD simulation of a Sav-based homology model that had been refined by manually adjusting the alignment. The results demonstrate a continuous, open pore and suggest the possibility of lateral openings on the cytoplasmic side of the channel.

We and others have recently called attention to the thermal sensitivity of ΔF508 CFTR channel function, a property that leads to a dramatic attenuation of channel function at 37ºC. This mutant phenotype seems to be a likely target for drug discovery. More recently we studied the thermal sensitivity of gating-impaired, G551D CFTR channels which have been successfully targeted in patients using the Vertex compound, VX-770. We were surprised to find that these channels also exhibited profound thermal sensitivity of channel function, a result that raised questions about the mechanistic basis for the efficacy of VX-770 in patients. We discovered, however, that VX-770 and other CFTR potentiators protected G551D channels from thermal inactivation, whereas the same compounds were without effect on the thermal inactivation of ΔF508 CFTR channels. This finding should set the stage for a search for molecules that can protect ΔF508 CFTR channel from thermal inactivation. (Supported by NIH and CFF)

P. 01

Rescue of NBD2 mutants N1303K and S1235R by small molecule correctors and transcomplementation.

Daniele Rapino[1], Inna Sabirzhanova[2], Miqueias Lopes-Pacheco[2], William B. Guggino[2], Liudmila Cebotaru[3]

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Although mutations in NBD 1 are most commonly associated with CF, disease causing mutations also occur in NBD2 of CFTR. Here we study two disease causing mutations in NBD2: N1303K, associated with severe disease and S1235R which causes mild or borderline symptoms. The goal is to gain more information on the impact of these mutations on CFTR processing and to devise ways to rescue them. Inhibition of proteasomes with MG132 or aggresomes with tubacin is able to rescue the immature band B and mature band C of N1303K and S1235R, indicating that degradation of both mutants is via proteasomes and aggresomes. VCP, the protein involved in translocation of mutant proteins to the proteasome and HDAC6, a protein involved in trafficking of mutant proteins to the aggresome, also bind to the NBD2 mutants indicative of their degradation in the proteasome and aggresome. There is no effect of the lysosome inhibitor (E64). We tested the effect of small molecule correctors in promoting maturation of NBD2 mutants. We found that several known correctors alone or in combination increased the maturation of B to C band of N1303K and S1235R. The best correction occurred with N1303K when a combination of the CFFT correctors, C4 plus C18 or C3 plus C4 was applied. In pull-down assays, we found that a number of chaperones, including HSP27, 40, 70 and 90 bind to the N1303K and S1235R mutants. Importantly, following treatment with the combination of correctors, chaperone binding was reduced especially in the N1303K mutant. This strengthens considerably our conclusion, that C3+C4 rescue both NBD2 mutants by reducing chaperone binding thereby promoting maturation. We next tested whether NBD2 mutants could be rescued by transcomplementation. Indeed transfection of the CFTR truncation, ∆27-264, into cell lines stably transfected with either of the NBD2 mutants was able to rescue them. This is the first time that the use truncated forms of CFTR proved to be effective in correcting NBD2 mutants. Our results suggest that the N1303K mutation has a profound effect on NBD2 processing compared to S1235R. Small molecule correctors are able to increase the maturation of band B to C in NBD2 mutants. In addition the truncation mutant, ∆27-264, is able transcomplement both NDB2 mutations. Our results suggest that patients bearing either the N1303K or S1235R mutations may be good candidates for both corrector and gene therapy. Funded by NACF Foundation.

Some corrector compounds that directly modify the conformational stability of full-length F508del-CFTR stabilize and/or induce its active channel form after biosynthetic rescue

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F508del-CFTR, the most common CF-causing mutation in North America and Europe, impairs conformational maturation and stability of the CFTR protein, reducing its functional expression at the surface of cells in which it is expressed. “Corrector” compounds, such as the structurally related C18 (VRT-534) and VX-809 (lumicaftor) can rescue the biosynthetic maturation and forward trafficking of this mutant to the cell surface, to some extent. We propose that at least some corrector compounds will bind directly to the mutant CFTR protein to enhance its conformational stability and induce the channel-competent form of the protein once it has trafficked to the cell surface. We employed purified and reconstituted full-length Wt, F508del- and G551D-CFTR to test our hypothesis.

Purified full length F508del-CFTR protein in detergent solution aggregates rapidly whereas Wt-CFTR is significantly more stable in this *in vitro* system. Direct interaction of purified F508del-CFTR with small molecules including C18, VX-809 and the corrector VRT-325, but not an inactive analog of VRT-325, result in reduced aggregation which we interpret as a stabilizing effect on the conformation of the protein.

Acute treatment of purified full length Wt-, F508del and G551D-CFTR reconstituted into proteoliposomes with C18 but not vehicle enhanced the channel activity of the protein. Both the ATP-dependent and ATP-independent channel activities were enhanced by the treatment of C18, and the ATP-independent channel activity was inhibited by the CFTR-specific inhibitor, CFTRinh-172.

Together, these studies show that full length CFTR protein is a direct target of at least some corrector compounds and their binding can modulate regions involved in gating. These compounds may bind directly to a meta-stable F508del-CFTR structure post-translationally, to stabilize and/or induce a channel active form of the mutant protein. These findings enhance our understanding of the mechanism of action of this clinically relevant class of pharmacological corrector compounds.

This work was supported by Cystic Fibrosis Canada and Operating Grants to C.E.B from the CIHR.

Restoration of NBD1 thermal stability is necessary and sufficient to correct ΔF508 CFTR folding and assembly

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CFTR (ABCC7), unique among ABC exporters as an ion channel, regulates ion and fluid transport in epithelial tissues. Loss of function due to mutations in the CFTR gene causes cystic fibrosis (CF). The most common disease-causing mutation, ΔF508 results in misfolding of the protein and clearance by cellular quality control systems. The deletion of F508 from the first nucleotide binding domain (NBD1) has two major impacts on the protein: reduced thermal stability of the domain and disruption of its interface with membrane-spanning domains (MSDs). It is unknown if these two defects are independent and need to be targeted separately. To address this question we varied the extent of stabilization of NBD1 using different second site mutations and NBD1 binding small molecules with or without NBD1/MSD interface amino acid substitution. One of the latter substitutions, R1070W also is a CF associated mutation that is destabilizing but paradoxically when added to the ΔF508 mutant improves its maturation. This ΔF508/R1070W double-mutant, however remains short-lived functionally. Combinations of different NBD1 changes had additive corrective effects on ΔF508 maturation that correlated with their ability to increase NBD1 thermostability. These effects were much larger than those caused by interface modification alone and accounted for most of the correction achieved by modifying both the domain and the interface. Thus, NBD1 stabilization plays an essential and dominant role in overcoming the ΔF508 defect. The fact that small molecules discovered to date have minimal influence on NBD1 thermal stability may partly explain their relatively modest corrective abilities at physiological temperature. Analysis of the ion channel activities of the various corrected ΔF508 CFTR species revealed that combination of NBD1 stabilization and NBD1 interface modification by the R1070 substitution resulted in a locked-open channel that was constitutively active in the absence of the normally obligatory dependence on phosphorylation by protein kinase A. Thus, simultaneous targeting of both the domain and the interface, as well as being non-essential for correction of biogenesis, may disrupt normal regulation of channel function.

Discovery of novel tailored F508del-CFTR binder correctors based on 3D structure models of entire CFTR protein for treating Cystic Fibrosis

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Cystic fibrosis (CF) is an autosomal and recessive disease due to mutations in the gene encoding a chloride ion channel CFTR (cystic fibrosis transmembrane conductance regulator). Deletion of phenylalamine at position 508 leads to the most common mutation, resulting from a mistrafficking of the CFTR protein and its retention in the Endoplasmic Reticulum (ER), abnormal gating of CFTR channel and endocytosis. Numerous small chemicals called CFTR correctors (VX809, SAHA, Corr-4a, iminosugars...) have been shown individually - albeit partially - to restore F508del-CFTR defective trafficking and functionally rescue chloride transport default. Although the mechanism of action of these correctors is still largely unknown, recent evidence suggests direct interaction of VX809 on the first transmembrane domain TMD1 of CFTR. Here, based on models refinement of the open and closed forms of CFTR, we used molecular docking and virtual screening to identify new active molecules able to bind and interact with identified F508del-CFTR pockets. This strategy led to (1) design novel tailored correctors following molecular docking prediction, allow to bind specifically in F508del-CFTR pockets, (2) synthetize these putative idealized compounds and (3) test them on the functional rescue of F508del-CFTR. We used our Bioscreen platform using a simple flux test combined to patch-clamp experiments in whole cell configuration on F508del-CFTR cell lines and human airway epithelial CFBE cells to screen these candidates for their potential correction efficacy. Preliminary experiments showed that after 24h of incubation some of these molecules restored the F508del-CFTR response to a cocktail of activators (forskolin + genistein) in CF cells with submicromolar $_{EC50}$ values. In a second set of experiments, using patch-clamp technique in whole-cell configuration on Hela F508del-CFTR, we tested 24h incubation of these novel correctors in combination with VX809 to investigate putative synergistic effects of two binder correctors on F508del-CFTR rescue. We demonstrated with this combination that the amplitude of the current densities recorded in response to activation was not significantly different compared to incubation with VX809 alone. Our next step will be to test second site suppressor mutations in combination with these novel molecules to investigate putative synergistic effects on F508del-CFTR folding and stability. Results will be presented opening perspectives on the development of new tailored correctors based on 3D structure model of entire CFTR protein to target the root cause of CF.

This work is supported by “Vaincre La Mucoviscidose”, “Mucovie” and FEDER funds.

P. 05

The membrane fluidity as well as the cytoskeleton modulate the activity of CFTR in response to stretch

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A wide range of CFTR expressing epithelia are permanently in motion and exposed to mechanical forces that can cause deformation and thus stretch the cell membrane. The present study questioned whether CFTR is a mechano-sensitive ion channel and if stretch affects CFTR-mediated currents. There is evidence that elements of the cytoskeleton and membrane fluidity may modulate the anchorage of ion channels in the membrane and thus interfere with the detection and transmission of mechanical forces. Therefore, we investigated if the cytoskeleton and membrane fluidity contribute to the mechanosensitivity of CFTR channels.

Human CFTR (hCFTR) was heterologously expressed in *Xenopus laevis* oocytes and its activity was measured by the *two-electrode-voltage-clamp* technique. CFTR-mediated currents were elicited by application of 5 µM forskolin and 100 µM IBMX (F/I) and the F/I-induced current $_{(IF/I)}$ was measured. Membrane stretch was applied by increasing the volume of the oocytes by injection of an intracellular analogous solution. To examine a putative involvement of cytoskeletal elements in mechanosensing of CFTR actin and tubuline filaments were both stabilized (phalloidin, paclitaxel) and destabilized (cytochalasin D, colchicine). The contribution of membrane composition in the strain-induced CFTR activation was investigated by increasing and decreasing the membrane fluidity by incubation in methyl-3-cyclodextrin and water-soluble cholesterol.

In control experiments, hCFTR was activated by a two-time application of F/I. The second $_{IF/I}$ was significantly reduced by 74 ± 1% ($p< 0.01$) compared to the first $_{IF/I.}$ Injection of different volumes (55.2 nl, 110.4 nl, 147.2 nl) increased the subsequent $_{IF/}$ up to 475 ± 48%. Stabilization of actin filaments with phalloidin significantly reduced hCFTR activity after strain exposure (27 ± 10%; $p< 0.01$). Similar results were observed by stabilization of microtubules with paclitaxel ($p< 0.01$). By contrast, destabilization of actin filaments (cytochalasin D) and microtubules (colchicine) did not impair hCFTR activity after the stretching stimulus. Increasing the membrane fluidity of CFTR-expressing oocytes with methyl-3-cyclodextrin reduced the stretch-induced CFTR activity significantly ($p< 0.01$). Similar effects were observed by decreasing the membrane fluidity with cholesterol ($p< 0.01$).

The present data showed that an increased membrane stretch augmented CFTR-mediated currents. Alteration of the cytoskeletal elements and membrane fluidity and thereby modulating the anchorage of CFTR channel in the cell membrane decreased the CFTR response to stretch. This indicates a contribution of both cytoskeleton and membrane composition in mechano-sensing of CFTR channel.

P. 06

Glucocorticoids distinctively modulate the CFTR-channel with possible implications in lung development

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During fetal development the lung is filled with fluid that is the product of an active chloride secretion accomplished by epithelial cells. The fetal lung liquid leads to lung expansion and is indispensible for lung growth. Studies showed that NKCC1 and the Na,K-ATPases at the basolateral membrane participate in chloride secretion. Little is known about the apical chloride channels responsible for the secretion, yet growing evidence suggests an involvement of CFTR. CFTR expression is developmentally regulated with a high expression in early fetal development and a decline in late gestation. Postnatal lung adaptation is characterized by the switch from placentary to pulmonary gas exchange and thereby removal of lung liquid by alveolar sodium absorption. This process is triggered by hormones that stimulate sodium channels, but little is known on how hormones like glucocorticoids affect pulmonary chloride channels. Since the rise of fetal cortisol levels correlates with the decrease in fetal CFTR expression a connection may be assumed and might correlate with the switch from lung proliferation to alveolar differentiation. Therefore the aim of this study was to analyze the influence of glucocorticoids on pulmonary chloride channels. Alveolar cells from fetal and adult rats, A549 cells and bronchial Calu-3 cells were studied with qPCR and Ussing chambers to determine the mRNA-expression and channel activity of CFTR. In fetal and adult alveolar cells, glucocorticoids strongly reduced CFTR mRNA-expression and channel activity. The reduction of CFTR mRNA-expression and activity was prevented by mifepristone, a glucocorticoid-receptor inhibitor. In addition, glucocorticoids also reduced CFTR-expression in human A549 cells, excluding a species-specific effect. In Calu-3 cells CFTR mRNA-expression was also reduced, yet not to the same extent, whereas channel activity was increased. These results demonstrate that glucocorticoids strongly reduce CFTR expression while their effect on CFTR activity is dependent on the physiological function of the cells. The analysis of another apical chloride channel, TMEM16A, revealed a glucocorticoid-induced reduction of mRNA-expression in alveolar cells and an increase in Calu-3 cells. Therefore, in addition to CFTR, glucocorticoids regulate TMEM16A-expression in a cell-type specific manner. Furthermore, the basolateral chloride transporter NKCC1 was reduced by glucocorticoids in alveolar and bronchial cells alike. The results demonstrate that a rise of the glucocorticoid level is able to reduce the CFTR-expression and is likely the cause for the decline during late gestation in preparation of the transition from prenatal to postnatal breathing. Therefore, the results may be relevant for diseases associated with a hypoplastic lung or an impaired adaptation to air breathing.

P. 08

Study of long-range regulatory mechanisms of the *CFTR* gene

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The cystic fibrosis transmembrane conductance regulator *(CFTR)* gene was identified in 1989. The regulatory mechanisms controlling its complex expression are still not fully understood. Although, 1949 mutations have been identified, many cases of cystic fibrosis remain still of unknown origin.
The aim of our project is to study long-range regulatory mechanisms of the *CFTR* gene.
We first developped the Chromosome Conformation Captures (3C) approach to map these potentials regulatory elements which could interact specifically with the *CFTR* gene by tri-dimentional folding mechanism.
Subsequently, we enhanced our analyses with a high-throughput adaptation of 3C: the 3C-Carbon Copy (5C) technology. This approach allows the analysis of millions chromatin interactions.
A first region of 783kb, comprising the *CFTR* gene, was covered with 5C primers. Interactions between these regions and the *CFTR* promoter are analysed by next generation sequencing with the Ion PGMTM.
These analyses are performed with primary epithelial cells, which express the gene and primary skin fibroblasts, wich do not express the gene.
Results obtained from 8 epithelial's libraries and 6 fibroblastic's libraries, isolated from healthy controls, are reproducible. Some regions seem to specifically interact with the promoter.
Thanks to these studies, we want to describe an expression dependent conformation of the *CFTR* locus.
These analyses will be pursued on patients affected by cystic fibrosis in whom either a single mutation or none was found in the *CFTR* locus.

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P.09

Rattlesnake phospholipase A_2, a new potentiator for CFTR: structural and functional studies

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Abstract

The snake venom group IIA secreted phospholipases A_2 ($sPLA_2$s) from the *Viperidae* family are multifunctional proteins that exhibit a wide range of toxic and pharmacological effects. These $sPLA_2$s catalyse the hydrolysis of the ester bond of phospholipids and also selectively interact with various proteins and regulate their activity. It has been previously reported that interaction between crotoxin (a venom $sPLA_2$ from *Crotalus durrissus terrificus*) and presynaptic receptors blocks the release of acetylcholine. We recently showed that the weakly toxic basic CB subunit of crotoxin binds at nanomolar affinity to the nucleotide binding domain 1 (NBD1) and ΔF508NBD1 of CFTR, and increases CFTR-Cl^- channel current (Faure et al Toxicon 75:212; 2013).

Aim

The aim of the present study was to further investigate the potentiating activity of CB on CFTR protein kinase A dependent Cl^- currents (I_{CFTR}) and to identify the binding interface of the two proteins.

Methods

I_{CFTR} were measured by patch clamp (whole cell and inside-out configurations) and voltage-clamp techniques in HeLA cells and *X. laevis* oocytes expressing CFTR, and by "*ex-vivo*" short-circuit-current in mice colon. Binding between NBD1 and CB isoforms was measured using surface plasmon resonance (SPR) and the ΔF508-NBD1-CB binding interface was studied by molecular docking simulations.

Results

The results from all functional assays showed an increase of I_{CFTR} after addition of 1-10nM basic PLA_2 subunit of crotoxin to the perfusate containing 100µM cptAMPc (for whole cell I_{CFTR} patch-clamp and Isc experiments, or 10nM PKA plus 1mM ATP-Mg for single channel measurements in inside-out configuration). In all cases the CFTR current was attested by inhibition with 10µM Inh-172. Three-dimensional molecular docking simulations between the CB and ΔF508NBD1 structures, guided by the SPR experimental data with CB isoforms, allowed us to propose a potential binding interface of the two proteins. Competition experiments by SPR showed that keratin 8 and CB compete for binding to NBD1/ΔF508NBD1.

Summary and Conclusion

In the present work using four different functional assays we demonstrate that the CB subunit of crotoxin is a novel potentiator for CFTR. The site of interaction between CB and ΔF508NBD1 mapped by theoretical methods may be similar to the site of interaction between keratin 8 and ΔF508NBD1 (Odolczyk et al EMBO Mol Med 5: 484-1501 2013). A search for CB-derived peptides with potentiator activity is currently underway. The characterization of the binding interface between CB and CFTR provides an original perspective for the design of novel CFTR drugs.

Glucocorticoids enhance the CFTR-activity in primary tracheocytes

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Glucocorticoids exert many physiological functions including regulation of pulmonary ion channels. Different ion channels within the airway epithelia mediate lung fluid production during fetal development and its absorption prior to birth. In the fetal lung active fluid secretion induces lung expansion which is important for lung proliferation. Therefore, an impairment of prenatal secretion may result in diminished lung development. Studies suggest that the CFTR-channel is involved in the fetal lung fluid secretion. In preparation to birth the respiratory epithelia converts to the absorptive phenotype concomitant to the rise of fetal glucocorticoid levels. Glucocorticoids stimulate the fluid absorption by enhancing the epithelial Na^+-channel (ENaC) and thereby initiate lung fluid clearance. However, it is yet unknown how its counterpart CFTR is affected by glucocorticoids, which might also be relevant for postnatal lung transition. In addition, in the postnatal lung, the conducting segments of the airways are dependent on a balanced interaction of secretive and absorptive ion channels. Disturbances can affect the mucociliary clearance (MCC), which is based on the correct depth and thickness of the periciliary liquid layer. In case of defective CFTR, the MCC is impaired, leading to chronically inflamed airways. Therefore, the analysis of glucocorticoid effects on the secretion process in the upper airways in relation to MCC is important since anti-inflammatory therapies with glucocorticoids are common for chronic airway inflammatory conditions, such as infections and allergies. Thus, the goal of this study was to observe the effects of glucocorticoids on CFTR-activity and mRNA-expression. Tracheocytes are an excellent model to study the regulation of absorption and secretion with regard to MCC, because they express both CFTR and ENaC. Therefore primary rat tracheal cell cultures were developed and routinely grown to confluent monolayers on permeable supports. Short circuit currents $_{(Isc)}$ were measured in Ussing-Chambers. Amiloride, forskolin and $CFTRi_nh172$ were used to distinguish between the contributions of different ion channels expressed by tracheocytes. The incubation with dexamethasone resulted in increased amiloride-sensitive I_{sc} (0,068 ± 0,013 µA/cm² to 0,15 ± 0,019 µA/cm²; p< 0,001), forskolin-induced I_{sc} (8,28 ± 0,68 µA/cm² to 12,53 ± 1,73 µA/cm²; p< 0,05) and $CFTRi_nh172$-sensitive I_{sc} (1,77 ± 0,26 µA/cm² to 3,94 ± 0,59 µA/cm²; p< 0,01). These results demonstrate that glucocorticoids enhance the CFTR- and ENaC-activity in tracheal epithelia. The response of the tracheocytes to glucocorticoids is different from distal airway and alveolar epithelia. While cells from distal airway origin showed a decreased CFTR-mediated current, the CFTR-activity was increased in the tracheal epithelia. On the other hand, the ENaC-activity was increased in both the distal airway and tracheal epithelia, yet ENaC-mediated currents are comparatively small in the tracheal epithelia and the CFTR-activity manifold higher. Further analysis will determine the effect of glucocorticoids on channel expression and the signaling pathway leading to the elevated channel activity. In conclusion, tracheal epithelia demonstrate a balanced response to glucocorticoids rather than a complete shift from secretion to absorption, since absorptive and secretive processes are both stimulated by glucocorticoids, which is probably beneficial to maintain efficient MCC under various conditions.

Impact of the F508del mutation on ovine CFTR, a Cl- channel with enhanced conductance and ATP-dependent gating

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Cross-species comparative studies are a powerful approach to understand cystic fibrosis transmembrane conductance regulator (CFTR) processing and function (1, 2). In this study, we investigated the single-channel activity of ovine CFTR and the impact of the F508del mutation using excised inside-out membrane patches from CHO cells transiently expressing CFTR constructs at 37 ºC as previously described (2, 3). Like human CFTR (3), ovine CFTR formed weakly inwardly rectifying Cl^- channels regulated by PKA-dependent phosphorylation and intracellular ATP. However, for three reasons ovine CFTR was noticeably more active than human CFTR. First, the single-channel conductance was increased (human, gamma = 8.55 ± 0.12 pS; ovine, gamma = 10.01 ± 0.21 pS; n = 6 and 8; $p < 0.05$). Second, ovine CFTR had a higher open probability (P_o) (human, $P_o = 0.45 \pm 0.02$; ovine, $P_o = 0.59 \pm 0.02$; n = 6 and 19; $p < 0.05$) because the frequency and duration of channel openings was increased. Third, ATP more strongly stimulated ovine CFTR channel gating (human, Kd = 0.167 mM; Po(max) = 0.57; ovine, Kd = 0.074 mM; Po(max) = 0.70 from Michaelis-Menten fits to mean data; n = 5 - 11 at each ATP concentration except at ATP (0.03 mM), where n = 3). Previous work by Ostedgaard et al. (1) demonstrated that the F508del mutation has a less severe impact on the single-channel activity of murine and pig CFTR. This was strikingly evident with ovine CFTR. The F508del mutation reduced the P_o of ovine CFTR by only ~30% to be equivalent to that of wild-type human CFTR (human: wild-type, $P_o = 0.45 \pm 0.02$, F508del-CFTR, $P_o = 0.06 \pm 0.01$; $p < 0.05$; ovine: wild-type, $P_o = 0.59 \pm 0.02$, F508del-CFTR, $P_o = 0.40 \pm 0.02$; $p < 0.05$; n = 6 - 19; $p = 0.074$ for human wild-type CFTR vs. ovine F508del-CFTR). However, the F508del mutation had a severe effect on the thermal stability of ovine CFTR. Like human F508del-CFTR, ovine F508del-CFTR Cl^- channels deactivated completely within 10 ~ 15 minutes in excised inside-out membrane patches at 37 ºC (n = 8). We conclude that ovine CFTR forms a regulated Cl^- channel with enhanced conductance and ATP-dependent channel gating compared to human CFTR. The F508del mutation has minimal impact on ovine CFTR channel gating, but strongly destabilises the channel at physiological temperatures. Our data suggest that F508del's impacts on gating and thermal stability are distinct effects. Supported by the CF Foundation, CF Trust, National Institutes of Health and the Strategic Scholarships Fellowships Frontier Research Networks, Office of the Higher Education Commission of Thailand.

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P.12

Purification and characterization study of the thermal stability of CFTR expressed in *S. Cerevisiae*

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F508deletion and G551D are by far the most frequent cystic fibrosis-causing mutations and are the targets for the CFTR drugs VX-809 (a corrector) and VX-770 (a potentiator) respectively. F508 deletion destabilizes its domain (NBD1) and probably also the interaction between transmembrane domains and NBD1. The G551D mutation is in the signature sequence in NBD1 in CFTR and therefore may affect ATPase activity by Walker A and B residues in NBD2. It has much lower open probability than wild-type channels, locking the CFTR protein in a closed state, which may be advantageous for biophysical and structural studies.
Human F508del CFTR and G551D CFTR were expressed in *S. cerevisiae* and purified in detergents n-dodecyl β-D-maltopyranoside (DDM) or lyso-phosphatidyl glycerol (LPG). Coumarin maleimide binding and thermal gel analysis were used to probe the thermal stability of the mutations versus WT CFTR in the presence of the two detergents and drugs. ATPase activity was also measured after removal of the detergents and reconstitution with lipid. We show that the mutations have opposite effects on the thermal stability of CFTR, with destabilization of the global CFTR thermal stability caused by F508 deletion. This leads to a simple model for the effects of the mutations at physiological temperatures and possible routes for therapy via small molecule correctors.

NBD1 Cotranslational Folding Intermediates as Targets for CF Drug Discovery

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The primary molecular defect in most CF patients is caused by CFTR misfolding Moreover, the efficiency of NBD1 folding itself limits CFTR trafficking, and is markedly compromised by the ΔF508 mutation. An important goal in CF therapeutics, therefore, is to understand how NBD1 acquires and maintains its folded state and to devise pharmacological strategies to improve its folding efficiency. Using fluorescence resonance energy transfer (FRET) we show that NBD1 folds cotranslationally as the nascent polypeptide emerges from the ribosome. Folding occurs sequentially via compaction of three distinct subdomains: the N-terminal, alpha-helical, and alpha-beta core. The timing of these folding events is critical as premature alpha-subdomain folding prevents subsequent formation of the beta-sheet core. To coordinate these events, the ribosome modulates intrinsic folding propensity of the nascent polypeptide by: first, delaying alpha-subdomain folding; second, facilitating intercalation of beta-strands within the parallel b-sheet core, and third balancing kinetics of synthesis and folding via selective codon usage. These studies pinpoint core formation as a critical step in de novo NBD1 folding. We next used the algorithm of Spencer et al, (J Mol Biol. 2012;422:328-35) to introduce synonymous codon substitutions into full length CFTR that are predicted to increase (or decrease) the alpha-subdomain and alpha/beta core translation rate (residues 525-593). When expressed in HEK cells, the “fast” coding sequence increased aggregation of immature (band B) CFTR resulting in delayed accumulation of immature CFTR (band B) in the insoluble cell lysate fraction. In addition, “fast” NBD1 CFTR that matured to band C, showed increased reactivity to the 7D12 monoclonal antibody epitope in the alpha-helical subdomain, indicating that the translation rate can co-translationally impact folding outcome.

The ability to access structural intermediates that exist only transiently during CFTR synthesis provides a potential means to pharmacologically manipulate the cotranslational folding pathway. To exploit these findings, we developed a high throughput screening assay, wherein immobilized ribosome nascent chain complexes (RNCs) can be imaged by high content microscopy in a 384 well format. This approach allows quantification of FRET efficiency on agarose beads containing as little as 1.8 attomole of NBD1 (1 million molecules). Solid-state FRET measurements recapitulate nascent chain folding observed in solution and can detect perturbations that both improve and inhibit NBD1 folding at specific stages of synthesis. This approach provides a novel tool to investigate normal and abnormal co-translational folding pathways and establishes a new CF drug discovery platform for screening small molecules that improve nascent NBD1 folding in CF patients. (Supported by CFFT and NIH DK51818).

Characteristics of stabilizing mutations of CFTR by FSEC, thermal gel analysis and CPM

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The cystic fibrosis transmembrane regulator (CFTR) protein is one of the most interesting members of the ATP-binding cassette (ABC) family as it acts as a chloride channel rather than a transporter. Mutations in CFTR are responsible for Cystic Fibrosis. The most common mutation (F508 deletion) destabilizes the protein such that little reaches the site of action (the plasma membrane). We wish to discover mutations which stabilize the protein in order to produce CFTR suitable for structural studies which could lead to better drugs. Similarly, our studies of CFTR stability may help in the understanding of the F508del-induced defect and lead to better therapeutic strategies. Here we describe our initial work with CFTR expressed in yeast (S. cerevisiae) where mutations can be rapidly generated and screened. We utilize fluorescence size-exclusion chromatography (FSEC) and thermal gel analysis to test CFTR stability before purification. Purified CFTR can also be tested by the thiol-specific fluorochrome N-[4-(7-diethylamino-4-methyl-3-coumarinyl)- phenyl] maleimide (CPM). We discuss several stabilizing mutations in CFTR and general strategies for obtaining a well-behaved, monodisperse, highly soluble and stable protein for structural studies.

P. 15

LMTK2 Mediated Phosphorylation Regulates CFTR Endocytosis in Human Airway Epithelial Cells

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Background: CFTR is a cAMP-activated, Cl^--selective ion channel expressed at the apical surface of fluid-transporting epithelia and CFTR-mediated Cl^- secretion is regulated by adjusting activity and abundance of CFTR at the plasma membrane (PM), mostly through clathrin-mediated endocytosis. Nonetheless, the protein interactions that control these processes in epithelial cells have only been partially explored. Previously, we showed that kinases have a key role in this process - as both SYK and WNK4 affect CFTR levels at the membrane [1,2].

Lemur Tyrosine Kinase 2 (LMTK2) is a transmembrane serine/threonine kinase playing a role in membrane trafficking. LMTK2 also directly binds to myosin VI, a motor protein known to facilitate CFTR endocytosis [3]. Although LMTK2 was previously shown *in vitro* to phosphorylate the S^{737}-residue in the regulatory domain (RD) of CFTR [4], it remains unknown whether also *in vivo* and if it impacts CFTR endocytic traffic.

Aim: To determine whether LMTK2 regulates CFTR endocytosis in human airway epithelial cells and if phopshorylation of S^{737} is involved in the process.

Methods: Western blot (WB), co-immunoprecipitation (co-IP), cell surface biotinylation and endocytosis assays. Human airway epithelial cells: CFBE41o- and Calu-3.

Results: Data demonstrate that endogenous LMTK2 localizes at the PM, including the apical membrane domain in both cell types. Endogenous LMTK2 and CFTR co-IP and LMTK2 phosphorylates the CFTR-S^{737}. Partial depletion of endogenous LMTK2 or overexpression of an LMTK2 fragment with impaired kinase activity increases CFTR abundance at the PM by attenuating its endocytosis. Compared to wt-CFTR, the CFTR mutant with the serine to alanine substitution in the LMTK2 phosphorylation site CFTR-S^{737A} also shows increased PM abundance and decreased endocytosis. Furthermore, depletion of endogenous LMTK2 in cells expressing F508del-CFTR also increases the short-circuit current when compared to mock-transfected cells.

Conclusion: Altogether, our results demonstrate that in human airway epithelial cells LMTK2 facilitates CFTR endocytosis and reduces its cell surface density by a mechanism that involves phosphorylation of CFTR-S^{737}, also contributing to the inhibitory effect of the S^{737} phosphorylation on CFTR mediated Cl^- secretion. These findings are particularly relevant as CFTR-S^{737} is also a substrate for PKA and AMPK. Additionally, modulation of LMTK2 activity may also be of therapeutic interest, contributing to the stabilization of F508del-CFTR at the plasma membrane.

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EPAC (Exchange protein activated by cAMP) 1 is involved in membrane complexes with CFTR and NHERF-1

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Background: Ion transport through CFTR is stimulated when cAMP levels in the subcortical compartment increase through PKA activation, thus triggering CFTR phosphorylation and channel opening. However, PKA is not the sole cAMP sensor in the cell: exchange proteins directly activated by cAMP (EPACs) also respond to direct binding to this second messenger. EPACs are guanine nucleotide exchange factors (GEF's) for small GTPases of the Rap family regulating cell-to-cell and cell-matrix adhesion, cytoskeleton rearrangements and cell polarization, processes which are dysregulated in CF. In pancreatic and neuronal cells, EPACs have also been shown to regulate exocytosis. In response to cAMP binding, EPACs shift to the plasma membrane (PM), to which it anchors through ezrin-radixin-moesin (ERM) proteins [1], previously shown to link CFTR to the actin cytoskeleton and to facilitate cAMP-driven CFTR activation by tethering PKA in its close proximity. This suggests that activation of both CFTR and EPACs might be spatially and temporally coincident, but little is known on the involvement of EPACs in CFTR biology.

Aim: To characterize the interaction between the predominant isoform of EPAC and CFTR and to elucidate its impact on CFTR biogenesis, processing, trafficking and function.

Methods: Western blot (WB), co-immunoprecipitation (co-IP), immunofluorescence with CFTR, EPAC1, Ezrin, NHERF1, Calnexin, α-Tubulin and E-cadherin Abs. Lung epithelial cell lines: A549 cells expressing wt- or F508del-mCherry-CFTR under a Tet-ON promoter and CFBE cells (stably expressing wt- or F508del-CFTR).

Results: WB show that Epac1 and its effector Rap1A are expressed in the above cell lines. Localization of EPAC in A549 cells in resting conditions is mostly nuclear or perinuclear, with some staining detected at the PM. Co-IP show that EPAC1 and CFTR interact in A549 and CFBE cells (expressing both wt- or F508del CFTR) and NHERF1-siRNA (but not Ezrin-siRNA) prevents this interaction. Finally, treatment of A549 cells with 8-pCPT-2'-O-Me-cAMP-AM (a membrane-permeable EPAC-specific agonist not activating PKA) leads to an increase in both wt-CFTR steady-state levels and CFTR-EPAC interaction in A549 cells, without affecting the efficiency of processing of CFTR at steady-state (band C/band B ratio).

Conclusion: Our results suggest an involvement of EPAC1 in late stages of CFTR biogenesis and support the hypothesis that EPAC1 may have a positive impact on CFTR membrane traffic, making it a novel potential therapeutic target for CF.

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Sumoylation modulates CFTR biogenesis

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The small heat shock protein (sHsp), Hsp27, selectively directs mutant cystic fibrosis transmembrane conductance regulator (CFTR) to degradation via poly-SUMOylation and ubiquitylation by the SUMO-targeted ubiquitin ligase (STUbL), RNF4 (1). Since the common CFTR mutant, F508del, destabilizes nucleotide binding domain 1 (NBD1), in which it resides, we assessed the conformational basis of its recognition for SUMO-dependent degradation. A membrane-tethered NBD1 (2) expressed in vivo recapitulated the properties of the full-length protein, including selective degradation of the F508del mutant (vs WT) during Hsp27 over-expression and its preferential modification by SUMO-2. This SUMO paralog exhibits poly-chain formation and a requirement for RNF4 targeting, as found for full-length (FL) CFTR. Also similar to FL CFTR, in vitro assays with purified NBD1 and SUMOylation components showed preferential modification of F508del NBD1 by SUMO-2, which was stimulated by Hsp27.

On its way to denaturation, NBD1 undergoes the transition to an intermediate conformation, detected as an increase in intrinsic tryptophan fluorescence (3). Conditions that de-stabilize NBD1, including reduced ATP concentrations or elevated temperature enhanced its SUMO modification, whereas factors that stabilize the NBD, e.g. the introduction of revertant or solubilizing mutations, reduced SUMO modification of the mutant NBD. The intrinsic fluorescence of F508del NBD1 increased during thermally-induced NBD1 unfolding, and the time-course of this conformational transition was paralleled by SUMO modification of the NBD. Mutation of a consensus SUMOylation site at K447 blocked the ability of Hsp27 to promote the degradation of membrane tethered F508del NBD1 as well as its SUMOylation. These findings indicate that Hsp27 detects the transition of mutant NBD1 to a non-native conformation, thus inducing its SUMO-2 modification and STUbL-mediated degradation.

We interrogated a protein array to detect proteins that would interact with mono-SUMO-1 or poly-SUMO-3, which might identify targets for SUMO-dependent modulation of CFTR biogenesis. Positive control results from the array included the STUbL, RNF4, and several components of the SUMO modification and deSUMOylation pathways, such as the SUMO E1 and E2 and several SUMO proteases (SENPs) that remove SUMO from its modified substrates. An interesting hit was PIAS4, which is a SUMO E3, a class of enzymes that select substrates for modification and catalytically facilitate the process. In contrast to the action of Hsp27, the over-expression of PIAS4 stabilized WT and mutant CFTR expression, and PIAS4 over-expression had the capacity to elicit the expression of mature F508delCFTR. As opposed to Hsp27, PIAS4 elicited the modification of F508del by SUMO-1, the paralog that does not form poly-chains. These preliminary findings suggest that different components of the SUMO modification pathway may either facilitate CFTR biogenesis via SUMO-1 modification or promote CFTR degradation via SUMO-2 conjugation, leading to recognition by a STUbL. These antagonistic pathways may determine the fate of WT or mutant CFTR proteins. [Supported by the NIH and CF Foundation Therapeutics]

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Hierarchy of CFTR folding and domain assembly understood through studies of the CFTR2 database collection

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CFTR folds to a large extent during its synthesis (co-translational), but needs significant post-translational domain assembly to complete its native functional structure. The hierarchy of this assembly process is still not known. This knowledge is important to fully understand the impact of CFTR mutations on its structure and to elucidate how investigational corrector drugs aid misfolded conformers.

Radiolabeling approaches in combination with limited proteolysis and selective immunoprecipitations provided us the resolution to follow CFTR's conformational changes in live cells with time. By studying the CFTR2 mutant collection as well as structure-based designed non-CF mutations we manage to provide key insights into the early and late folding events of CFTR.

While NBD1 folds co-translationally, and misfolds in the ΔF508 mutant during synthesis, MSD1 and NBD2 attained their final protease resistant structures late, coinciding with appearance of CFTR in the Golgi complex.
Specific CFTR2 mutants located in NBD1 and NBD2 showed how the folding of these two domains and MSD1 were connected. While both mutants traveled to the Golgi complex with similar efficiency, the protease susceptibility of NBD1, NBD2, and MSD1 differed, suggesting that some late domain assembly steps were not a prerequisite for release by the endoplasmic reticulum protein quality control machinery.
We demonstrated that NBD1 and NBD2 fold independent from each other, and that MSD1 is involved in both early and late domain folding and assembly events. Our data show which domain interactions are crucial for proper folding of CFTR, and suggest a hierarchical CFTR folding model

VX-809 stabilizes the early steps of F508del-CFTR biogenesis and folding

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Background: Cystic Fibrosis (CF) disease is mostly caused by the F508del mutation which impairs protein folding, leading to its retention in the endoplasmic reticulum (ER) and premature degradation and thus preventing most of the mutant from reaching the plasma membrane (PM). Nowadays, pharmacological therapies are emerging for CF disease, namely small molecules, such as VX-809 that rescue the mutant CFTR localization and function. Although VX-809 is still under clinical trial[1], the first results were more modest than expected. A recent study has pointed out to the possible binding of this compound to the NBD1:ICL4 interface[2], a critical contact site in CFTR structure.

Aim: To assess the effect of small molecule correctors on the early steps of mutant CFTR biogenesis, function and membrane stability, we investigated the effects of CFTR correctors: VX-809, C4 (compound 4a) and C3 (VRT-325) as well as their additive/synergistic effects with low temperature.

Methods: We used metabolic pulse-chase analysis in BHK cells stably expressing F508del-CFTR with anti-CFTR 596 Ab to assess CFTR turnover rate and maturation efficiency, cell surface biotinylation to quantify CFTR endocytosis and efflux iodide technique to measure levels of CFTR activity.

Results: Incubation with C3, C4 and VX-809 confirmed the described rescue of the mutant protein - as shown by the appearance of its fully-glycosylated form (band C) - but VX-809 also elicits a significant stabilization of the immature core-glycosylated form (band B), that is not observed for C3 and C4. Furthermore, VX-809 increases the biosynthesis of CFTR from its very early time points (from t= 20min).

Moreover, as previously shown by biochemical data[2], we found that all the 3 correctors (VX-809, C3 and C4) further increase the function of F508del-CFTR rescued by low temperature.

Additionally, preliminary data from assays of CFTR internalization of wt-CFTR expressed in CFBE cells suggest that VX-809 decreases CFTR endocytosis.

Conclusions: Altogether, these results suggest that VX-809 has an extended effect upon CFTR - by increasing the stability of both immature form of F508del-CFTR at an early phase of its biogenesis, and the mature form of the protein at the PM. Understanding the impact of these small compounds on CFTR processing will be crucial to improve the efficacy of CF therapies *in vivo*.

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Mechanisms for Rescue of Misfolded CFTR from Premature Degradation by ER Quality Control

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Cystic Fibrosis (CF) is a fatal genetic disorder associated with defective hydration of lung airways due to the loss of chloride transport through CFTR. CFTR contains two membrane-spanning domains (MSD), two nucleotide-binding domains (NBD) and a regulatory domain, such that channel assembly requires multiple inter-domain contacts. The most common CF-causing mutation F508del occurs in NBD1, and results in misfolding and premature degradation of F508del-CFTR. There are also a number of rare mutations that occur in other domains that cause CFTR misfolding and thereby give rise to CF. We are defining mechanisms for CFTR misfolding caused by F508del as well as additional rare CFTR mutants. The goal is to identify steps in the CFTR folding pathway that can be modulated by folding correctors to permit escape of specific CFTR mutants from ERQC machines and thereby promote their surface function. An update on studies related to ERQC of CFTR and mechanisms for correction of CFTR misfolding will be provided.

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The role of Keratin 8 in Alpha-1-antitrypsin trafficking and/or degradation

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Background
We have previously reported that Keratin 8 (K8) affects the trafficking of delF508-CFTR from the endoplasmic reticulum to the plasma membrane by interacting directly with delF508-NBD1 (Odolczyk et al., EMBO Mol Med 2013, Colas et al., HMG 2012). Down-regulation of K8 in cells corrects delF508-CFTR processing defect and restores its function. We hypothesized that K8 may interact with other abnormally folded proteins preventing their correct trafficking and function.
Aim
To test this hypothesis we investigate the interaction between K8 and alpha-1-antitrypsin (AAT1), an inhibitor of proteases of inflammatory cells. AAT1 is secreted by hepatocytes and airway epithelial cells into the extracellular environment. Mutants of AAT1, null Hong Kong (AAT1 NHK) and Z (AAT1 Z), are abnormally folded and degraded by the proteasome. This leads to reduced AAT1 secretion and causes, ultimately, alpha-1-antitrypsin deficiency disease.
Methods
HeLa cells transiently transfected with cDNAs of AAT1 (WT, NHK and Z) were used. The potential interactions or complex formation were tested using Western Blot analysis, immunofluorescence (IF), co-immunoprecipitation (CoIP), proximity ligation assay (PLA) and surface plasmon resonance.
Results
WT and AAT1 mutant variants co-immunoprecipitated with K8. Preliminary result from surface plasmon resonance technic did not show a direct interaction between K8 and AAT1 WT. Proximity ligation assay experiments suggested that both NHK and Z mutants were localized in close proximity to K8 filaments (< 40 nm), in contrast to WT AAT1. Immunofluorescence experiments showed that K8 filaments network was changed in cells expressing mutants as compared to the K8 network of WT AAT1 expressing cells. Reduction of K8 expression by shRNA led to the cellular accumulation of mutant AAT1 variants.
Conclusions
The results suggest that K8 complexes formed with misfolded NHK and Z AAT1 mutants have different characteristics than complex of wild type AAT1. This complex formation may play a role in mutated AAT1 trafficking defect and degradation.

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TRPV6 is upregulated by PLC-PI(4,5)P2 pathway in CF cells

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Abnormal calcium increase observed in the genetic disease cystic fibrosis (CF) has been well described since the 60's. Looking for new actors of this harmful dysregulation, we examined ion channels belonging to the transient receptor potential vanilloid (TRPV) channels family as emerging target to explain abnormal increase of Ca^{2+} influx. TRPV5 and TRPV6, display properties namely high Ca^{2+} selectivity and constitutive activity, that make them upcoming actors in CF abnormal calcium influx.

The first objective of this study was to investigate the possible involvement of TRPV5 and TRPV6 in the increase of Ca^{2+} influx in CF cells. 16HBE14o-, CFBE41o- cells and freshly isolated human airway epithelial cells from CF (homozygotes F508del-CFTR) and non CF patients were used. The expression of TRPV5 and TRPV6 channels was verified by Western Blot and constitutive Ca^{2+} influx was measured using Fluo-4 AM probe. Constitutive Ca^{2+} influx is 1.5 times higher in CF cells compared to non CF cells. Measurement of Ca^{2+} influx after applying the specific inhibitor of TRPV6, SOR-C27 and TRPV6 siRNA strategy showed a decrease of constitutive Ca^{2+} influx in non CF and more importantly in CF cells. These results suggest that TRPV6 is implicated in the abnormal increase of constitutive Ca^{2+} influx in CF cells.

It has been showed that TRPV6 undergoes Ca^{2+}-induced inactivation, protecting the cell from toxic Ca^{2+} overload. Indeed, Ca^{2+} influx through TRPV6 channel activates a Ca^{2+} sensitive PLC and the resulting depletion of membrane phospholipid phosphatidylinositol 4,5-bisphophate (PI(4,5)P2) and inhibits this channel. Moreover, TRPV6 activity depends on the presence of PI(4,5)P2. We were then wondering if the absence of inhibition from PLC-PI(4,5)P2 pathway could be responsible for abnormal increase of TRPV6 activity in CF cells. For this purpose, we measured constitutive Ca^{2+} influx in the presence of the PLC inhibitor U73122 or its inactive analog U73343. Results showed that U73122 increased constitutive Ca^{2+} influx in non CF but not in CF cells. The inactive analog had no effect on constitutive Ca^{2+} influx in both cell lines. Addition of SOR-C27 showed a decrease of constitutive Ca^{2+} influx in 16HBE and more importantly in CFBE cells. These data demonstrated that PLC-PI(4,5)P2 pathway is implicated in abnormal increase of TRPV6 activity in CF cells.

We will examine in future experiments if inhibition of PLC activity itself or a different quantity of PI(4,5)P2 is responsible for the increase of TRPV6 activity in CF cells using CF and non CF human airway epithelial cells of bronchial origin from Epithelix.

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SLC6A14 elicits CFTR-mediated chloride and fluid secretion in CF airway

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Background: SLC6A14 is associated to the severity of CF disease (Sun 2012). This transporter mediates the uptake of cationic and neutral amino acids across the apical membrane of airway epithelial cells (Galietta 1998) and we previously demonstrated that L-arginine, a substrate for SLC6A14, enhanced constitutive CFTR-mediated Cl^-secretion in human non-CF and CF primary bronchial epithelial cultures. We now postulate that SLC6A14 activation with L-arginine also effects transepithelial fluid transport in human airway epithelial cells.

Method: Primary human bronchial epithelial cells from CF (CFBE) and non-CF subjects (NBE) were obtained from lung transplants (Drs. Keshavjee and Zabner, University of Toronto and Iowa). Transepithelial L-arginine transport was measured as appearance of ^{3}H-L-arginine at the basolateral side of human airway epithelial cells following addition of L-arginine and ^{3}H-L-arginine to the apical side in an Ussing chamber. Airway surface liquid (ASL) height was measured following application of a PBS fluid bolus with texas red dextran using confocal microscopy. ASL height was assessed in presence of aprotinine to inhibit ENaC activity. L-arginin (Arg) was used as substrates and α-methyltryptophan (αMT, Karunakaran 2008) as inhibitor for SLC6A14.

Results: In NBE and CFBE cells transepithelial Arg transport was reduced by 60% in absence of Na^+ (p=0.02) as well as in presence of αMT (p=0.02) indicating that SLC6A14 is a main transport system for Arg across the airway epithelium. Our preliminary results show that SLC6A14 not only enhance CFTR-mediated $Cl^-_{leq,}$ but also affect airway epithelial fluid secretion. In NBE cells addition of Arg had no effect on the ASL height measured at 24 hrs (p=0.27). However, in CFBE cells addition of Arg led to a significant increase in the ASL of CFBE cells (5.4 ± 0.57 µm versus 8.2 ± 0.52 µm, p=0.003). This increase in ASL was inhibited in the presence of αMT. Ongoing investigations are evaluating intracellular pathways to explain the effect of SLC6A14 on CFTR function.

Conclusion: SLC6A14 is an important membrane constituent in airway epithelial cells. Activation of SLC6A14 increases constitutive CFTR-mediated $_{leq}$ as well as epithelial fluid secretion, which in turn leads to an improvement of the ASL height in CF human airways. Thus, SLC6A14 may be useful as a future drug target supporting our attempts to overcome the airway epithelial defect in CF.

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Regulation of the chloride channel ANO1 by microRNAs in Cystic Fibrosis patients

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Introduction: Impairment of the activity of the CFTR chloride channel observed in cystic fibrosis (CF) patients is the main cause of the deterioration of lung function. This lung disease is the leading cause of morbidity and mortality in CF patients. In 2008, three groups have identified a novel chloride channel: the channel ANO1 (TMEM16a), proposed as a potential therapeutic target to restore chloride efflux in the lung in CF. Previous works from our laboratory have also shown that the activity and expression of ANO1 were reduced in a CF context (Ruffin et al., BBA Molecular Basis of Disease, 2013). Today, the origins of these decreases remain unknown. Some studies showed that CFTR is regulated by miRNAs which are small non-coding RNAs regulating negatively gene expression post-transcriptionally. One hypothesis is that miRNAs could have a major role in the deregulation of ANO1.
Objective: The objective of this work is to understand the origin of the reduced expression of ANO1 in CF cells by studying the role of miRNAs.
Methods: To answer this objective, we performed a gene candidate approach using bioinformatics databases to study miRNAs that could target ANO1. To initiate this project, we evaluated the expression levels of miRNAs candidates by RT-qPCR in bronchial epithelial cells lines and in CF and non-CF human lung explants. To validate the link between ANO1 and miRNAs, we performed functionality experiments studying the miRNAs candidates binding to the 3´UTR of ANO1 in condition of over-or under-expression of miRNAs, and quantifying the expression of ANO1 in these conditions.
Results: The bioinformatic study allowed us to identify different miRNAs including miR-9 and miR-144 as potential regulators of ANO1. In one hand, the study of the expression of miR-9 in CF versus non-CF context did not show difference of expression of miR-9 and in condition of over- or under-expression of miR-9, we did not observe regulation of ANO1. On the other hand, we observed a significant decrease in the expression of ANO1 in condition of over-expression of miR-144 but no difference in luciferase activity in the same conditions.
Conclusion: Our results suggest that miR-9, alone, do not regulate ANO1 in our experimental conditions and that miR-144 could indirectly regulate ANO1. It would be interesting to study through whom miR-144 regulates ANO1. Moreover, to go further, we make a miRNOME and transcriptome approach on primary air-liquid interface cultures of human airway epithelia CF and non-CF. The results of this dual approach must be received soon and will allow us to explore all deregulated miRNAs in the CF context.

Study of the SLC26A8 association with CFTR wild type and mutant proteins

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The Solute Carrier 26 (SLC26) members are transmembrane proteins mediating the transport of a broad range of anions, including chloride, bicarbonate, sulfate and oxalate. SLC26 proteins are most often localized at the plasma membrane of the epithelial cells where they are involved in the composition of the secreted fluids in the body. Interestingly, the existence of a physical and functional interaction between several members of the SLC26 family and the CFTR channel has been well documented. In particular, we have shown that CFTR interacts *via* its R domain with SLC26A8 (also known as TAT1; Testis Anion Transporter 1), a member of the SLC26 family exclusively expressed in the male germ cells and involved in sperm motility and capacitation events. SLC26A8 physical interaction with CFTR results in a strong stimulation of CFTR conductance. Furthermore, we have identified three heterozygous missense mutations in *SLC26A8* by screening a cohort of 146 men presenting with asthenozoospermia (i.e. reduced sperm motility) c.260G>A (p.Arg87Gln), c.2434G>A (p.Glu812Lys), and c.2860C>T (p.Arg954Cys)] and observed that these mutations abrogate CFTR stimulation, suggesting that the CFTR/SLC26A8 complex is essential for the regulation of anions fluxes in the sperm.

Taking into account their association and functional cooperation with the CFTR channel in most of the affected tissues in cystic fibrosis patients, SLC26 proteins constitute potential targets for the development of novel therapeutic strategies. Here by analyzing the stability, the localization and the conductance of CFTR proteins, we will discuss the impact of SLC26A8 expression on two mutant forms of CFTR (CFTRDeltaF508 and CFTRG551D), as a preliminary step towards this therapeutic objective.

Paracellular and transcellular transport of ions in human bronchial epithelium

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Defect of water and ion transport caused by mutation in single *cftr* gene leads to the cystic fibrosis - CF. *cftr* gene code anion channel known as Cystic Fibrosis Transport Regulator (CFTR). Defective CFTR protein leads to very viscous secretion. There are three contradictory hypotheses of how viscous mucus is produced in CF patients. First hypothesis claims that there is decreased water transport to apical side of epithelial cell monolayer, the second there is increased water absorption of water from the apical side and the third there is decreased bicarbonate transport in CF patients. There are multiple ions which are transported across the epithelial monolayer i.e. sodium, potassium, chloride, hydrogen and bicarbonate ions. Until this time there was no method to simultaneously monitor all ions transport through epithelium. In our laboratory we built and successfully tested the multielectrode biosensor system which can measure transport of all the ions.

In experiments the 16HBE-14a cell line was used. It forms tight junctions and is characterized by high transepithelial electrical resistance (TER). Cells were seeded onto Costar Snapwell inserts (0.45 µm pore size, 1 cm^2 surface area) and grown submerged in culture medium until the TER of cell monolayer was about 1000 Ω. To polarize the cells, the medium from the upper side of the insert was taken off to maintain the air contact at the apical side of monolayer and started the experiments, when TER decreased to about 400 Ω.

To better understand the mechanism of electrolyte transport in epithelial lung cells, the sodium and chloride ions transport was measured at first. Using our potentiometric electrode system was equipped with two chloride, two sodium and the reference electrode on each side of cell monolayer. In the first experiment, the sodium gradient across the cell layer was provided (the low sodium fluid on apical side and high on the basolateral side). When the solution flow was stopped, sodium ions were transported across the monolayer from basolateral to apical side. Sodium transport was not changed after introduction of 10µM amiloride (sodium channel blocker).

In the second experiment the chloride ions gradient was provided (low chloride on apical side, high chloride concentration on basolateral side). We observed the electroneutral transport of chloride ions from basolateral to apical side. Then the solution was supplemented with 50µM of glybenclamide (CFTR channel blocker) and 50µM of DIDS (other apical chloride channels blocker) three was no chloride transport but the transepithelial potential reached -25 mV. Thus in polarized epithelium of 16HBE-14a cell line , sodium is transported by paracellular route while chloride by transcellular route - defective in CF.

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Role of microRNAs in human airway epithelium differentiation: Characterization of miR-449 as a central player in multiciliogenesis conserved in vertebrates.

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The airway epithelium is the first line of defense which protects the respiratory tract against frequent external aggressions (inhaled toxic particles, pathogens, allergens,...). This epithelium lining the surface of the airways is constituted by basal cells, goblet cells and multiciliated cells (MCCs) which exhibit hundreds of motile cilia. The coordinated beating of these motile cilia is crucial to the mucociliary clearance essential for airway cleansing. Cystic fibrosis (CF) is a genetic disease characterized by chronic pulmonary infections which lead to airway epithelium remodeling, a goblet cell hyperplasia and a loss of MCCs. As a result, the mucociliary clearance is impaired, ultimately provoking respiratory failure. The formation of motile cilia (multiciliogenesis) requires sequentially a cell cycle arrest, followed by a massive multiplication of centrioles which then migrate and dock into a dense apical actin network before axoneme elongation of each cilium. A better understanding of complex mechanisms governing multiciliogenesis could help to develop new regenerative therapeutic strategies in CF for restoring airway epithelium integrity and mucociliary clearance.
MicroRNAs (miR or miRNAs) are small non-coding regulatory RNAs implicated in numerous biological processes and more recently associated with several chronic airway diseases.
Using two distant models of mucociliary epithelium (*in vitro* with the human airway epithelium and *in vivo* with the the *Xenopus* embryonic epidermis) we have shown that miRNAs of the miR-449 family were specifically expressed in precursors and mature MCCs. Protector oligonucleotides, targeting miR-449 binding site on several targets (Notch pathway and small GTPase R-Ras), indicate that miR-449 promote the cell cycle arrest, control both the amplification of centrioles and the apical actin remodeling, and allow MCC precursors to differentiate. Our findings demonstrate that miR-449 miRNAs are key regulators of vertebrate multiciliogenesis which act as “chefs d'orchestre” by finely controlling several pathways to trigger MCC differentiation.

Proteomic analyses of BALF from young CF children reveal altered lipid, cyclic nucleotide, and Iron signaling and metabolism during inflammation versus non-CF disease controls

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CF lung disease begins in infancy, and is characterized by mucus plugging, infection with inflammation, air trapping, and structural lung changes (eg: bronchiectasis) that are readily detectable by three years of age. Early markers of lung disease that are specific for CF, however, are lacking. This information is critical to our understanding of what initiates CF lung disease, and to identify proximal targets for intervention. This lack of specificity may in part represent examination that has been limited to candidate-based analysis of biofluids, coupled with comparisons with inadequate disease controls. We hypothesized that using an unbiased and blinded approach, proteomic analysis of BALF from young CF patients without chronic Pseudomonas infection compared with non-CF disease controls (ages birth-5 years) would identify disease pathways and biomarkers associated with early CF lung disease. Banked specimens from the CCHMC BALF Biorepository were selected based diagnosis (CF vs non-CF controls with chronic tracheostomy), age (< 4 yrs), microbiology (all CF patients were *Pseudomonas aeruginosa* culture negative) and matched for gender. All samples underwent common processing and were stored at -80C until analysis. Samples from each patient were assigned identification numbers, blinded, and randomized such that the researchers performing the proteomic analyses were prevented from having any information about the experimental subjects. Once proteomic analyses were concluded, the blind was partially broken so as to reveal which samples belonged in the same group, allowing for the comparison of data between cohorts, without revealing the identities of the groups. Protein was precipitated from BALF supernatants, resolubilized, and subjected to 1D and 2D gel SDS PAGE. Segments of the gels were excised and subjected to in-gel protein digestion, peptide fragments extracted and analyzed by LC-MS/MS running in data dependent mode. Quantitation was performed using gel densitometry (quantitative) and MS spectral counting (semi quantitative). In addition to identifying proteins that were significantly different in CF vs non-CF samples, signaling pathway analyses were conducted with the GeneGo software package. While a number of identified differential expressions validate findings by other groups, we identified a number of novel differences. These data support the notion that altered signaling specific to the CF condition occurs early in lung disease. Pathway analysis revealed that the most significant differences in CF vs non CF belong to pathways that reflect increased cellular metabolism, increased lipid and alcohol catabolism, and the increased catalysis of cyclic nucleotides such as cAMP. Transcriptional regulation analysis revealed significant differences in pathways regulated by CREB1, SP1, and androgen receptor in CF vs non CF. Our results indicate that CF BALF contains protein signatures that demonstrate increased metabolism, increased catabolism and an alteration in pathways that regulate lipid, alcohol, and iron metabolism during infection. These significant differences in CF vs non CF infected controls suggests that the CF BALF signature can be segregated from non CF disease controls, exhibiting different biomarkers that delineate pathways that may be informative about early disease pathogenesis and also serve as potential therapeutic targets.

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The mode of action of ADAM17 in resolution of inflammation in CF lung disease

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Background

We found in F508del CFTR mutant mice ($Cftr^{tm1eur}$) that lung injury causes induction of IL-6 and Amphiregulin (AREG) compared to wt. Recent studies link AREG with TGFß1-stimulated collagen accumulation in challenged murine lungs, leading to fibrosis. Further, both AREG/EGFR and IL6/IL6R/Gp130 pathways are involved in the resolution of lung inflammation and injury through the STAT3 transcription factor, which has recently been identified as a modulator of CF lung disease in humans.
Both AREG and IL6R are activated and released from the cell surface by the membrane bound protease ADAM17, which is regulated by extracellular and intracellular signaling.

Aim

We aimed to establish whether ADAM17 plays a pivotal role in CF lung disease and define the druggable targets in the molecular pathways up and downstream of this regulator.

Methods and Results

We addressed these issues in Primary Human Bronchial Epithelial cells in Air-Liquid Interface culture (HBEC-ALI) and the CF mutant immortalized cells CFBE41o-. In HBEC-ALI IL6R and AREG are shedded for 90% to the basolateral compartment. In both models AREG shedding is inhibited 80% ($P< 0.001$) by a specific inhibitor of ADAM17 (TMI-2, Wyeth). In HBEC-ALI IL6R is inhibited by 40% ($P< 0.01$), because a splice variant exists that is not a substrate of ADAM17.

We showed here that ADAM17 is a highly regulated enzyme in bronchial epithelial cells. Cigarette smoke treatment of normal HBEC-ALI, which is known to reduce CFTR activity, increases IL6R production by 50% and AREG shedding twofold. The ROS scavenger, N-acetylcysteine, reduced ADAM17 activity by 90% in CFBE cells ($P< 0.05$) and by 25% in HBEC-ALI ($P< 0.04$ tree different donors analysed in triplicates) suggesting a relationship between these two phenomena.
ADAM17 activity is tightly regulated by tyrosine phosphorylation. The tyrosine kinase inhibitor (genistein) reduced AREG shedding by ~80% ($P< 0.05$) in CFBE41o- cells, whereas an inhibitor of protein tyrosine phosphatases (orthovanadate) significantly increased AREG production. Furthermore, ADAM17 shedding activity depends on EGFR activity. AG 1478, a tyrosine kinase inhibitor that is specific for EGFR (ErbB1), reduced AREG production (~80%) to the same extent as an inhibitor of the kinase downstream of EGFR, MEK1/2 (U0126) and the ADAM17 inhibitor TMI-2 mentioned above.

Conclusions

In bronchial epithelial cells AREG and IL-6R released by ADAM17 act mainly in the basolateral compartment, what may affect epithelial (autocrine) and subepithelial receptors (transsignalling), involved in epithelial repair, inflammation and fibrosis. Our data further suggest that CFTR deficiency and stimulated ROS production affect ADAM17 activity. We show that phosphorylation is the crucial mechanism that regulates ADAM17 dependent shedding in this system. Since ROS is known to inactivate phosphatases, we suggest that ROS may affect ADAM17 activity by increasing its phosphorylation.
We propose that the druggable ADAM17/AREG/IL-6R/STAT3 pathway is a valid target for further study of experimental therapeutics in CF. We currently investigate changes in ADAM17 activity after induction of CFTR expression in the CF mutant CFBE41o- cells.

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Cx26, Notch and PPARg-dependent pathways during human airway epithelial cell repair

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Being subjected to continuous assaults, the pseudostratified airway epithelium undergoes a coordinated repair process in order to maintain its protective function. The mechanisms regulating this process, which may be deregulated in Cystic Fibrosis (CF) cells, are poorly known. Studies have shown the implication of Notch and peroxisome proliferator-activated receptor g (PPARg) pathways in the differentiation of epithelial cells. Furthermore, physical integrity of this tissue is essential to maintain its barrier functions. Since gap junctions, composed of connexin (Cx) proteins, contribute to homeostasis and barrier functions of the airway epithelium, we evaluated the possible relationship between Cx26, PPARg and Notch signaling pathways during repair of human airway epithelial cells (HAECs). HAECs from non-cystic fibrosis (NCF) and from CF patients were grown at the air-liquid interface to generate well polarized epithelia. The expression and/or localization of transcription factors and Cxs were monitored by qPCR and immunofluorescence (IF) in repairing HAECs, 24hrs after mechanical cell ablation (termed 'wounding') and at later times when the ablated area was covered (wound closure). Hes1 gene expression was used to monitor the activation of Notch signaling pathway during repair. PPARg activation was monitored by measuring the expression of HPGD; the enzyme providing its endogenous ligand 15-ketoprostaglandin E2 (15kPGE2) and the expression of paraoxonase2 (PON2). Wounding triggered proliferation and an increase in Cx26 expression, which returned to basal levels after wound closure, in both NCF and CF cells. Hes1 expression was shown to be unaltered during repair in NCF cells; however, in CF cells, its expression, and therefore Notch activation, was decreased 24h after wounding ($p < 0.05$). HPGD expression was also unaltered in NCF cells during repair; however, in CF cells, the basal levels were lower compared to NCF cells ($p < 0.05$). The size of Cx26 plaques revealed by IF was markedly decreased upon PPARg activation with 15-kPGE2 (7.25 ± 0.4 AU vs vehicle: 12.7 ± 1.1 AU; $p < 0.05$); however, PPARg inhibition with its pharmacological inhibitor GW9662 maintained Cx26 plaques (11 ± 0.5 AU). While Notch's pharmacological inhibition with DAPT had no effect on Cx26 expression or on its plaques formation, modulation of Cx plaques size by PPARg activator was more pronounced in the presence of DAPT (vehicle: 12.7 ± 1.1 AU; DAPT+15kPGE2: 0.03±0.02 AU, $p < 0.05$;). These results reveal that Notch and PPARg signaling pathways are deregulated in human airway CF cells. Moreover, they show that the expression of Cx26, which is a marker of repairing HAECs, is negatively regulated by PPARg activation. We suggest that Notch activation is crucial during the early phases of HAEC repair and that PPARg signaling pathway has a dominant effect on Notch to induce proper differentiation at later stages of the repair process. Altogether, our results show that Notch, PPARg and Cx26 are interrelated to finely tune the HAEC repair process. Since these pathways are deregulated in CF, future studies will evaluate if these defects alter the reestablishment of an integrated airway epithelium.

In vivo imaging of NF-kB activation in a mouse model of acute lung inflammation: effect of Azythromycin

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Azithromycin (AZM) is recommended in the treatment of chronic inflammatory diseases like diffuse panbronchiolitis, cystic fibrosis and COPD due to documented anti-inflammatory effects, although the mechanisms are still under investigation.
Our work aimed at comparing effects of this macrolide *in vivo* and *in vitro* on activation of NF-kB.
We utilized the CF airway cell line 16HBE14o-AS3. Expression of the pro-inflammatory markers dependent on NF-kB activation as tumor necrosis factor (TNF) a, interleukin (IL) 8, IL-6 and intercellular adhesion molecule (ICAM) 1 were evaluated by qPCR in cells treated with lipopolysaccharide (LPS) in the presence or absence of AZM.
We found that LPS 10ug/ml increases mRNA expression of IL-8, TNF-a, IL-6 and ICAM-1 respectively of about 2.8, 3, 2.7 and 3.4 folds (n=5, $p < 0.001$). Pre-treatment with AZM 10uM inhibited the LPS-induced increase of expression of IL-8, TNF-a and IL-6 of about 30%, 40%, 35% and 35% respectively (n=5, $p < 0.05$), while no effect on ICAM-1 induction was observed.
Next, *in vivo* bioluminescence (BLI) imaging of NF-kB activation was applied as a non-invasive read-out for AZM anti-inflammatory effect in a mouse model of LPS-induced lung inflammation. Luc induction was detectable as early as 2h after LPS challenge and peaked at 4 h after treatment, with a 10-fold induction over baseline. The signal dropped down at 24 h.
Pretreatment with AZM at 100-600 mg/kg resulted in 8 and 4.5 fold of induction at peak respectively, causing a 29-56% inhibition of Luc signal when compared with the LPS-control mice.
AZM reduced total white cells and neutrophil recruitment to the airways 24 h after LPS challenge with an efficacy comparable to that observed for the inhibition of the NF-kB BLI signal. A selected panel of cytokines up-regulated after LPS challenge were significantly down-regulated by AZM in a dose-dependent manner.
We have shown that AZM inhibits LPS-induced lung inflammation and this anti-inflammatory activity is correlated with the inhibition of NF-kB activation in the lung as detected by *in vivo* BLI. This approach offers new opportunities to non invasive measure the efficacy of anti-inflammatory compounds in a vast array of animal models of disease, including CF

The involvement of vascular endothelium in cystic fibrosis lung inflammation

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The vascular endothelium plays a key role in the inflammatory response. It, in fact, releases a number of mediators regulating leukocyte recruitment and extravasation as well as the vascular tone. Although we previously documented endothelial dysfunction in cystic fibrosis (CF) patients, whether endothelial cells are affected by the molecular defect of CF remains to be determined.
To address this issue, we first assessed CFTR expression and activity in human umbilical vein endothelial cells (HUVEC) as well as in pulmonary aorta endothelial cells (PAEC), using western blotting, confocal microscopy and YFP fluorescence microscopy. We then evaluated the impact of CFTR blockade in HUVEC and PAEC exposed to inflammatory cytokines and subjected to physiological shear stress to mimic in vivo conditions. Real-time video recording consistently showed that CFTRinh-172, a selective CFTR inhibitor, determined dramatic morphological changes of cell monolayers, fully evident after 25 min flow. These included non-apoptotic membrane blebbing, localized at the apical surface and the cellular edges, as well as shrinking, cell detachment from the substrate, dismantling of cell-cell contacts and widening of inter-endothelial spaces. This was associated with alteration of VE-cadherin and p120-catenin membrane distribution as well as with Paxillin accumulation in the bleb neck. Consistent with this, CFTR blockade stimulated the release of microparticles (MP) from endothelial cells. This observation paralleled the finding that CF patients presented increased number of circulating endothelial cells and of endothelial-derived MP. CFTR blockade also stimulated IL-8 release by HUVEC, while suppressing insulin-induced nitric oxide generation, likely as a consequence of reduced phosphorylation of endothelial nitric oxide synthase and AKT. Notably, agents that increase cAMP levels, in particular the combination of phosphodiesterase inhibitors and β2-adrenergic agonists, prevented a number of dysfunctional responses of endothelial cells to CFTR inhibition. Together, these results uncover novel mechanisms of CF lung inflammation as well as potential biomarkers and innovative therapeutic approaches to CF lung disease.

Does genotype influence on occurance of hyponatraemic hypochloraemic dehydration in children with cystic fibrosis

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Objective: Infants and young children with cystic fibrosis (CF) may be at increased risk of hyponatraemic hypochloraemic dehidration (HN HC DH) with metabolic alkalosis when they sweat excessively. The presence of severe CF transmembrane conductance regulator (CFTR) mutations are predisposed factors for the development of metabolic alkalosis with hypoelectrolytemia. The purpose of our study was to investigate the prevalence of this sweat-related metabolic disorder in our CF center and the influence of CFTR mutation on it.
Methods: We investigated the medical records of all children diagnosed with cystic fibrosis who are under follow-up in the CF center in Pediatric clinic of Ljubljana and were hospitalised due to hyponatraemic, hypochloraemic dehydration in the period from 2007-2012. Data analysis included clinical and laboratory features.

Results: A total of 4 children (7.2 %) from the Ljubljana CF center (55 patients in total, up untill their 19th birthday) were eligible for the study. All four were homozigous for the ΔF508 mutation. One boy has had two episodes of hyponatraemic hypochloraemic dehidration. In one girl the episode occurred before she was diagnosed with CF. Two children had episodes in summer (in common three episodes) in hot weather, two in autumn at respiratory exacerbation. Median age at hospitalisation was 7 months (range 4-34 months).
Conclusions: In our study dehidration with hypoelectrolytaemia occured only in children who were homozigous for the ΔF508 mutation. It might be possible that excessive ion transport in sweat glands and lost of chloride and potasium is more pronounced in this mutation and that children with this mutation are » physiologically« more susceptible for HN HC DH.
Vomiting and anorexia are warning signs and should alert parents and physicians to the possibility of this complication which can be prevented by proper hydration and salt replacement. If untreated, it can cause seizures, arrythmias and even respiratory failure.

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Whole genome sequencing provides evidence of within-patient evolution and adaptation to the lung of *Mycobacterium abscessus*

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Aim

Mycobacterium abscessus is an emerging cause of infection in patients with cystic fibrosis. It can be difficult to treat and consequently many patients develop chronic infection. Our aim was to look for evidence of within-patient evolution of *Mycobacterium abscessus*, using whole genome sequencing.

Methods

Whole genome sequencing (average depth of coverage 111 fold) was performed on 155 isolates of *Mycobacterium abscessus* from 22 patients with cystic fibrosis. Genetic diversity across the whole genome was analysed. Colony morphology was documented for all isolates and antibiotic susceptibility testing performed on a subset. Clinical data was collected for all 22 patients - including treatment regimens, lung function (FEV1), and markers of systemic inflammation (C-reactive protein).

Results

The genetic diversity between isolates (measured by pairwise SNP distance) from the same individual increased with the length of infection (r^2=0.78, p-value = 0.0007). Genetic diversity within samples (assessed by the number of minority variants) also increased the longer the individual had been infected (r^2=0.72, p-value < 0.0001).

We found that genetic diversity correlated with bacterial load (using time to liquid culture positivity as a surrogate). Peaks in genetic diversity and bacterial load correlated with clinical exacerbations (evidenced by increases in CRP and/or drop in FEV1). Conversely effective treatment was associated with a drop in genetic diversity and bacterial burden.

Phenotypically we saw evidence for within-sample diversity of both rough-smooth morphotype and antibiotic susceptibility and, as expected, found heterozygosity within genes associated with antibiotic resistance and colony morphology.

We also detected convergent evolution of *Mycobacterium abscessus* isolated from different individuals, with repeated accumulation of non-synonymous SNPs in a number of genes thought to control virulence.

Conclusions

We have demonstrated for the first time that the genetic diversity of *Mycobacterium abscessus* increases over the course of infection and may play an important role in the development of antibiotic resistance and modulation of virulence. This genetic diversity may also explain the lack of correlation between laboratory antibiotic susceptibility testing and the clinical response of patients to specific treatments.

Pseudomonas aeruginosa reduces the expression of CFTR in airways via post translational modification of NHERF1

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P. aeruginosa infections of the airway cells decrease apical expression of both wild-type (wt) and F508del CFTR through the inhibition of apical endocytic recycling. CFTR endocytic recycling is known to be regulated by its interaction with PDZ domain containing proteins (Swiatecka-Urban A. *et al.* 2002, Guerra L. *et al*. 2005, Kwon SH. *et al*. 2007, Bomberger J.M. *et al*. 2011). Recent work has shown that the PDZ domain scaffolding protein NHERF1 finely regulates both wt and F508delCFTR membrane recycling (Favia M. *et al*. 2010, Monterisi S. *et al*., 2012). Here, we investigated the effect of *P. aeruginosa* infection on NHERF1 post-translational modifications and how this affects CFTR expression in bronchial epithelial cells and in murine lung.

Infection reduced expression CFTR and, in parallel, increased phosphorylation and ubiquitination of NHERF1, as a consequence of both bacterial pilin- and flagellin-mediated host-cell interaction, both in bronchial cells *in vitro* and in mice *in vivo*.

The ability of *P. aeruginosa* to down-regulate mature CFTR expression was partially reversed a) in NHERF1 knockout mice *in vivo* and b) in bronchial epithelial cells *in vitro* after silencing NHERF1 expression or by testing NHERF1 mutants impairing its phosphorylation at serines 279 and 301. These studies provide the first evidence that NHERF1 phosphorylation in response to infection may negatively regulate the assembly and function of multiprotein NHERF1 complexes which are relevant to stabilize CFTR on the plasma membrane. The identification of the transduction signals downstream Toll-like Receptors that could be involved in NHERF1 phosphorylation could highlight novel targets to block the potential interference of *P. aeruginosa* on the therapeutic efficacy of potentiator and/or corrector compounds.

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Macrophage Migration Inhibitory factor (MIF) accelerated biofilm formation, a potential novel therapeutic target.

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Introduction: A major unmet clinical need in cystic fibrosis (CF) is defining novel mechanisms for accelerated bacterial biofilm formation which can then form the basis for focused innovative antibacterial therapies. In the context of *Pseudomonas aeruginosa* (*PA*), chronic infection leads to progressive lung destruction. Macrophage migration inhibitory factor (MIF) is a pro-inflammatory mediator with unique tautomerase enzymatic activity which has previously been shown to play a key role in driving an aggressive inflammatory phenotype in clinical disease.1,2,3

Aim: The aim of this project is to investigate the role MIF enzymatic activity plays in PA biofilm formation and the potential therapeutic efficacy of novel small molecular weight inhibitors targeting this enzymatic activity.

Method: Using the PA01 strain of *Pseudomonas aeruginosa*, biofilm formation with and without MIF (100ng/ml) was studied *in vitro* 96 well plate culture systems and flow cell system using a GFP tagged PA01. We also investigated the effect MIF had on antibiotic resistance of PA01 biofilms. qPCR was performed on RNA extracted from 24hr PA01 cultures with and without MIF (100ng/ml) to analyse genes involved in biofilm formation and cellular growth. We have established an animal model of bronchiectasis of PA01, to examine the effects that small molecular weight inhibitors, of MIF enzymatic activity, will have on infection and inflammation in the mouse lung.

Results: Results have shown that MIF significantly enhances biofilm formation of PA01 ($p < 0.01$) both in 96 well plates and pellicle formation. This MIF enhanced biofilm formation results in increased antibiotic resistance against Tobramycin (50µg/ml) ($p < 0.01$) in comparison to controls. In addition we have found a significant early induction of specific quorum sensing gene, AlgT, previously shown to be responsible for the conversion to a mucoid phenotype[4],($p < 0.05$). In the flow cell analysis of biofilms grown in the presence of MIF (100ng/ml) after 24hrs the structures were significantly larger in mass ($p < 0.05$) and more established than controls.

Conclusion:
We describe, for the first time, how the gram negative organism, *Pseudomonas aeruginosa,* hijacks a human cytokine, MIF, for its own survival advantage. Specifically, it accelerates biofilm formation contributing to enhanced antibiotic resistance. This work supports our contention that MIF inhibition represents a valid therapeutic target potentially as an adjunct therapy to maximize the effectiveness of antibiotics.

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P. aeruginosa-induced apoptosis in airway epithelial cells is mediated by gap junctional communication in a JNK-dependent manner

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The response of the cystic fibrosis (CF) airway epithelium to the opportunistic pathogen *Pseudomonas aeruginosa* is characterized by altered inflammation and apoptosis. Gap junctions (GJs) mediate horizontal communication between cells and are thought to contribute to a coordinated response of the airway epithelial cells to infection. Here, we examined innate immune recognition and epithelial responses at the level of the gap junction protein connexin43 (Cx43) in polarized human airway epithelial cells upon infection by PAO1 strain. We report that PAO1 activates cell surface receptors to elicit an intracellular signaling cascade leading to enhancement of functional expression of Cx43. The latter increased was not observed in cells infected with a PAO1 mutant strain lacking flagellin. Expression of Cx43 involved an opposite regulation exerted by JNK and p38 MAPKs. PAO1-induced Cx43 expression was reduced by inhibitors of p38 and strongly enhanced by JNK inhibition. As expected, PAO1 triggered a pro-inflammatory (evaluated by IL-8 release) and a pro-apoptotic (evaluated by cleaved caspase-3 and annexin V detection) response. In the presence of a JNK inhibitor, PAO1-induced airway epithelial cells apoptosis was increased. Interestingly, the latter effect was prevented by lentiviral expression of a Cx43-specific shRNA and by pharmacological blockade of gap junctional communication. Gap junction blockade, however, did not change PAO1-evoked IL-8 release. Thus, PAO1-induced apoptosis in airway epithelial cells is mediated by gap junctional communication and JNK signaling appears to act as a negative regulator of Cx43 expression. Moreover, we found that JNK activity was up-regulated by pharmacological inhibition of the cystic fibrosis transmembrane conductance regulator (CFTR). Conversely, restoration of CFTR function in a CF airway cell line (CF15 cells), by low temperature correction or adenoviral expression of CFTR, reduced the activation of JNK signaling. Finally, pharmacological inhibition of CFTR activity was associated with decreased Cx43 expression and reduced apoptosis. These results indicate that Cx43 expression is a component of the response of airway epithelial cells to innate immune activation by regulating the survival/apoptosis balance. Defective CFTR could alter this equilibrium with deleterious consequences on the CF epithelial response to *P. aeruginosa*.

Antiviral activity of azithromycin in cystic fibrosis airway epithelial cells

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Introduction. Virus-associated pulmonary exacerbations, most predominantly caused by rhinoviruses (RV), contribute to cystic fibrosis (CF) morbidity. However, there are only few therapeutic options to treat and/or prevent virus-induced CF pulmonary exacerbations. Recent evidence suggests that the macrolide antibiotic azithromycin (AZM) has antiviral properties in RV-infected primary human bronchial epithelial cells. We hypothesize that AZM induces antiviral mechanisms in primary CF bronchial epithelial cells.

Methods. Primary bronchial epithelial cells from 11 CF children (median [range] age: 8.21 [1.14-14.98] years) were pretreated with AZM and infected with the minor group virus RV1B. Viral RNA, interferon (IFN)λ2/3, IFNI3, IFN-stimulated genes (ISGs; oligoadenylate synthetase, MxA, viperin), RV1B surface receptor (low density lipoprotein) and pattern recognition receptors (PRRs; retinoic acid inducible gene I, melanoma differentiation associated gene 5, toll-like receptor (TLR)3, TLR2) expressions were measured by RT-qPCR. Pro-inflammatory cytokines and IFNI3 production were assessed by ELISA. Cell death was evaluated by Flow cytometry.

Results. After AZM pretreatment, RV1B load was 7-fold reduced in CF bronchial cells compared to untreated cells

(median [interquartile range] 10^6 copies/reaction: 8.13 [5.28-34.4] vs. 56.8 [18.4-81.5]; p=0.001). The decreased RV1B load was not due to AZM-induced cytotoxicity in CF bronchial cells. Furthermore, AZM pretreatment significantly increased RV1B-induced IFNs and ISGs, and also RV1B surface receptor and PRRs mRNA expressions in CF bronchial cells. Interestingly, while stimulating antiviral responses, AZM pretreatment did not significantly prevent virus-induced inflammatory cytokines production.

Conclusion. AZM pretreatment reduces RV load in primary paediatric CF bronchial epithelial cells *in vitro*, possibly through inducing a robust antiviral response including the induction of IFNs and ISGs. This study points to the potential of AZM as a novel therapeutic approach to treat and/or prevent RV-induced CF pulmonary exacerbations

Innate immune response of human bronchial epithelial cells to infection by *Aspergillus fumigatus*

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Introduction

Aspergillus fumigatus (*A.f*) is an opportunistic fungus that is frequently recovered in the sputum of cystic fibrosis (CF) patients. Inhalation of spores results in fungal growth inside the lung and hematogenous dissemination. Although it is generally accepted that alveolar macrophages phagocyte and kill conidia while neutrophils lyse hyphae of germinated spores, we demonstrated, in a previous study (Balloy *et al*., 2008), that respiratory epithelial cells (REC) can sense germinated spores of *A.f* and thereby be activated at least through two different pathways: i) a MyD88 independent one, and thus independent of most of Toll-like receptors, leading to IL-8 synthesis *via* PI3 kinase, p38 MAPK, ERK1/2, AP-1 and NF-kB activation, and ii) a MyD88 dependent one and involving NF-kB activation too. Here, we studied in more details those two pathways of the innate response induced by *A.f*.

Methods

The human bronchial epithelial cell line BEAS-2B was infected with *A.f* conidia. Cytokine concentrations in cell culture medium were determined by ELISA or multiplex analysis. To assess the role of different pathways, target proteins were knockdown using specific siRNA or their biological activities dampened by pharmacological inhibitors. siRNA efficiency was controlled by measuring target mRNA expression by Taqman PCR.

Results

Through a multiplex analysis of 27 inflammatory mediators, we observed that only IL-8, IL-6 and MCP-1 were significantly synthesized by *A.f*-stimulated REC and this independently of the MyD88 pathway as deduced from the use of specific siRNA.

We then looked for the involvement of Syk, a immunoregulatory protein tyrosine kinase involved in most antifungal responses. We observed that Syk was required (siRNA and piceatanol) for *A.f* -induced PI3 kinase, p38 MAPK, ERK1/2, NF-kB activation and cytokines synthesis. Whereas Syk activation is often associated with C-type lectin-like receptors such as Dectin-1, Dectin-2 or Mincle, we demonstrated here that Dectin-2 and Mincle were not expressed in our epithelial cell line. Concerning Dectin-1, involved in the recognition of *A.f* b-glucan by alveolar macrophages, it is expressed at a very low level in REC activated by *A.f* or not, and its knockdown did not modified cytokine synthesis.

Conclusions

The study demonstrates that Syk mediates kinases and NF-kB activations and cytokine synthesis in *A.f*-stimulated REC. This pathway is independent of MyD88, and does not involve C-type lectin-like receptors such as Dectin-1, Dectin-2 or Mincle.

Future studies will look for which receptor is involved in the recognition of *A.f* and cytokine synthesis. As filamentous fungi may contribute to the local inflammatory response, and therefore to the progressive deterioration of the lung function in CF, results could provide potential targets for designing therapeutic agents against *A.f* infection.

Pseudomonas aeruginosa eradicates Staphylococcus aureus in airways by manipulating the host immunity : a role for a host bacteridical protein

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Airways of young cystic fibrosis (CF) patients are mainly colonized by *Staphylococcus aureus* (SA) while *Pseudomonas aeruginosa* (PA) predominates in adults. However, the mechanisms behind this infection switch are unclear. The type-IIA secreted phospholipase A2 (sPLA2-IIA) is a host enzyme endowed with bactericidal activity. Here, we showed that sPLA2-IIA levels increased in expectorations of CF patients in age-dependent manner. These levels were sufficient to kill SA but had only marginal effects on PA strains. Recombinant human sPLA2-IIA efficiently killed SA clinical isolates collected from CF patients but had limited effects on PA isolates. The latter induced sPLA2-IIA expression by both cell lines and primary bronchial epithelial cells (BECs) isolated from CF patients. Using bacterial mutants we identified the PA virulence factor involved in the induction of sPLA2-IIA expression by BECs. Animal models of lung co-infection by SA and PA demonstrated the role of sPLA2-IIA in the selective airways clearance of SA. The presence of PA in airways enhanced SA clearance by increasing sPLA2-IIA production. We conclude that sPLA2-IIA induction in airways cells by PA may contribute to SA eradication in CF airways. This highlights a new concept suggesting that a bacterium can eradicate another bacterium by manipulating the host immunity.

Identification of TecA, the *Burkholderia cenocepacia* type 6 secretion system effector protein affecting eukaryotic cytoskeleton architecture

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The Type 6 Secretion System (T6SS) is a versatile weapon widespread among Gram-negative pathogens and symbionts. Some T6SS deliver toxins to kill or inhibit the growth of susceptible bacteria, while others have evolved to target eukaryotic cells. Deletion of *atsR*, a negative regulator of virulence factors in *B. cenocepacia* K56-2, increases T6SS activity. Also, macrophages infected with K56-2 *1atsR* display dramatic alterations in their cytoskeleton architecture with the formation of unusual “beads on a string-like” structures. This phenotype relies on the T6SS to affect the activation of multiple Rho family GTPases by an unknown mechanism. Systematic deletion of each gene within the T6SS cluster and *vgrGs* in K56-2 ΔatsR identified critical core components and accessory proteins of the T6S machinery but failed to uncover an effector protein affecting the host cytoskeleton. Screening of a K56-2 *1atsR* transposon library in our macrophage infection model identified a mutant with an insertion in BCAM1857, which was unable to elicit cytoskeletal rearrangements. BCAM1857 encodes a small protein of unknown function and is unique to *B. cenocepacia* species. *Burkholderia multivorans* ATCC17616 Δ*ats*R*BM* has an active T6SS but lacks a BCAM1857 homolog. While it was initially unable to induce cytoskeletal rearrangements upon macrophage infection, expression of BCAM1857 was sufficient to make ATCC17616 Δ*ats*R*BM* induce the formation of “beads on a stringlike” structures. Based on these findings we propose that BCAM1857 (named TecA) is the T6SS effector protein affecting the cytoskeleton architecture in macrophages infected by *B. cenocepacia*.

Polymicrobial interactions in in vitro biofilms of cystic fibrosis co-isolates *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Burkholderia cepacia* complex

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Chronic lung infections are the leading cause of morbidity and mortality in patients with cystic fibrosis (CF). It is known that the key CF pathogens *Pseudomonas aeruginosa* (PA), *Staphylococcus aureus* (SA) and *Burkholderia cepacia* complex (Bcc), when present in biofilms, are highly resistant to antibiotic treatments and are protected against immune responses in CF airways, but is unclear how they interact and respond to other biofilm bacteria to modulate the virulence of the whole consortium and contribute to pathogenesis. Therefore, to understand the dynamics of the interactions between these species, we examined a multispecies biofilm containing PA, SA and Bcc strains that were co-isolated from a single sputum sample from a CF patient. These isolates were used to grow biofilms in a drip flow reactor (DFR) under low-shear force close to the air liquid interface with a continuous flow (0.4 ml/min) of media at 37°C for 3- and 6-days, an d we compared the growth and morphology of individual isolates in mono-, dual- and multispecies biofilm using plate counts and SEM. The results showed that these isolates formed mono-, dual- and multispecies biofilms in the DFR, but their growth varied remarkably in 3 and 6 days-old mono-, dual- and multispecies biofilms. The growth of PA and SA was significantly increased in all combinations compared with their monospecies in 3-day-old biofilms, whereas the growth of PA and SA was significantly increased only in the combinations of PA+Bcc and SA+PA+Bcc, respectively, compared with their monospecies in 6-day-old biofilms. In contrast, growth of Bcc was significantly decreased in all the combinations compared with its monospecies in 3-and 6-day-old biofilms. SEM revealed that rods and cocci co-existed in close proximity in the dual- and multispecies biofilms. Furthermore, a mathematical model of pure resource competition was applied to bacterial growth log value data assuming that SA, PA and Bcc compete only for resources. In the multispecies biofilm, both SA and PA grew better than when they were growing singly, and Bcc grew very poorly in contrast to the predictions of the competition model, where slight reduction in the growth of PA and SA and better growth of Bcc was predicted. This deviation of the predictions of the competition model from the data results indicates that interactions between these bacterial isolates involve mechanisms other than the depletion of a single resource. In conclusion, our study suggests that the DFR can be used as an *in vitro* model to grow multispecies biofilms and study polymicrobial interactions of CF lung infections, and that interspecies interactions alter the behavior of bacterial species in a biofilm, most likely through a quorum-sensing mechanism. This mechanism may allow the bacteria to adapt, survive and persist during chronic infection in the lungs of CF patients. These insights may be important for understanding the role of interspecies interactions in promoting the persistence of these bacteria and in enhancing the virulence of the consortium in biofilms during chronic CF lung infections.

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Linking the CFTR to the Cystic Fibrosis Mucus Phenotype

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The basic defect in cystic fibrosis (CF) is well characterized, but the link between defects in the CF transmembrane conductance regulator (CFTR), the causative gene of CF disease, and the phenomenon of stagnant mucus is not well understood. It has been shown that the ileal mucus in CF mice adheres to the epithelium, is denser, and is less penetrable than that of wild-type mice and that apical addition of 115 mM NaHCO3 to mucus partially normalizes this mucus phenotype (1, 2). Using this knowledge of effects on ileal mucus, we develop an airway experimental set-up. In the airway, CFTR is localized apically not to MUC5AC-expressing goblet cells, but to the neighboring ciliated epithelial cells. This is similar to the small intestine where the MUC2 secreting goblet cells are localized adjacent to the CFTR expressing enterocytes. Using bead-tracking and mass spectrometry analysis, we study the effects of therapies on the mucociliary transport rate, Muc5ac and Muc5b secretion, and mucus clearance patterns in the bronchotracheal tree of both wild-type and cystic fibrosis porcine models as well as in rat airway explants. Scanning electron microscopy is used to visualize the properties of the mucus layer, and transmission electron microscopy is used to study mucin secretion in detail. Preliminary results suggest the importance of bicarbonate in the proper unpacking and secretion of mucins. We hypothesize that the restoration of bicarbonate to the apical surface of the epithelium in combination with osmolytes may induce proper mucin unpacking in CF epithelia, and therefore could relieve the mucus obstruction that causes clinical problems for cystic fibrosis patients.

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Primary human airway epithelial cells expanded with feeder cells and ROCK inhibitor for screening novel GSNO reductase inhibitors and CFTR correctors

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The current cell culture technology limits our ability to indefinitely expand primary human airway epithelial (HAE) cells while retaining their cell-specific phenotype and function. Controlled experiments of primary normal and cystic fibrosis (CF) -derived HAE cells determined that they retain primary culture morphology and functional properties for only a few passages (up to passage 2), thereby limiting the number of critical *in vitro* experiments performed. Therefore, the ability to markedly increase the supply of functionally relevant primary HAE cells derived from patients with various CF mutations (i.e. ΔF508, G551D, R117H, etc.) will allow for a significant increase in additional future studies designed to evaluate essential therapeutics for mutation specific CFTR correction or potentiation. Recent studies demonstrated that primary normal and tumor cells could be isolated and cultured in combination with irradiated fibroblast/feeder cells (mouse 3T3 fibroblasts, J2 sub clone) and an inhibitor of the rho kinase (ROCK) signaling pathway (Y-27632, "Y"). Under these co-culture conditions, cells were shown to proliferate indefinitely with characteristics similar to that of adult stem cells. Cells were then grown onto membrane porous cell supports in the absence of feeder cells and ROCK inhibitor, and shown to revert back/differentiate to their original cell phenotype. Utilizing this co-culture system, we have successfully expanded normal as well as CF-derived HAE cells from patients expressing the ΔF508 mutation (up to passage 6). These cells were seeded onto membrane porous inserts where they maintained an air/liquid interface, retained their cell specific morphology, and expressed functional ion transport characteristics similar to well-differentiated HAE cultures. These newly expanded and well-differentiated HAE cultures were examined histologically by hematoxylin and eosin (H&E) staining as well as alcian blue-Periodic Acid Schiff (AB_PAS) staining. Ussing chamber analyses had been performed using these newly expanded primary HAE cells (up to passage 6) to confirm retention of characteristic HAE ion transport properties. Moreover, we utilized these newly expanded cells for screening of our lead novel GSNO reductase inhibitors and CFTR correctors to identify their capacity to increase CFTR-mediated Cl^- transport. In addition, we have initiated studies for identifying changes in airway surface liquid (ASL) height using these newly expanded normal and CF HAE cells by confocal microscopy, another *in vitro* based model for screening lead novel compounds. Collectively, we anticipate that by expanding our supply of normal and CF-derived HAE cells, we significantly increase our ability to perform functional *in vitro* cell based screening assays designed to target CFTR corrective/potentiative therapies.

Abnormal mucus secretion in multiple organs of a F508del CFTR murine model

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Prevention and resolution of mucous obstruction in lung, pancreas, liver and intestine is considered a priority in the treatment of CF. To facilitate preclinical studies involving various experimental drugs, including correctors and potentiators, we have studied mucus secretion in different organs of our F508del CFTR mouse model ($Cftr^{eurtm1}$). Liver and pancreas obstruction are rare in this mutant strain (Wilke et al 2011). Lethal perinatal intestinal obstruction (meconium ileus equivalent) is observed in 10-50% of homozygous F508del mice on normal chow. Mucus secreted by intestinal explants of mutants of this strain is abnormally sticky (Hansson et al 2012). We show here that in 85% of the nasal cavities of homozygous F508del mutant mice (N=12) abnormal secretions (mucous blebs) in the ciliated airway epithelium are present that appear to originate from hyperplastic goblet cells, whereas this was observed in only 10% of wild type littermates mice (N=12/15). Therefore abnormal secretions are common in F508del CFTR mutant mouse upper airways. The mouse lung bronchi are lined with clara- and ciliated cells, similar to distal bronchioles in human. In unchallenged normal and F508del CFTR mutant mice, mucus plugging and air-trapping are not evident in microCT scans of unchallenged mutant mice under isoflurane anesthesia (N=20), only few goblet cells are observed in the proximal bronchi. However, mutant mice (BL/6) do show a twofold higher Muc5AC content in lavage compared to wild type littermates (N=11/8, $P< 0.01$). During repair of naphtalene induced airway injury we observe a tenfold higher induction of the goblet cell marker Gob5/Clca3 mRNA in mutant mice compared to normal (N=7/7, $P< 0.001$), confirmed by immunohistochemistry. This reflects a strong tendency of CFTR deficient murine airway progenitor cells in situ to differentiate towards a mucus secreting phenotype under stress, likely related to the development of goblet cell hyperplasia observed in human and pig CF airways. The molecular basis of this phenomenon is under study. The mouse salivary glands are of particular interest because their CFTR dependent secretion activity is severely compromised in F508del CFTR mutant mice (Wilke, 2011). Using the 3G11 antibody, and using CFTR KO^{cam} mice as control we could show that CFTR is expressed primarily in the ductal and serous epithelial cells in wildtype mice, whereas this was severely reduced in F508del mutant mice. Histological analysis (PAS/AB) of the three major glands and showed for the first time that mucous plugging of the parotic gland ducts was very frequent (50 +/- 10 % of all duct cross sections) in F508del mutant mice whereas this was not observed in wild type littermates ($P< 0.01$, N= 10). In the submandibular and sublingual glands this was not observed, consistent with a much less intense staining for charged mucins in the mucous acini of these glands. We conclude that the F508del CFTR mouse model shows a quantifiable multi-organ mucus secretion defect, similar to the human condition, that can be used to study the etiology of this of CF-related pathology and potential therapeutic interventions.

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VX-809 and VX-770 treatment in rectal CF organoids expressing various CFTR genotypes

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Introduction: The potentiator VX-770 (Ivacaftor), registered for CF patients carrying CFTR-G551D, is now clinically being assessed for non-G551D CFTR mutants. We have recently developed an assay that quantitates residual CFTR function and efficacy of CFTR-restoring drugs in primary intestinal organoids from CF individuals. Here, we used CFTR-function measurements in organoids derived from over 20 CF patients to (i) identify CFTR alleles that benefit from VX-770 and VX-809 and (ii) compare function of drug-restored mutant CFTR to wild-type CFTR.

Methods: We measured CFTR activity with or without CFTR restoration by VX-809 and VX-770 in organoids expressing class I to V CFTR mutations, including stop, F508del, N1303K, A455E, S1251N, R117H and (TG)13(T)5. Two different CFTR-dependent readouts were used: (i) rapid forskolin-induced swelling (FIS) of organoids and (ii) steady-state organoid lumen size that have dynamic ranges at low or high CFTR function, respectively. The steady-state organoid lumen size during culture (without forskolin addition) discriminates clearly between healthy control (cystic, big lumen) and CF (not cystic, small lumen), and increases in CF organoids upon overnight treatment with CFTR-restoring compounds.

Results: Using the FIS assay, we observed a dose-dependent relation between rapid organoid swelling and forskolin concentration, which is different for organoids expressing alleles ranging from class I to V. VX-770 treatment enhanced swelling in all class II-V expressing organoids, while VX-809 treatment was effective for specific mutations. No FIS or enhanced swelling by these drugs was observed in class I expressing organoids. Lumen sizes of CF organoids upon overnight incubation with restoring drugs indicated that some mutants are restored up to levels of organoids expressing two wild-type CFTR alleles, whereas others are restored below or up to levels of F508del/wild-type organoids.

Conclusion: CFTR genotype-specific responses to VX-809 and VX-770 treatment can be detected using organoid-based CFTR function measurements. Organoid-based assays may therefore be used to identify patients that benefit from existing and newly developed CFTR-targeting drugs.

Approaches to invalidate CFTR in human airway epithelial cells

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In vitro models of the human airway epithelium are vital for Cystic Fibrosis research. Here, we describe approaches to invalidate *CFTR* expression in the Calu-3 epithelial cell line and in primary human airway epithelial cells (HAECs) by RNA interference and site-specific gene modification. In a first strategy, we used shRNA sequences targeting the CFTR mRNA (shCFTR). Efficiency of three shCFTR sequences, alone or in combination, was assessed in transduction and co-transduction experiments in Calu-3 cells using lentiviral vectors (LVVs) co-expressing the green fluorescent protein (GFP). The best efficiency was obtained with one particular combination of two shCFTR sequences. These were used to generate two Calu-3 cell lines knocked-down for *CFTR*. Two Calu-3 cell lines expressing a control shAlter were also generated. CFTR mRNA and protein expressions as well as variations of the transepithelial currents in Ussing chamber were measured in polarized GFP-positive Calu-3 cells grown on Transwell filters. *CFTR* knock-down (70% inhibition) was observed at the post-transcriptional and post-translational levels coupled with a 20% decrease of the transepithelial current response to the CFTR inhibitor GlyH101 following cAMP-induced activation in both shCFTR cell lines. In view of these results, we applied this strategy to primary HAECs. Cells from three non-CF patients were transduced with the identified combination of LVVs, FACS sorted and then grown at the air-liquid interface for at least 30 days. The average of GFP-positive sorted cells was 67%. Normal transepithelial resistance values and differentiation to a ciliated airway epithelium was obtained in two cultures out of five. Analyses are still being performed to assess CFTR mRNA and protein levels but Ussing chamber data did not show variations of the transepithelial current responses. In a second strategy, we are investigating the consequences of site-specific gene modification using the CRIPSR/Cas9 system. Two target sequence-guide RNA structures were designed to induce double-strand breaks in the region of *CFTR* exon 2 and were then vectorized in a LVV alongside the Cas9-GFP fusion protein cassette. These tools were first validated in 293T cells. Their efficiency is now being evaluated in Calu-3 cells transduced at different MOIs and FACS sorted. Analyses of the resultant cell lines are ongoing to determine the gene modification activity and its effects on the CFTR mRNA and protein levels before further trials on primary HAECs. In summary, we have developed two new Calu-3 cell lines knocked-down for *CFTR* by co-transduction of two shCFTR LVVs. The characterisation of these cell lines regarding the signalling pathways involved in chemokine secretion, proliferation and migration is underway. However, the shRNA approach was not efficient to knock-down *CFTR* in primary HAECs.
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Dysbiosis of the fecal microbiome in cystic fibrosis transmembrane conductance regulator deficient mice

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Background and aims:
Dysbiosis of the gut microbiota has been recognized as an important contributor in many diseases, affecting the immune state and metabolic homeostasis, components that are altered in cystic fibrosis (CF) patients. In addition to lung and pancreatic disease, CF patients can develop an intestinal disease which includes obstructions, intestinal inflammation, small intestinal overgrowth, malabsorption and malnourishment. Mouse models of cystic fibrosis have been created that develop intestinal disease, including the *Cftr*^{tm1UNC} model which has a null mutation in *Cftr*. A limited number of studies in CF patients have indicated dysbiosis of the microbiota of fecal samples, which includes decreased diversity and altered prevalence of specific bacteria. However, the composition of the fecal microbiome has not been investigated in CF mouse models. This study was undertaken to characterize the fecal microbiome in BALB/c *Cftr*^{tm1UNC} mice.

Methods:
At 12 weeks of age, fecal samples from BALB/c *Cftr*^{tm1UNC} mice and wildtype littermates were collected and fecal bacterial DNA was isolated from 50mg of the sample. The fecal microbiome was characterized by 454 pyrosequencing of the V4-V6 region of the 16S rRNA gene and analyzed using an Operational Taxonomical Unit (OTU) approach.

Results:
In fecal samples from both CF and wildtype mice, the majority of the identified bacteria were from the phylum Bacteroidetes and Firmicutes, with bacteria in the phylum of Verrucomicrobia, Proteobacteria, Actinobacteria, Tm7 and Tenericutes also detected. The relative abundances of specific individual OTUs were significantly different between CF and wildtype littermates including 20 of the 50 most prevent OTUs. These OTUs corresponded to groups of *Bacteroides*, Porphyromonadaceae, *Blautia*, *Anaerostipes*, Alphaproteobacteria, Lachnospiraceae, *Clostridium*, Erysipelotrichaceae, Proteobacteria, *Bifidobacterium* and *Alistipes*. In addition to changes in bacterial groups, the overall bacterial community structure was altered with CF mice having a significantly lower number of observed OTUs and reduced diversity compared to samples from littermates. Finally, Bray Curtis dissimilarities between fecal samples were measured which caused samples from CF mice to cluster separately from those of wildtype mice.

Conclusions:
The feces of CF mice, when compared to wildtype littermates, presented with reduced diversity and altered relative abundances of specific bacteria. Thus, this data suggests that CF mice, similarly to CF patients, have dysbiosis in the fecal microbiome, increasing the usefulness of this model.

The FXR-FGF15 axis is impaired in mice with a deficiency for CFTR

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Background: With the improved treatment of the pulmonary complications of cystic fibrosis (CF), gastrointestinal problems are rapidly becoming the major cause of morbidity and even mortality in CF. The gastrointestinal disorders include: fat malabsorption, CF related liver disease (CFLD), impaired growth, and impaired reabsorption of bile acids.
The FXR-FGF15 axis is an important pathway in the regulation of bile acid homeostasis as well as for liver growth and regeneration. Liver regeneration after partial hepatectomy is impaired in mice in which either the nuclear receptor FXR (FXR KO mice) or Fibroblast growth factor 15 (FGF15 KO mice) is inactivated.
Hydrophobic bile acids such as cholic acid (CA) are strong activators of the FXR-FGF15 axis. Previously we found that chronic CA administration induces a liver growth and proliferation response in controls that is absent in cystic fibrosis (CF) mice, suggesting that the FXR-FGF15 axis is impaired in these mice. (unpublished data)
Methods: Here we determined whether the effect of dietary CA supplementation on liver growth is dependent on the presence of FXR in the intestine. We determined liver growth upon dietary CA supplementation in tissue specific intestinal FXR -/- and control mice. Intestinal (villin-CRE) specific FXR knockout (iFXR$^{-/-}$) mice and wild-type (WT) littermates (n=7, age 9-12 weeks) were administered a semisynthetic, low fat diet (AB 4063 02) supplemented with 0.5% of cholic acid (CA) for 15 days. After this period mice were sacrificed, body and liver weights were measured and hepatic lipid composition was determined.
Results: After 15 days of CA treatment there was no difference in body weight between WT and iFXR$^{-/-}$ mice (23.0 vs. 22.8 g respectively; NS). There was a significant increase in liver weight in WT compared to iFXR$^{-/-}$ mice (1,4 vs. 1,2 g respectively; P=0.03). Body to liver weight ratio in WT was significantly higher compared to iFXR$^{-/-}$ mice (0.07 vs. 0.06 respectively; $p< 0.001$). The hepatic triglyceride content was equal between WT and iFXR$^{-/-}$ mice (5.3 vs. 5.4 µmol/g of liver respectively; NS).
Conclusion: Intestinal FXR is essential for liver growth in response to dietary CA exposure. The liver proliferation upon CA administration was, likewise as in CF mice, missing in intestinal FXR -/- mice. CA induced liver growth is not due to changes in hepatic lipid composition. Current results underline the important role of intestinal FXR in the regulation of bile salt metabolism and the hepatic response to prolonged hydrophobic bile salt exposure. Our data suggests that intestinal FXR signaling is impaired in CF mice which may be critical in the development of gastrointestinal complications of CF patients. Our current studies are directed to understanding the mechanism by which CFTR modulates the FXR-FGF15 axis and ultimately in improving the gastrointestinal complications of CF patients.

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MiRNAs: New targets for CFTR restoration?

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- P.Phe508del is the most common mutation in CF patients and results in the absence of almost all CFTR proteins in cell membrane. However when the p.Phe508del protein reaches the membrane, the channel is active.
- Our group is interested in the identification of regulatory elements that control the CFTR mRNA level. Characterization of miRNAs acting only in lung adult cells allowed us to envision new therapeutic tools. We used an experimental model based on reconstituted airway epithelium from biopsy of nasal cells (from patients and

controls). This model allows to test molecules for the correction of CFTR mutation consequences. So, we treated p.Phe508del homozygous airway epithelium with locked nucleic acids oligonucleotides (LNA) which specifically block binding of miR-101, miR-145 or miR-384 to the CFTR mRNA.
- Treatment with these oligonucleotides led to an increase in the CFTR mRNA and CFTR protein levels. In CF bronchial cell lines (CFBE cells, p.Phe508del homozygous), these oligonucleotides restored the CFTR channel activity. In parallel, from the same CF and non-CF airway epithelia, we assessed the miRNAs expression pattern. We highlighted the overexpression of five miRNAs in CF patients with one that affect the CFTR 3'UTR stability. We will elucidate their other targets of these miRNAs.
- Finding new regulatory players controlling the CFTR mRNA level and/or involved in CF physiopathology help us to envision new tools for CF therapy.

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Chromatin remodeling mediated by the FOXA1/A2 transcription factors activates CFTR expression in intestinal epithelial cells

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The forkhead box A transcription factors, FOXA1 and FOXA2, function as pioneer factors to open condensed chromatin and facilitate binding of other proteins. We showed previously that these factors are key components of a transcriptional network that drives enhancer function at the cystic fibrosis transmembrane conductance regulator (CFTR) locus in intestinal epithelial cells. The CFTR promoter apparently lacks tissue-specific regulatory elements and expression of the gene is controlled by multiple cis-acting elements, which coordinate gene expression in different cell types. Here we show that concurrent depletion of FOXA1 and FOXA2 represses CFTR expression and alters the three-dimensional architecture of the active locus by diminishing interactions between the promoter and intronic cis-acting elements. Reduction of FOXA1/A2 also modifies the enrichment profile of the active enhancer marks H3K27ac and H3K4me2 across the CFTR locus and alters chromatin accessibility at individual cis-elements. Moreover, loss of FOXA1/A2 suppresses the recruitment of other members of the transcriptional network including HNF1 and CDX2, to multiple cis-elements. These data reveal a complex molecular mechanism underlying the role of FOXA1/A2 in achieving high levels of CFTR expression in intestinal epithelial cells.

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Genetic and functional correction of the human ΔF508 CFTR locus using designer nuclease technology

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Considerable effort has been invested to develop a sustainable gene therapy for Cystic Fibrosis. Most of these approaches have been based on transfer of a normal *CFTR* gene copy to therapeutically relevant cell types. Another promising, alternative strategy is the targeted *in situ* correction of the mutated *CFTR* locus using designer nucleases, such as zinc-finger nucleases (ZFN).

In this study, we aimed at correcting the ΔF508 mutation using the ZFN technology in combination with an appropriately designed donor DNA to correct the mutated *CFTR* locus via homologous recombination. Activity of the *CFTR* exon 10-specific ZFN was verified by employing cleavage assays *in vitro* and *in cellula*. Human bronchial epithelial cells (CFBE41o$^-$), which are homozygous for the ΔF508 mutation, were nucleofected with ZFN expression vectors and a donor DNA carrying a *CFTR* super-exon encoding exons 10-24 and a puromycin resistance cassette. PCR-based genotyping confirmed targeted integration of the super-exon into *CFTR* exon 10 in the selected cell population. Subsequent clonal analysis revealed a gene targeting frequency of 8%. All genetically corrected clones carried a monoallelic targeted integration of the super-exon, reflecting a heterozygous genotype. Furthermore, corrected clones showed donor specific *CFTR* mRNA expression, as evaluated by semi-quantitative RT-PCR.

Finally, one gene corrected CFBE41o$^-$ clone has been evaluated for restored CFTR function. Ion transport measurements in Ussing chamber measurements confirmed functional transepithelial characteristics when activated with cAMP and inhibited via CFTRInh172, indicative of restored CFTR channels.

In conclusion, our data demonstrate that designer nucleases are a powerful tool to genetically and functionally correct mutations in the CFTR model cell line CFBE41o$^-$. This proof of principle study will pave the way for subsequent genome editing strategies in patient-derived stem cells for future therapeutic applications.

Strategy to correct >80% of CF-causing mutations using CRISPR/Cas9 and mini-gene constucts

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Correction of individual CF-causing mutations requires homologous recombination (HR) between target gene and a donor molecule containing the correct sequence. Repair efficiency is substantially increased when a double-stranded break (DSB) is created close to the target mutation, using site-specific nucleases such as ZFNs, TALENs or CRISPR/Cas9. We have previously described the correction of the ΔF508 mutation in human cell lines using ZFNs (Lee et al., 2012).

In this study we describe use of the CRISPR/Cas9 system (Mail et al., 2013) to correct CF mutations. The specificity of this system derives from the Watson-Crick base-pairing of a 20-mer guide RNA (gRNA) which recruits the Cas9 nuclease to create the DSB. We designed a gRNA expression vector (pGUIDE) which allows cloning of two 20-mer oligos encoding the gRNA in a single step.

Our first gRNA was designed to create a DSB in exon 10 approximately 90 bp upstream of the ΔF508 mutation. Cells were co-transfected with this expression vector, a donor plasmid and a Cas9 expression plasmid, and DNA repair measured 72 hours later by deep sequencing analysis. Correction of the ΔF508 mutation was observed in 2.8% of alleles without selection. A similar level of repair was also observed in cells treated with our ZFNs and the same donor plasmid. Deep sequencing analysis revealed that the repair extends to ~100 bp either side of the DSB suggesting this approach could correct any of the ten other mutations that occur in exon 10.

As this direct repair approach is limited to correcting mutations in exon 10, we have developed a mini-gene recombination strategy with the potential to repair mutations anywhere in exons 10-24. The mini-gene approach involves the incorporation of exons 10-24 as a partial cDNA sequence preceded by a splice acceptor. If correctly recombined into intron 9, then the mini-gene should splice to the endogenous exons 1-9. This strategy has previously been used to replace exons 2-8 of the F9 gene to restore haemostasis in haemophilic mice (Li et al, 2011).

We have constructed two mini-gene constructs, one to be used with existing ZFNs which cleave in intron 9, 203 bp upstream of ΔF508 and one to be used with a novel gRNA targeting a region towards the centre of intron 9. In cells where recombination has occurred, we have designed our donors such that they will express GFP, thus allowing quantification of repair.

Successful gene correction using a CFTR mini-gene would result in normal spatiotemporal expression of the corrected CFTR gene, is permanent for the lifetime of the cell and is not subject to gene silencing and therefore offers a number of advantages over the conventional cDNA addition strategy for gene therapy.

New drug delivery system in treatment of Cystic Fibrosis using siRNA knock-down of the Aha-1 gene

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Introduction: Cystic fibrosis is a genetic disease with mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. The most common mutation is the deletion of F508, which causes a misfolding of the CFTR protein and thereby a degradation in the ubiquitin-proteasome-system in the cell. Earlier research has shown that inhibition of the hsp90 co-chaperone, Aha-1, locates the misfolded CFTR to the plasma membrane, where the function of the channel is restored (Sun et al, 2008). Our aim is to use the RNA interference (RNAi) mediated by 22 nucleotides long small interfering RNAs (siRNAs) inside the cell to degrade mRNA with complementary sequences. In this way siRNA targeted against Aha-1 mRNA will interfere with Aha-1 protein expression and restore CFTR in the plasma membrane.

Methods: To achieve this goal both *in vitro*, *ex vivo* and *in vivo* experiments are carried out. The CuFi-1 cells (Cystic fibrosis cell line with del F508 mutation) are seeded out in 12-wells plates and transfected with particles composed of siRNA formulated in different types of chitosan. RNA isolated from the cells are analysed with qPCR and a knock down is registered. To set up an *ex vivo* experiment primary lung epithelial cells from healthy pigs are harvested and seeded out on semipermeable membranes and transfected with chitosan/siRNA particles.

To optimize delivery and lung distribution in a human-like anatomic setting we established a nanoparticle delivery protocol for pigs. A pig model (60kg) was established where particles are distributed into the lungs of an anaesthetised pig (20mL/h Fentanyl and 15mL/h Propofol i.v.) by the use of an ultrasonic nebuliser attached to the ventilator. 5mL 1:100 Infrared 715/755 Fluospheres (Life Technologies, #F8799) were injected as described above to investigate the distribution method of the ultrasonic nebuliser. After killing the pig (4mL/kg Pentobarbital), lungs are taken out and kept on ice prior to scanning in an In Vivo Imaging Scanner (IVIS).

Chitosan particles are known to promote mucus adhesion and efficient knockdown has been demonstrated in a mouse model (Howard et al. 2006; Nielsen et al. 2010). To move this delivery platform to the pig model we are currently performing a comparative in vivo study where biodistribution of chitosan formulated Cy7-labelled siRNA is studied over time in the pig.

Results: A knock down of Aha-1 is seen in the CuFi-1 cell line and validation of knockdown in primary lung epithelial cells is currently ongoing. Fluorescent nanoparticles are observed to be distributed throughout the lungs by the use of the ultrasonic nebulizer and to a better extent when using a catheter to inject intra tracheal. This distribution method works very well and is easy to use in the anaesthetised pig.

Conclusion: A highly efficient Aha-1 target for siRNA knockdown has been established *in vitro* and an air-liquid interphase cell culture system has been established for primary lung epithelial cells. An efficient treatment protocol for siRNA delivery to pig lung has been established and RNAi experiments are in progress.

rAAV2/5-based gene therapy model for cystic fibrosis

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Introduction: Gene therapy holds promise for the treatment of CF in a curative, mutation independent manner. Viral vectors exploit the natural mechanism of viruses to enter and persist in the host cell and therefore are efficient gene transfer agents. In this study, we aimed to develop a mouse model for CF gene therapy using adeno-associated viral vector serotype 5 (rAAV2/5), which efficiently transduces the respiratory epithelium.

Results: To cure genetic disorders by gene therapy, life-long correction and thus stable and efficient gene expression is required. In a first step, we developed a generic mouse model for pulmonary gene transfer using rAAV2/5. To achieve long-term gene correction in actively dividing tissue, such as the airway epithelium, repeated administration of a non-integrating rAAV vector is required. We hypothesized that an immune response against the vector particle would be prevented following perinatal (i.e. fetal or neonatal) gene transfer due to the immaturity of the immune system, allowing repeated administration(s) in adult life. We administered rAAV2/5-fLUC to fetal and neonatal mice by intra-amniotic injection and intranasal instillation, respectively, which resulted in a comparable BLI signal emerging from the lungs (3.4 ± 0.6 E+06 p/s and 1.5 ± 0.3 E+06 p/s, respectively). However, the luciferase signal decreased 5-fold over the first 3 months post-injection. Low immunoreactivity (i.e. low level neutralizing antibodies (nAb) against the vector particle) allowed successful readministration and resulted in stable gene expression up to 6 months. As a control, we studied stability of gene expression in an adult setting, where a single vector dose was sufficient to obtain stable gene expression in the lungs up to 6 months. However, in this group higher titers of nAb were observed compared to the fetal or the neonatal group.

In a next step, we aimed to translate the reporter gene model in a therapeutic model for CF. First, we had to overcome the size limit of rAAV (~ 5 kb). Therefore, we functionally validated a truncated CFTR construct with a partial deletion in the regulatory domain (CFTRΔR) described by Ostedgaard and colleagues (*1*). Upon stimulation with forskolin and genistein, HeLa cells overexpressing CFTRΔR showed an efflux of $^{125}I^-$ which was comparable to WT CFTR. Next, we cloned the shortened CFTRΔR cassette (including a truncated CMV promoter and minimal poly-adenylation signal (*1*)) into our AAV transfer plasmid. We obtained vector titers comparable to control rAAV2/5 vectors. rAAV2/5-CFTRΔR was shown to be functional on the mRNA level by qPCR in transduced HEK293T cells.

CONCLUSIONS AND FUTURE PERSPECTIVES: Using reporter genes we obtained long-term gene expression in the murine airways after initial fetal or neonatal rAAV2/5 administration combined with successful readministration, as well as upon single vector administration in an adult setting. This allows translation to a gene therapy model for CF using rAAV2/5-CFTRΔR.

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Novel correctors of F508del-CFTR are additive to VX-809 in primary cultures of human lung cells homozygous for F508del or A561E

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Background:

The most advanced investigational drug to treat CF patients with F508del-CFTR is VX-809 (Vertex), a molecule that works by rescuing F508del-CFTR from its abnormal intracellular localization to the cell surface probably by binding to the NBD1:ICL4 interface, a critical contact site in CFTR structure[1]. A second pocket in F508del-CFTR vs wt-CFTR was nevertheless postulated[1]. Although VX-809 exhibited ~25% rescuing efficacy in CFTR activity *in vitro*, there was no significant effect on lung function in Phase II clinical studies. Therefore a compound correcting F508del-CFTR with an additive profile to VX-809 would be of significant impact on the majority of CF patients.

Aim:

To assess the effects of novel F508del-CFTR correctors (B9, E12, Sygnature Discovery) and their additivity to VX-809. Ultimately, these compounds may be explored in triple drug combination therapy (two correctors and one potentiator).

Methods:

The effects of 2 novel correctors (B9, E12) and their additivity to VX-809 were investigated in F508del-CFTR-transduced CFBE cells and primary cultures of human bronchial epithelial cells F508del- or A561E-homozygous patients by measurements of genistein-inducible equivalent short-circuit current $_{(\Delta Ieq\text{-}SC\text{-}Gen)}$ in Ussing chamber. Monolayers transepithelial resistance was (±SD) 1550±40 0/cm^2.

Results:

Data for $_{\Delta Ieq\text{-}SC\text{-}Gen}$ in F508del-cells were (mean ±SD μA/cm^2): 0.836±0.173 (VX-809,n=3); 0.275±0.013 (B9,n=3); 0.461±0.095 (E12,n=3); 0.103±0.084 (DMSO,n=3). Additivity of novel compounds with VX-809 were (mean $\Delta I_{eq\text{-}SC\text{-}Gen}$ ±SD μA/cm^2): 1.47±0.233 (B9,n=3); 2.77±0.846 (E12,n=3), the latter is statistically significant vs VX-809 alone.

For A561E-cells, $_{\Delta Ieq\text{-}SC\text{-}Gen}$ were (mean ±SD μA/cm^2): $_{\Delta Ieq\text{-}SC\text{-}Gen}$ were (mean ±SD μA/cm^2): 1.28±0.205 (VX-809,n=4); 1.25±0.105(B9+VX-809,n=2); 1.137±0.13 (E12+VX-809, n=3); 0.174±0.025 (DMSO, n=3). Neither B9 nor E12 were significantly additive to VX-809.

Conclusions:

These data show that: 1) A561E is rescued by VX-809; 2) B9 and E12 rescue F508del-CFTR activity to a lesser extent than VX-809; 3) E12 significantly adds to VX-809 in rescuing F508del-CFTR. E12 seems to rescue F508del-CFTR by a different mechanism of action than VX-809. It thus has potential to be further developed into a corrector drug.

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Identification of novel, efficacious F508del CFTR correctors to treat cystic fibrosis

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Background: Cystic fibrosis (CF) is a multi-faceted disease caused by dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR). The most common mutation, found in 90% of CF patients, is a deletion of phenylalanine at position 508 (*F508del*-CFTR). The correctors currently in clinical development targeting CFTR processing may be limited in effectively treating severe CF airways disease. Therefore, discovery and development of more efficacious correctors of *F508del*-CFTR continues to be a critically important goal of the CF community. N30 Pharmaceuticals has developed and begun clinical testing of S-nitrosoglutathione reductase (GSNOR) inhibitors to treat CF patients. As part of our continued effort to find a cure for CF, we set about to discover new, targeted *F508del*-CFTR correctors with improved efficacy to be used alone or in combination with our potent GSNOR inhibitors. In this study we describe new developments in our CFTR corrector program.

Methods: We utilized SAR analysis and high throughput screening (HTS) to design and synthesize novel *F508del*-CFTR correctors. The new non-GSNOR inhibitor compounds were studied in cell-based systems. Fischer rat thyroid (FRT) cells stably expressing both YFP and *F508del*-CFTR and CFBE 41o- cells (CFBE-dF) transiently transfected with YFP and *F508del*-CFTR were used for HTS assessment of compound activity. Cells were incubated for 24 h in the presence of test compounds or vehicle controls. CFTR activity was measured in response to the addition of 20 µM forskolin and a CFTR potentiator (P3, 3 uM, CFFT), and YFP quenching by iodide influx was measured. Data were obtained for n = 5 replicates for each condition. Western blots measuring “B to C-band” maturation in FRT and HeLa cells expressing *F508del*-CFTR were used to confirm the corrector activities. Moreover, Ussing chamber short-circuit current (I_{sc}) assays were performed using CF human airway epithelial cells to assess functional correction of CFTR Cl^- channel activity. Pharmacokinetic and toxicology assessments also were conducted to explore drug metabolism and safety profiles.

Results: In the YFP based assays, several novel correctors have been identified. The lead compounds had EC_{50}s of < 10 nM, which represents a greater than 10-fold improvement compared to the most potent correctors currently available. Western blot and I_{sc} assays have confirmed the efficacy and potency of these novel correctors. The lead correctors also have demonstrated excellent ADME/PK and safety profiles in exploratory studies.

Conclusions: SAR and HTS assessments in YFP based assays resulted in the identification of highly efficacious, novel *F508del*-CFTR correctors. These correctors induced *F508del*-CFTR maturation in cell lines, correction in human airway epithelial cells, and exhibited acceptable PK and safety profiles. Further characterization of the lead compounds as potential drug candidates is ongoing, as are combination studies with N30 Pharmaceutical's lead GSNOR inhibitors.

OligoG normalizes the CF mucus phenotype

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Cystic fibrosis (CF) is a recessive genetic disease caused by nonfunctional chloride and bicarbonate ion transport via CFTR. In the lungs of these patients, dense, intractable mucus collects because it is insufficiently cleared by the mucociliary clearance system, something that leads to lung infections and lung damage.

We have previously shown that the gel-forming MUC2 mucin is packed in an orderly way that allows unpacking by calcium removal and raised pH (1). Using an explant system (2), we found that the mucus of the small intestine in mice without a functional CFTR channel (CftrΔF508) is, in contrast to normal mucus, attached to the epithelium. This phenotype could be reverted to a non-attached phenotype by apical solutions containing about 100 mM bicarbonate (3).

OligoG CF-5/20 (AlgiPharma, Sandvika, Norway) is a natural product derived from brown algae alginate comprised of mainly guluronate oligomers, with average length of 13 monomers. Previous studies have shown that this oligomer alters the rheology of mucin/alginate gels, mucin/DNA gels and CF sputum. Because of this, OligoG is being tested as an inhalation therapy on CF patients. We have now tested OligoG on mouse ileum CF mucus. Explants from the small intestine of CftrΔ508 mutant mice were mounted in the horizontal Ussing-type chamber (2). OligoG (1%, 1.2%, 1.5%, 2%, 3% or 6%) was added to the apical buffer, pH of 7.4. The attachment of the already formed mucus was assessed by comparing the total mucus thickness before and after aspiration. OligoG at 1.5% or higher transformed the mucus to a non-adherent normal phenotype without increase in mucus thickness. At 1% OligoG the mucus remained attached and at 1.2% an intermediate phenotype was observed. The effects are likely due to OligoG's known ability to chelate calcium. These observations represent an indication of how OligoG could work in cystic fibrosis patients through a normalization of mucus layers in both the gut and potentially the lungs, at a concentration that could be achieved.

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The dual-acting small-molecule CFFT-004 rescues F508del-CFTR expression and function in recombinant cells and primary cultures

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To overcome the processing and channel gating defects of F508del-CFTR requires small-molecules that traffick the mutant protein to the cell surface (CFTR correctors) and enhance channel gating (CFTR potentiators). Interestingly, some dual-acting small-molecules have been identified with both CFTR corrector and CFTR potentiator activities. Here, we investigate the mechanism of action of the dual-acting small-molecule CFFT-004. We tested its effects on the single-channel behaviour of F508del-CFTR using excised inside-out membrane patches from recombinant BHK cells. As controls, we studied low temperature-rescued F508del-CFTR and C18, an analogue of VX-809, the first CFTR corrector to be clinically evaluated. Low temperature-rescued F508del-CFTR has a severe gating defect with greatly reduced open probability (P_o) (wild-type: 0.42 ± 0.04; F508del-CFTR: 0.05 ± 0.01; n = 8 and 21; $P < 0.05$) because the duration of long closures separating channel openings is greatly increased. To investigate corrector activity, cells were incubated with CFFT-004 (5 μM) at 37 ºC for 24 h, then thoroughly washed before membrane patches were excised and CFTR channels studied at 37 ºC. The P_o of CFFT-004-rescued F508del-CFTR channels was 4-fold higher than low temperature-rescued F508del-CFTR (CFFT-004: 0.20 ± 0.02; n = 12; $P < 0.05$) because channel openings were more frequent and prolonged. To test for dual-activity, CFFT-004 (5 μM) was acutely added to the intracellular solution following the activation of CFFT-004-rescued F508del-CFTR channels by PKA and ATP. Acute addition of CFFT-004 (5 μM) further enhanced the P_o of CFFT-004-rescued F508del-CFTR by augmenting the frequency of channel openings (correction: 0.20 ± 0.02; correction+potentiation: 0.27 ± 0.04; n = 9; $P < 0.05$). In contrast to CFFT-004, C18 exhibited corrector, but not corrector-potentiator activity. To begin to explore the specificity of CFFT-004, we studied A561E-CFTR, the second most common CF mutation in Portugal, possessing a similar mechanism of CFTR dysfunction as F508del-CFTR. The activity of CFFT-004 (5 μM)-rescued A561E-CFTR channels was equivalent to that of low temperature-rescued A561E- and F508del-CFTR channels, suggesting that drug action might be mutation-specific. Consistent with this idea, analysis of CFTR expression in recombinant BHK cells by Western blotting revealed that treatment with CFFT-004 (5 μM) at 37 ºC for 24 h failed to promote the maturation of A561E-CFTR. However, CFFT-004 (5 μM) achieved minimal maturation of F508del-CFTR (1% band C relative to wild-type CFTR; n = 7), suggesting differential effects of the small-molecule on F508del-CFTR trafficking and channel gating. Finally, we investigated the effects of CFFT-004 on primary cultures of human bronchial epithelial (HBE) cells from patients homozygous for F508del or A561E by Western blotting and equivalent short-circuit current measurement. Corrector treatment with CFFT-004 (5 μM) at 37 ºC for 24 h augmented F508del-CFTR, but not A561E-CFTR expression and function in HBE cells. Moreover, acute addition of CFFT-004 (5 μM), to test for dual-activity, further enhanced F508del-CFTR, but not A561E-CFTR current. We conclude that CFFT-004 is a dual-acting small-molecule that rescues F508del-CFTR expression and function in recombinant cells and primary cultures. Supported by the CF Trust and PTDC/SAU-GMG/122299/2010 and BioFig (PEst-OE/BIA /UI4046/2011) grants from FCT/POCTI.

Trimethylangelicin (TMA) promotes F508del-CFTR functional rescue in CF airway cells

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Cystic fibrosis transmembrane conductance regulator (CFTR) carrying the F508del mutation is retained in the endoplasmic reticulum and fails to traffic to the cell surface where it functions as a PKA activated chloride channel. Pharmacological correctors that rescue F508del CFTR trafficking may overcome this defect. However, the rescued F508del CFTR still displays reduced chloride permeability necessitating a combined administration of correctors and potentiators of the gating defect. We previously found that 4,6,4'-trimethylangelicin (TMA), besides inhibiting the expression of the IL-8 gene in airway cells in which the inflammatory response has been challenged with *P. aeruginosa*, also potentiates the cAMP/PKA-dependent activation of wt CFTR or F508del CFTR that has been restored to the plasma membrane [1]. In the present study, we present data demonstrating that long preincubation with nanomolar concentrations of TMA also effectively rescues F508del CFTR-dependent chloride secretion in CFBE41o- cells overexpressing F508del CFTR (CFBEΔF). This TMA-dependent functional rescue of F508del CFTR was accompanied by a significant increase of the amount of fully glycosylated mature band C and confocal immunolocalization analysis confirmed that TMA rescued F508del CFTR is significantly expressed on the apical cell surface. Further, the F508del CFTR-dependent chloride secretion rescued by TMA preincubation was further increased by acute addition of the CFTR potentiator, VX-770 or by the acute addition of TMA itself. These results were verified in primary cell monolayers derived from CF patients homozygous for F508del mutation, where nanomolar concentrations of TMA produced a significant increase in (i) cAMP-stimulated chloride secretion and (ii) F508del-CFTR expression on the apical membrane of polarized monolayers. The correction effect of TMA seems to be selective for F508del CFTR and persisted for 24 hours after washout. Taken together, the results suggest that TMA, besides its anti-inflammatory property, displays dual corrector and potentiator activities and, therefore, could be a possible compound for a single drug therapy of cystic fibrosis.

[1] Tamanini, A. et al. (2011) Trimethylangelicin reduces IL-8 transcription and potentiates CFTR function. *Am J Physiol Lung Cell Mol Physiol* **300**, L380-390

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SPLUNC1-Derived Peptides for the Treatment of Na+ hyperabsorption in Cystic Fibrosis (CF) Lung Disease

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CF lung disease is characterized by airway surface liquid (ASL) dehydration that leads to mucus plugging and chronic infection that are a major source of CF patient morbidity. Na^+ hyperabsorption through the epithelial Na^+ channel (ENaC) is a major contributor to CF ASL dehydration. Whilst small molecule inhibitors of ENaC exist, they typically have short durations of action, or cause off target effects. We have previously shown that short palate lung and nasal epithelial clone 1 (SPLUNC1) is an important regulator of ASL volume that is non-functional in CF human bronchial epithelial cultures (HBECs; 2). However, a peptide derived from SPLUNC1 (called S18), is functional in both normal and CF HBECs for >24 h following a single dose (2, 3). To further develop S18 as a potential therapeutic, we tested this peptide *in vitro* and *in vivo*. HBECs were obtained as described (1). FRAP was performed using a Leica SP5 confocal microscope (1). Mass spectrometry was performed as described (3). IACUC approval was obtained for all murine experiments. Sterile saline was added IP to adult mice and urine collected in metabolic cages over time. S18 and amiloride were added at 250 nmol/g body weight. Inhaled ENaC antagonists such as amiloride cross the lung, enter systemic circulation and cause diuresis and hyperkalemia. Thus, we added 100 µM S18 mucosally to CF HBECs. Over a 24 h period, S18 was undetectable serosally by LC/MS/MS (Orbitrap; all n=3). Since murine models of lung disease are good test-beds for therapeutics, we added S18 vs. vehicle to murine tracheal cultures from WT or SCNN1B overexpressing mice. ASL height was significantly lower in SNN1B than WT MTE cultures (4.5 ± 0.2 vs 10.7 ± 2.9 µm; n=6). 100 µM S18 significantly ($p< 0.05$) increased ASL height in both groups (WT, 21.6±0.7; SNN1B, 16.8±2 µm; all n=6), suggesting that mice are a valid model to test S18 efficacy. We next evaluated the effects of S18 on murine renal function. Peritoneal addition of saline significantly increased murine urine output over 8 h (n=12). Inclusion of amiloride into this bolus doubled the rate of urine excretion (n=12). In contrast, S18 had no effect on urine output (n=12). CF lungs are characterized by mucus plugging. Thus, we also tested whether S18 sticks to mucus. Using FRAP, we found that S18 diffused identically in saline solution as in mucus (n=3), suggesting that inhaled S18 will not become trapped in mucus and can diffuse as needed in CF lungs. Here we demonstrate that S18 does not bind to mucus, does not get transported across airway epithelia and does not affect renal function. The effects of S18 on murine models of lung disease remain to be determined. However, our data suggests that inhaled S18 may be beneficial for the treatment of CF lung disease. Funded by NIH HL108927 and the NC Biotechnology Center.

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Development of small molecules to correct the defective chloride transport in cystic fibrosis

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Cystic fibrosis is a genetic disease caused by mutations in the chloride channel CFTR. Several mutations are known but the most common is phenilalanine 508 deletion (F508del). This mutation impairs the folding and trafficking of CFTR protein. The mutant protein is arrested in the endoplasmatic reticulum and degraded by the ubiquitin/proteasome system. If targeted to the plasma membrane with rescue maneuvers, F508del-CFTR shows also a reduced channel activity (gating defect). Nowadays, two classes of molecules have been studied in order to correct the F508del defects: correctors, which ameliorate the trafficking to plasma membrane, and potentiators, that act by improving the gating.We synthesized over 60 compounds belonging to the chemical class of amminoarilthiazoles (AATs). These molecules have shown in previous studies to have dual activity as corrector and potentiator on mutant CFTR (Pedemonte at al, 2011). Synthesis of all compounds involved the reaction of a-halocarbonyl compounds, opportunely modified, with thiourea derivatives. Structural characterization of synthesized compounds was done by means of analytical HPLC techniques coupled with electrospray mass spectrometry. Novel AATs have been tested on cells co-expressing the F508del-CFTR and the halide sensitive yellow fluorescent protein (HS-YFP). The HS-YFP assay allowed rapid determination of F508del-CFTR function by measuring iodide influx.Two active compounds have been identified: FC G and FC105Br. These AATs are more active than En277I, the compound previously identified by our group (Pedemonte at al, 2011). Importantly, co-treatment of cells with AATs and the investigational drug VX-809 elicited a strong synergic effect. This finding indicates that the two types of molecules act with a different mechanism of action, possibly by binding to separate binding sites on CFTR protein.In conclusion, the results obtained will be useful to develop novel drugs able to correct mutant CFTR. Structure-activity relationship studies using QSAR will be performed, in order to understand the chemical features that could be modified to improve AATs activity and synergy with VX-809.This work was supported by grants from the Italian Foundation for Cystic Fibrosis (FFC #2/2012).

Structure-activity relationships (SAR) of neoglycoconjugates derived from deoxynojirimycin modulating the metabolism of sphingolipids as possible anti-inflammatory agents for CF lung disease

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A great deal of hope has followed the recent FDA approval of ivacaftor (Kalydeco) for the treatment of CF basic defect. However, CF patients who already have lung disease or those for whom effective genotype-specific treatments are not yet available, need better antibiotic, mucus-altering therapies together with anti-inflammatory drugs to slow the decline of pulmonary function. Sphingolipids (SLs) play an important regulatory role in CF with respect to pulmonary infections and inflammation. Given the emerging importance of SLs in various pathologies, novel drugs are being developed to selectively target different enzymes involved in SL metabolism. In this regard, iminosugars open up exciting new opportunities for therapeutic agent discovery. Among the most famous iminosugars, miglustat restores the defective F508del CFTR function (Norez, 2006) and reduces the inflammatory response to *P.aeruginosa* (Dechecchi, 2011). Miglustat inhibits different enzymes involved in SL metabolism that could lead to eventual off-target effects, rising concerns about the efficacy of the treatment. Starting from the lead iminosugar deoxynojirimycin (DNJ), a small library of neoglycoconjugates with an adamantane mojety (AMP-DNJ) has been synthesized and characterized for biological activity as well as correction of defective F508del-CFTR function (Guisot, 2011). This study is aimed to perform a Structure-Activity Relationships (SAR) on these compounds as anti-inflammatory agents in CF bronchial cells, by modulating SLs, in order to identify compounds that could provide novel therapeutic options for CF lung inflammation and generate knowledge about relevant targets, pathways and chemical structures that may be used as starting points for a drug discovery campaign. CF bronchial cells were treated with ranging doses of AMP-DNJ derivatives and then infected with *P.aeruginosa*. As a read-out of the effect of these molecules on inflammatory response, the expression of the chemokine IL-8 was measured. Compared to miglustat, very similar maximal inhibition of 50% was found in CF bronchial cells treated with the lead iminosugar AMP-DNJ. Interestingly IC50 value (2-9nM) was considerably lower than that obtained with miglustat (2µM). By varying the length of the alkyl chain linking DNJ and AMP, we found that shorter conjugates were similarly effective in reducing the inflammatory response to *P.aeruginosa* in CF bronchial cells, being IC50 values obtained with each compound very similar (0.4-1 µM). These results demonstrate that AMP mojety improves the potency of inhibition, thus leading to increase the selectivity for the molecular target. The length of the alkyl chain between the iminosugar and AMP seems to be crucial, since potency of shorter compounds is lower than that observed with AMP-DNJ. Our findings further support the use of modulators of the metabolism of SLs for CF lung inflammation and provide evidence that these iminosugars are effective even at low dosages, thus limiting potential adverse effect.

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Vessel associated progenitor cells in the cell based Therapy of the Cystic Fibrosis lung disease

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Cystic Fibrosis (CF) is caused by mutations in the CFTR gene. Lung disease, characterized by airway obstruction, inflammation and chronic bacterial infection, is the leading cause of death. At variance with gene and pharmacological therapies, no one efficacious cell-based approach has been proved to date.
We are developing a cell therapy based on transplantation of mouse mesoangioblasts (mMABs) in mouse models of CF. MABs are small vessels-associated progenitor cells that we demonstrated to be able to cross the vessel wall upon intra-arterial injection, undergo skeletal muscle differentiation and rescue skeletal muscle dystrophy in mice and dogs. Our preliminary results show that MABs engraft lung, tracheal and intestinal epithelium for up to 4 months in healthy and CFTRF508del mice. As additional evidence supporting the use of MABs in CF, we observed that MABs express, in vitro, the CFTR channel.
In parallel, we are investigating on mMABs ability to participate to the epithelium and/or to its stem cell niche. In the end, we suppose that MABs might be recruited by epithelium and contribute to its homeostasis, so that MABs would be promising for the development of an efficacious in vivo cell therapy for the cure of CF.

Phosphodiesterases type-4 as novel target to control neutrophilic lung inflammation in cystic fibrosis.

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Background. The respiratory insufficiency represents the major cause of morbidity and mortality in patients with cystic fibrosis (CF). Lung disease develops over time because of neutrophilic inflammation. Unfortunately, current therapies for CF lack of specific approaches to counteract exaggerated recruitment and tissue damaging activities of neutrophils migrated into the lungs.
Hypothesis and objectives. The present study was undertaken to test the impact of inhibitors of phosphodiesterases type-4 (PDE4), a class of drugs, which is currently tested in clinical trials in patients with chronic obstructive bronchopathy, on normal and CF neutrophil activities potentially relevant to the pathogenesis of pulmonary disease in CF.
Results show that selective PDE4 blockade curbed neutrophils extracellular traps (NETs) release, preserved morphological integrity and restrained myeloperoxidase into the cytoplasmic granules of healthy or CF neutrophils challenged with bacterial endotoxin for 18 hours under adhesion on fibrinogen-coated surfaces. Moreover, PDE4 inhibitor-treated neutrophils, remaining morphologically intact for several hours, underwent physiological apoptosis accompanied by reduced release of Annexin-V-binding microparticles and reduced synthesis of interleukin-8. Selective inhibitors of Src Family Kinases (SFK), phosphoinositide-3 kinase (PI3K)-p110beta and PI3K-p110delta, but not of PI3K-p110alpha/gamma or PI3K-p110gamma reduced neutrophil "nettosis" and protected neutrophils morphological integrity.
PDE4 blockade prevented time-dependent phosphorylation of downstream effectors of SFK and PI3K such as Pyk2-Tyr579/Tyr580 and Akt-Ser-473, uncovering a novel mechanism of action of PDE4 inhibitors on "nettosis". Notably, PDE4 blockade showed higher pharmacological activity in CFTRinh-172-treated respect to untreated normal neutrophils, suggesting an unexpected functional interaction between CFTR and PDE4-regulated signalling.
Conclusions. Overall our results demonstrate that PDE4 inhibitors sustain biochemical signals that may drive neutrophilic inflammation towards physiological resolution. This strongly supports a role of PDE4 as a novel target to "correct" the neutrophilic inflammation in CF.

PTC124 is unable to significantly induce read-through of premature stop codons (PTC) in primary intestinal organoids of CF patients

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Approximately 10% of patients with a genetic disease have premature termination codons (PTC). It is known from literature that aminoglycosides can facilitate read-through of PTC leading to functional protein expression. PTC124 has been developed recently to facilitate read-through in a well-tolerated and oral bioavailable way. Only limited to no efficacy in a phase III clinical trial was observed in CF patients with a PTC in the CFTR gene. We used a highly sensitive functional CFTR assay in primary intestinal organoids from CF patients with PTC to assess CFTR-restoring capacity of PTC124 and aminoglycoside G418 in vitro. Efficacy of PTC124 was compared with aminoglycoside G418 (a published PTC read-through inducing agent) and analyzed after o/n incubation, followed by forskolin-induced swelling of organoids that is fully CFTR-dependent. Organoid swelling was measured and quantified by confocal microscopy and imaging software.

Single treatment with PTC124 was not able to induce swelling in PTC-containing organoids, whereas G418 was able to restore CFTR function to some extent but not in organoids lacking PTC, demonstrating read-through. Moreover, VX-770 and VX-809 further enhanced G418-induced CFTR function in organoids expressing PTC and a CFTR frame shift mutation, suggesting functional interactions between these drugs. In organoids compound heterozygous for F508del and several different PTC, PTC124 activity was only detected in the presence of a W1282X mutation and co-treatment with VX-770 and VX-809. In other organoids with different PTC, no additive effects of PTC124 were measured on top of VX-770 and VX-809 treatment. The most optimal PTC read-through conditions with G418 and CFTR-targeting drugs VX-770 and VX-809 resulted in comparable swelling levels of VX-809 treated F508del homozygous organoids. Since the latter treatment showed no clinical efficacy in a phase II clinical trial, our data suggest that more potent PTC read-through drugs are required to induce clinically significant benefits in CF patients. Furthermore, read-trough agents can be combined with CFTR-targeting drugs to enhance their effect, and may show subject-specific or mutation-specific efficacy.

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CFTR impairment in response to ethanol consumption and in alcoholic pancreatitis

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Background. Excessive ethanol consumption is one of the most common causes of acute and chronic pancreatitis. It is also well documented that genetic defects of CFTR can lead to pancreatitis, however, the role of CFTR in the pathogenesis of alcohol-induced pancreatitis has not been investigated
.

Methods. We examined the effects of ethanol, fatty acids and fatty acid ethyl esters on CFTR function and expression in human (volunteers, patients and cell lines) and in animal models (guinea pigs and CFTR -/¬mice).

Results. Sweat chloride concentration was increased to 62.7mM in alcohol intoxicated patients (blood alcohol 74.2mM) but not in healthy volunteers (blood alcohol 23.3mM, sweat chloride 47mM). Marked loss of CFTR expression was found in pancreas specimens from patients with acute or chronic alcohol-induced pancreatitis. In functional studies, we detected strong inhibitory effects of alcohol and fatty acids on CFTR activity and HCO3- secretion in pancreatic ductal epithelial cells. The inhibitory effect was mediated by sustained intracellular calcium overload, decreased cAMP levels and mitochondrial dysfunction. We reproduced the alcohol-induced decrease in CFTR expression in cultured pancreatic epithelial cells and in vivo in guinea pigs. This alteration was caused by a combination of reduced mRNA levels, decreased cell surface stability and folding defect of CFTR. Finally, deletion of CFTR lead to more severe pancreatitis in CFTR knockout mice induced by ethanol and fatty acids.

Conclusions. The findings indicate that alcohol-induced loss of CFTR function is critical in the development of alcoholic pancreatitis; therefore, correcting CFTR function should offer significant therapeutic benefit

Ethanol and fatty acids inhibit the activity of CFTR chloride channel and anion exchangers in pancreatic ductal epithelial cells

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Background: Excessive ethanol (EtOH) consumption is one of the most common causes of acute pancreatitis, which has no specific treatment yet. Pancreatic ductal epithelial cells (PDEC) secrete HCO_3^- rich pancreatic fluid via Cl^-/HCO_3^- exchangers (CBE) and CFTR, which prevents acinar damage. No information is available about the effects of EtOH and EtOH metabolites (fatty acid ethyl esters and fatty acids) on PDEC, therefore our **aim** was to characterize these effects.

Methods: In our experiments human pancreatic epithelial cell line (Capan-1), guinea pig PDEC and human pancreatic tissue were used. Changes of intracellular pH (pH_i), Ca^{2+} concentration ($[Ca^{2+}]_i$), ATP $[(ATP)_i]$ and mitochondrial membrane potential ($\Delta\Psi$) of PDEC were measured by microfluorometry or confocal microscopy. We measured the effects of ethanol metabolites on CFTR Cl^- current of PDEC with patch clamp. The expression and localization of CFTR were analysed in PDEC and in human pancreatic tissue samples (from patients with alcohol induced acute (AP) or chronic pancreatitis (CP) or without pancreatic disease (NP)) with immunohistochemistry and RT-PCR.

Results: The administration of low EtOH concentration (10mM) stimulated pancreatic epithelial HCO_3^- secretion *in vitro* via IP_3 mediated $[Ca^{2+}]_i$ elevation. In contrast, both high concentration of EtOH (100mM) and palmitoleic acid (POA) (200µM) inhibited the HCO_3^- secretion of PDEC. Both the activities of the apical CBE and CFTR were decreased by 100mM EtOH or 200µM POA. The administration of 200µM POA induced sustained $[Ca^{2+}]_i$ elevation by releasing Ca^{2+} from the endoplasmic reticulum via IP_3 and ryanodin receptor activation and extracellular Ca^{2+} influx. Moreover, 100mM EtOH and 200µM POA depleted the $(ATP)_i$ and decreased $\Delta\Psi$. The inhibitory effects of EtOH and POA were mediated by sustained $[Ca^{2+}]_i$ elevation. We also showed that EtOH, POAEE and POA significantly decreased the expression of CFTR after 48h in PDEC. The expression of CFTR was significantly decreased on the luminal surface of pancreatic ducts in AP and CP patients.

Conclusions: These results suggest that one of the main targets of EtOH and fatty acids is CFTR. Both toxic factors inhibit pancreatic ductal HCO_3^- secretion as well. Restoration of CFTR function and the HCO_3^- secretion may be potential therapeutic possibilities in alcohol induced AP and CP

The buffer capacity of airway epithelial secretions

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The pH of airway epithelial secretions influences bacterial killing and mucus properties and is reduced by acidic pollutants, gastric reflux, and in respiratory diseases such as cystic fibrosis (CF). The effect of acute acid loads will depend on buffer capacity, however the buffering of airway secretions has not been well characterized. In this work we develop a method for titrating micro-scale (30 µl) volumes and use it to study fluid secreted by the human airway epithelial cell line Calu-3, a widely used model for submucosal gland serous cells. Microtitration curves indicated that HCO_3^- was the major buffer. Peak buffer capacity (**β**) increased from 17 to 28 mM/pH during forskolin stimulation, and was reduced by >50% in cystic fibrosis transmembrane conductance regulator (CFTR)-deficient Calu-3 cell monolayers, confirming an important role of CFTR in HCO_3^- secretion. Back-titration with NaOH revealed non-volatile buffer capacity which was due to proteins synthesized and released by the epithelial cells. Lysozyme and mucin concentrations were too low to buffer Calu-3 fluid significantly, although model titrations using porcine gastric mucins at concentrations near the sol-gel transition suggest mucins may contribute to the buffer capacity of ASL *in vivo*. We conclude that CFTR-dependent HCO_3^- secretion and epithelially-derived proteins are the predominant buffers in Calu-3 secretions.

Novel radiolabelled probes for in vivo detection of CFTR

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Molecular imaging allows the non-invasive visualization of a target macromolecule *in vivo* by virtue of its interaction with an imaging probe. Single-photon emission computed tomography (SPECT) and positron emission tomography (PET) are the most sensitive imaging modalities currently available. These techniques allow an early disease diagnosis and follow up of therapeutic strategies.

In recent years, some small molecules that correct the folding/trafficking defect of F508del-CFTR have reached a pre-clinical state. Although the ultimate endpoints to assess the efficacy of the pharmacological correction would be the benefits upon the clinical phenotype (chloride level in the sweat, nasal potential difference and lung function outcomes), there is no available methodology to detect the presence of normal or rescued CFTR at the membrane in living organisms. This detection would be particularly relevant in the lung, which is the main organ affected by CF. So, the aim of this work was the development of a non-invasive radiolabelled imaging probe for the detection of normal (and rescued F508del-CFTR) at the plasma membrane (PM) of human cells. This probe was based on a CFTR inhibitor, CFTR$_{inh}$-172a, known to interact specifically with CFTR at the region of the channel pore. The ^{99m}Tc radioisotope, used in SPECT, was the chosen radionuclide mainly due to its almost ideal physical properties, low cost and easy availability.

The CFTR inhibitor was radiolabelled with the *fac*-[^{99m}Tc(CO)3]$^{+}$ core, using the bifunctional chelator (BFC) approach. This strategy involved a three-component system constituted by a high affinity biomolecule (CFTRinh-172a), the radiometal (^{99m}Tc) and a BFC (L1), designed to bind to the radiometal and also to the biomolecule. CFTR$_{inh}$-172a was conjugated to the previously described L1 with a yield of 53%, resulting in the bioconjugate B1. The reaction of B1 with the [^{99m}Tc(CO)3(H2O)3]$^{+}$ precursor gave the corresponding ^{99m}Tc(I) complex, Tc1. To assess if the metal complex of CFTR$_{inh}$-172a still maintained its ability to interact with CFTR at the PM, a non-radioactive surrogate of the ^{99m}Tc(I) complex was synthesized through the reaction of B1 with the precursor Re(CO)3(H2O)3]$^{+}$, giving the rhenium complex Re1with a 55% yield. The inhibitory efficacy of Re1 was assessed through the iodide efflux assay in BHK cells expressing the wt-CFTR protein. At a concentration of 50 µM the compound inhibited approximately 56% of wt-CFTR activity.

Cellular studies in human bronchial epithelial cells expressing wt-CFTR or mutant F508del-CFTR will be performed. The amount of radiolabelled small molecule that can interact with the CFTR protein at the PM will be measured and the CFTR inhibitor will be tested as probe for detecting mutant-CFTR modulation.

In the future, this radioprobe can be a tool potentially useful for evaluation of pharmacological correction but also for the pre-selection of patients for pharmacological potentiator therapy.

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Radiolabelling of anti-CFTR antibodies for the detection of CFTR

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Therapies based in small organic molecules that correct the trafficking or gating defects of CFTR (termed correctors or potentiators, respectively) are emerging. However, there is no available methodology to detect the expression of normal or rescued CFTR at the membrane in living organisms, after treatment with correctors. Molecular imaging can thus be a valid option as techniques such as positron emission tomography (PET) or single photon emission computed tomography (SPECT) are widely used for diagnosis and follow up of therapeutic strategies in several diseases, for instance, cancer and in the cardiology and neurology fields.

The major goal of this work was the development of a non-invasive radiolabelled imaging probe for the detection of normal (and rescued F508del-CFTR) at the plasma membrane of human bronchial epithelial cells. This probe was based on an anti-CFTR antibody, ECL1, which recognizes the first extracellular loop of CFTR (residues 103 to 117) (Peters *et al.*, Methods Mol Biol 2011, 742:335-353). The ^{99m}Tc radioisotope, used in SPECT, was the chosen radionuclide mainly due to its almost ideal physical properties, low cost and easy availability.

The ability of ECL1 to recognize CFTR was first confirmed by western blot and immunoprecipitation, using baby hamster kidney (BHK) cells expressing wild-type (wt) CFTR. Radiolabelling of the antibody was then performed by a direct method, relying on the binding of ^{99m}Tc to sulfhydryl groups in its structure. The ECL1 antibody was firstly purified from rabbit serum, reduced to generate free sulfhydryl groups and then radiolabelled either with ^{99m}Tc(I) or ^{99m}Tc(IV). For radiolabelling with ^{99m}Tc(I), the highest radiolabelling yield obtained was 25%. After optimization with a control IgG, radiolabelling with ^{99m}Tc(IV) was also performed, although without detection of radiolabelled ECL1. After further optimization of the radiolabelling yield, the usefulness of ^{99m}Tc-ECL1 as a molecular imaging radioprobe will be explored in cellular studies using BHK-wt cells and human cell lines. In particular, the effect of the radiolabelling procedure on the intrinsic biological properties of the antibody, namely on the immunoreactivity and binding affinity to CFTR, will be assessed.

It is also envisaged the radiolabelling of other anti-CFTR antibodies that recognize the first extracellular loop of CFTR. These radioprobes will have the potential to be used on SPECT imaging to detect/visualize the expression of CFTR at the membrane of human cells.

In the future, these novel tools can be then used as imaging biomarkers and transferred as clinical tools to assess early therapy response in drug evaluation, thereby facilitating translational research.

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