2010
European Cystic Fibrosis Society

New Frontiers in Basic Science of Cystic Fibrosis

Conference Programme & Abstract Book

Carcavelos, Portugal

Chairpersons

Harry Cuppens, Michael Gray and J.P. Clancy

7-10 April 2010

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Welcome from the ECFS President

Dear Friends and Colleagues,

It is a great pleasure to invite you to the seventh European Cystic Fibrosis Conference entirely dedicated to Basic Science. This year we are delighted to welcome Prof. J.P. Clancy, Prof. Harry Cuppens, and Dr. Mike Gray as the conference Chairpersons.

Basic scientific discovery is critical to our understanding of CF and will contiue to underpin the development of new therapies. The success in translational research in the past 2 years with three completely novel, potentially disease modifying therapies entering clinical trials is a real testimony to the huge efforts of the CF scientific community. The ECFS is proud to provide 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. Your active participation will contribute to a productive exchange of information.

I extend to you a very warm welcome to this exciting conference.

Stuart Elborn
President
European Cystic Fibrosis Society

Conference Chairpersons' Welcome

We are very happy to welcome you to the 7th Basic Science of CF conference which in 2010 will again take place in Portugal, in the town of Carcavelos which is approximately 20 kilometers from the centre of Lisbon.

With this conference you can expect to hear about recent unpublished results and engage in discussions of data and ideas in an informal and interactive environment.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. You will have excellent opportunities to discuss your data, interact and discuss science 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 will also be two key-note lectures as well as four informal and interactive special interest group discussions. For two evenings 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 you all to an exciting conference of high scientific quality.

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J.P. Clancy
University of Alabama at Birmingham
United States of America

Harry Cuppens
University of Leuven
Belgium

Michael Gray
University of Newcastle
United Kingdom

2010 ECFS Conference
New Frontiers in Basic Science of Cystic Fibrosis

Portugal, 7-10 April 2010

Programme

Chairpersons: J.P. Clancy (Birmingham, Alabama, US), Harry Cuppens (Leuven, Belgium), Mike Gray (Newcastle, UK)

Wednesday, 7 April 2010 (Day 1)	
13:00-17:30	Registration, Light Meal Set-up of Posters – Room Safira
17:30-18:00	**Official opening of the meeting by the Conference Chairpersons** – Room: Esmeralda
18:00-19:00	**Opening keynote lecture** CFTR in the GI Tract: Studies in CF Mice and CF Patients – H. de Jonge (NL)
19:00-20:30	**Welcome Reception**
20:30-21:30	*Dinner*

Thursday, 8 April 2010 (Day 2)

07:30-08:45	*Breakfast*
08:45-10:30	**Symposium 1 – CFTR2010 - a space (or spatial) odyssey** **Chairs: R. Ford (UK) / D. Clarke (CA)**
08:45-09:10	Mass Spectrometry Studies of CFTR Phosphorylation - C. Bear (CA)
09:10-09:35	What can we Learn about CFTR (dys)functions from 3D Structures – I. Callebaut (FR)
09:35-10:00	CFTR 3D Structure: New Approaches - R. Ford (UK)
10:00-10:10	Abstract 2: Understanding CFTR Biogenesis Through The Deletion Of The Regulatory Extension (RE) And Regulatory Insertion (RI) – AC Da Paula (PT)
10:10-10:20	Abstract 4: Role of the C-terminal extremity of CFTR NBD1 in the control of CFTR activity – A. Billet (FR)
10:20-10:30	Abstract 43: Screening for F508del-CFTR Correctors Identified by Pharmacophore Modelling through Western blot and NBD1 Folding Assays – M. Palma (PT)
10:30-11:00	*Coffee break* & **Poster viewing** – Room: Safira
11:00-12:45	**Symposium 2 – WT and F508del CFTR trafficking – learning the rules of the road** **Chairs: G. Lukacs (CA) / M. Gentzsch (US)**
11:00-11:10	Abstract: 8: Preferential sumoylation of ΔF508 CFTRand NBD1 leads to protein degradation – R. Frizzell (US)
11:10-11:20	Abstract 10: F508del-CFTR increases intracellular Ca^{2+} signaling that causes enhanced calcium-dependent Cl^- conductance in cystic fibrosis – JR Martins (PT)
11:20-11:45	Sequential domain folding in wild-type and ΔF508 CFTR – I. Braakman (NL)
11:45-12:10	CFTR Folding in Vitro and in Vivo: Implications of Cooperative Domain Assembly – G. Lukacs (CA)
12:10-12:35	Repair of CFTR Folding Defects Caused by Cystic Fibrosis Mutations - D. Clarke (CA)
12:35-12:45	Abstract: 6: Mechanisms of F508del-CFTR Rescuing by Genetic Revertants, Low Temperature and Small Molecules – C. M Farinha (PT)
12:45-14:30	*Lunch*
14:30-16:15	**Symposium 3 – Ion transport networks in epithelia** **Chairs: K. Kunzelmann (DE) / B. Stanton (US)**
14:30-14:55	Physiology of Anoctamins - K. Kunzelmann (DE)
14:55-15:20	Slc26 anion transporters involvement in acid-activated duodenal HCO_3^- secretion - U. Seidler (DE)
15:20-15:45	Interaction of ENaC and CFTR - M. Gentzsch (US)
15:45-15:55	Abstract 15: Regulation of ENaC by the Stress Response Protein SERP1 – D. Faria (PT)
15:55-16:05	Abstract 34 : Development Of A Multi-Cellular Co-Culture Model Of Normal And Cystic Fibrosis Human Airways In Vitro – A. Bielemeier (UK)
16:05-16:15	Abstract 5: Spleen Tyrosine Kinase (SYK) phosphorylates CFTR NBD1 and interacts in vivo with CFTR – S. Luz (PT)
16:15-16:45	*Coffee Break* & **Poster Viewing** – Room: Safira

16:45-18:15	Room: Esmeralda **Special Group Discussion-I – Modulator insights into wt and F508del CFTR behavior** **Moderators: L. Galietta (IT) / I. Braakman (NL)** Room: Rubi **Special Group Discussion-II – CF biomarkers now and on the horizon** **Moderators: C. De Boeck (BE) / J.P. Clancy (US)**
20:30-21:30	*Dinner*
21:30-23:30	Evening Poster Session – Posters with Even Numbers – Room: Safira

Friday, 9 April 2010 (Day 3)

07:30-08:45	*Breakfast*
08:45-10:30	**Symposium 4** – **Mucus Biogenesis and disruption in CF** **Chairs: C. W. Davis (US) / M. Gray (UK)**
08:45-09:10	Airway Goblet Cells: Mucin Synthesis and Secretion – C. W. Davis (US)
09:10-09:35	How does Mucin Biogenesis Contribute to Mucus Accumulation in CF? - D. Thornton (UK)
09:35-10:00	A new Assay for Modulation of Ion Transport and Mucus Clearance in Cells and Tissues: Video-rate Ultrahigh-Resolution 3D Optical Imaging by Spectral Domain Optical Coherence Tomography - S. Rowe (US)
10:00-10:10	Abstract 13: Gap Junctions Contribute To Airway Surface Liquid Homeostasis in Human Airway Epithelial Cells – D. Losa (CH)
10:10-10:20	Abstract 14: The switch of intestinal Slc26 exchangers from anion absorptive to HCO3- secretory mode is dependent on CFTR anion channel function – A. Singh (DE)
10:20-10:30	Abstract 46: Proteomics Characterization of Nasal Epithelial Cells: Their Use for Biomarkers Discovery in CF – N. Charro (PT)
10:30-11:00	*Coffee break* & **Poster viewing** – Room: Safira
11:00-12:50	**Symposium 5 – Inflammatory signalling in CF lung disease** **Chairs: G. Cabrini (IT) / A. Mehta (UK)**
11:00-11:05	Introduction – A. Mehta (UK)
11:05-11:30	Inflammatory signalling in CF lung disease – G. McElvaney (IE)
11:30-11:55	CFTR Intractome and Inflammation – A. Edelman (FR)
11:55-12:20	Pharmacological Modulation of Chemotactic Signaling in CF Respiratory Models – G.Cabrini (IT)
12:20-12:30	Abstract 21: NF-κB Inflammatory Signalling is suppressed by CFTR in a number of Cell models – M. Hunter (UK)
12:30-12:40	Abstract 24: Modulation of sphingolipids metabolism reduces the neutrophil chemotaxis in human and murine respiratory models in vitro and in vivo.- M. Dechecchi (IT)
12:30-12:50	Abstract 22: IL-8 production by Calu-3 cells depressed for CFTR and in CF human airway epithelial cells - M. Chanson (CH)
12:50-14:00	*Lunch*

14:00-18:00	**Free Afternoon**
18:00-19:30	Room: Rubi **Special group discussion-III – Alternate restorative strategies for CF** **Moderators: F. Van Goor (US) / M. Mall (DE)** Room: Esmeralda **Special group discussion-IV – Inflammation in CF - getting to the routes by digging out the roots.** **Moderators: A. Mehta (UK) / M. Chanson (CH)**
20:00-21:30	*Dinner*
21:30-23:30	Evening Poster Session - Posters with Odd Numbers – Room: Safira

Saturday, 10 April 2010 (Day 4)

07:30-08:45	Breakfast
08:45-10:30	**Symposium 6 – Understanding CF microbial targets and the CF pulmonary microbiome** **Chairs: S. Elborn (UK) / J.P. Clancy (US)**
08:45-09:10	Tobramycin and FDA-approved iron chelators eliminate *P. aeruginosa* biofilms on cystic fibrosis airway epithelial cells – B. Stanton (US)
09:10-09:35	Clonal variation of *Pseudomonas aeruginosa* isolates from CF Airways - B. Tümmler (DE)
09:35-10:00	Anerobes and the sputum microbiome in CF - S. Elborn (UK)
10:00-10:10	Abstract 29: *Pseudomonas aeruginosa* Infection Drives Functionally Unopposed Matrix Metalloproteinase Activity In Lung Parenchymal And Inflammatory Cells.- J. Moffett (UK)
10:10-10:20	Abstract 28: Trappin-2, an antimicrobial peptide, induces a flagellin/TLR5-dependent modulation of alveolar macrophage & epithelial cell activity – D. Descamps (FR)
10:20-10:30	Abstract 30: The effect of budesonide in a refined rat model of respiratory infection with *Pseudomonas aeruginosa* – E. Growcott (UK**)**
10:30-11:00	*Coffee break* & **Poster viewing** – Room: Safira
11:00-13:05	**Symposium 7 – Modelling of CFTR defects** **Chair: J. Engelhardt (US) / M. Drumm (US)**
11:00-11:25	Preclinical Evaluation of Therapeutic Effects of ENaC Blockers on Cystic Fibrosis-like Lung Disease in Mice – M. Mall (DE)
11:25-11:50	Airway Disease in the *CFTR*-targeted Pig – D. Stolz (US)
11:50-12:15	Characterization of a NEW CFTR Knockout Ferret Model – J. Engelhardt (US)
12:15-12:35	Model Systems Based on Human Rectal Biopsies to Examine CFTR Modulators **–** J.P. Clancy (US)
12:35-12:45	Abstract 36: Generation Of New Lung-Specific Tet-Dependent Activator Mice For Tight And Quantitative Control Of Conditional Gene Expression In The Murine Lung – J. Duerr (DE)
12:45-12:55	Abstract 40: Plasma lipidomics reveals phenotype-associated differences within a cohort of F508del homozygous patients – M. Ollero (FR)
12:55-13:05	Abstract 45: Evaluation of safety and gene expression with a single dose of PGM169/GL67A administered to the airways of individuals with cystic fibrosis: The UK CF Gene Therapy Consortium 'Pilot Study' – U. Griesenbach (UK)

13:05-14:30	*Lunch*
14:30-16:15	**Symposium 8 – CF genomic modifiers and worldwide genetics** **Chair: C. De Boeck (BE) / B. Tümmler (DE)**
14:30-14:55	Modifier Genes in Cystic Fibrosis: Genome-Wide Association Study – M. Knowles (US**)**
14:55-15:20	Mouse Models as Tools to Evaluate CF Modifiers – M. Drumm (US)
15:20-15:45	CFTR2: Defining the clinical and functional consequences of CFTR mutations - G. Cutting (US)
15:45-15:55	Abstract 39: Histone deacetylases (HDACs) and IFRD1 in CF airway epithelial cell models - E. Blanchard (FR)
15:55-16:05	Abstract 37: Wobble splicing at a NAGNAG acceptor site induces a mild cystic fibrosis phenotype – A. Hinzpeter (FR)
16:05-16:15	Abstract 38: Abnormal CFTR mRNA processing: which mutations cause it, which are the consequences and how can it be treated – A. Ramalho (PT)
16:15-16:45	*Coffee Break* & **Poster Viewing**
16:45-18:15	**Symposium 9 - Now and Future modulators** **Chairs: M.D. Amaral (PT) / F. Van Goor (US)**
16:45-17:10	CFTR and CaCCs as Pharmacological Targets in Cystic Fibrosis – L. Galietta (IT)
17:10-17:35	High-content Fluorescence Microscopy siRNA Screens to Track Function/Traffic of ENaC & CFTR – M.D. Amaral (PT)
17:35-18:00	Identification and Validation of Novel Drug Targets to Treat Cystic Fibrosis: Modifiers of the Trafficking Defect of CFTRΔF508 – D. Fischer (NL)
18:00-18:10	Abstract 44: SIV vector pseudotyped with SeV-F/HN envelope proteins produces long lasting expression in the murine lung, is readministrable and transfects human airway models. – U. Griesenbach (UK)
18:10-18:20	Abstract 42: Effect of TS-04-95 on Primary Human Bronchial Epithelial Monolayers after a Repeated Dose Treatment – I. Uliyakina (PT)
18:20-18:30	Abstract 7: Effect of small molecule correctors on different mutants of CFTR – L. Cebotaru (US)
18:30-18:45	*Break*
18:45-19:45	**Closing Lecture** CFTR Modulators – Targets and Opportunities - JP Clancy (US) / S. Rowe (US)
20:30	*Dinner / social event*

Sunday 11 April 2010 – Delegates Depart

POSTER TITLES & AUTHORS

P.1 **CFTR Controls the Chloride Ion Homeostasis in Rat Spinal Motoneurons during Postnatal Development**
Alexey Ostroumov, Manuela Simonetti, Andrea Nistri

P.2 **Understanding CFTR Biogenesis through the Deletion of the Regulatory Extension (RE) and Regulatory Insertion (RI)**
Ana Carina Da Paula, Margarida D Amaral

P.3 **Trypsin-4 and Neutrophil Elastase Regulate CFTR Expression and Function in Cystic Fibrosis**
Mathieu Le Gars, Delphyne Descamps, Naziha Bakouh, Gabrielle Planelles, SS Hong, Pierre Boulanger, Michel Huerre, Michel Chignard, Jean-Michel Sallenave

P.4 **Role of the C-terminal Extremity of CFTR NBD1 in the Control of CFTR Activity**
Arnaud Billet, Mathilde Jollivet, Jean Paul Mornon, Isabelle Callebaut, Frederic Becq

P.5 **Spleen Tyrosine Kinase (SYK) Phosphorylates CFTR NBD1 and Interacts in vivo with CFTR**
Simão F Luz, Ana Mendes, Francisco M Romeiras, Paulo Matos, Peter Jordan, Margarida D Amaral, Carlos M Farinha

P.6 **Mechanisms of F508del-CFTR Rescuing by Genetic Revertants, Low Temperature and Small Molecules**
Carlos M Farinha, Marisa Sousa, Inna Uliyakina, Ana Carina Da Paula, Margarida D Amaral

P.7 **Effect of Small Molecule Correctors on Different Mutants of CFTR**
Liudmila Cebotaru, William Guggino

P.8 **Preferential Sumoylation of ΔF508 CFTR and NBD1 Leads to Protein Degradation**
Xiaoyan Gong, Annette Ahner, Bela Schmidt, Wael Rabeh, Gergely Lukacs, Patrick Thibodeau, Raymond A Frizzell

P.9 **Expression and Functional Roles of TRP Channels in Normal and CF Airway Epithelial Cells**
Samuel Hanrahan, Susan Stokesberry, Francine de Courcey, Lorcan McGarvey, J Stuart Elborn, Alex Zholos, Madeleine Ennis

P.10 **F508del-CFTR Increases Intracellular Ca^{2+} Signaling that Causes Enhanced Calcium-dependent Cl^- Conductance in Cystic Fibrosis**
Joana Raquel Martins, Patthara Kongsuphol, Joana Almaça, Fadi Aldehni, Luka Clarke, Rainer Schreiber, Margarida Amaral, Karl Kunzelmann

P.11 **CFTR Activates Calcium Activated Chloride Channels in *Xenopus* Oocytes through Extracellular Acidosis**
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P.13 **Gap Junctions Contribute to Airway Surface Liquid Homeostasis in Human Airway Epithelial Cells**
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P.14 **The Switch of Intestinal Slc26 Exchangers from Anion Absorptive to HCO_3^- Secretory Mode is Dependent on CFTR Anion Channel Function**
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P.15 **Regulation of ENaC by the Stress Response Protein SERP1**
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Luisa Alessio, Carlos M Farinha, Margarida D Amaral

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P.38 **Abnormal CFTR mRNA Processing: Which Mutations Cause it, Which Are the Consequences and How Can it be Treated**
Anabela S Ramalho, Margarida D Amaral

P.39 **Histone Deacetylases (HDACs) and IFRD1 in CF Airway Epithelial Cell Models**
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P.40 **Plasma Lipidomics Reveals Phenotype-associated Differences within a Cohort of F508del Homozygous Patients**
Mario Ollero, Giuseppe Astarita, Isabelle Sermet-Gaudelus, Ida Chiara Guerrera, Julien Colas, Daniele Piomelli, Aleksander Edelman

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P.42 **Effect of TS-04-95 on Primary Human Bronchial Epithelial Monolayers after a Repeated Dose Treatment**
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P.43 **Screening for F508del-CFTR Correctors Identified by *Pharmacophore Modelling* through Western blot and NBD1 Folding Assays**
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P.44 **SIV Vector Pseudotyped with Sev-F/HN Envelope Proteins Produces Long Lasting Expression in the Murine Lung, Is Readministrable and Transfects Human Airway Models**
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Uta Griesenbach, Jane C Davies, Deborah R Gill, Nia Voase, Gwyneth Davies, Tracy Higgins, Alastair J Innes, Chris A Boyd, David Porteous, Stephen C Hyde, Eric WFW Alton

P.46 **Proteomics Characterization of Nasal Epithelial Cells: Their Use for Biomarkers Discovery in CF**
Nuno Charro, Tânia Simões, Paula Pacheco, Carlos Lopes, Pilar Azevedo, António Bugalho de Almeida, King C Chan, Haleem J Isaaq, Timothy Waybright, Timothy D Veenstra, Josip Blonder, Deborah Penque

P-47 **Detection and Discrimination of CFTR Mutants by Flow Cytometry**
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P.48 **Analysis of CFTR Expression in Nasal Epithelial Cells by Flow Cytometry**
Marit A van Meegen, Suzanne W.J. Terheggen-Lagro, Lodewijk A.W. Vijftigschild, Cornelis K van der Ent, Jeffrey M Beekman

AWARD WINNERS

Novartis Young Fellows Travel Award:

Davide Losa, CH
Anurag Singh, DE
Julia Dürr, DE
Arnaud Billet, FR

Student Helper Award:

Diana Faria, PT
Joana Raquel Martins, PT
Simão Filipe Cunha da Luz, PT
Joana Almaca, PT
Luisa Alessio, PT
Patthara Kongsuphol, DE

UK CF Trust Young Investigator Travel Awards:

Samuel Robert Hanrahan
Mairi Hunter
Catriona Kelly
Paul James Buchanan
Joanne Moffett
Mark Williams

07 April – 18:00-19:00 **Room: Esmeralda**

Opening Lecture

CFTR in the GI Tract: Studies in CF Mice and CF Patients

Hugo R. de Jonge (NL)

Department of Biochemistry, Erasmus University Medical Center, Rotterdam, the Netherlands

The CFTR chloride channel is widely distributed in the GI tract of all species examined, including sharks, mice and humans. In the enterocyte, CFTR serves as the major apical anion channel accounting for electrolyte and water secretion in response to intestinal secretagogues, including neurotransmitters, endocrinic and paracrinic factors, bile salts and microbial enterotoxins. A major signaling pathway resulting in CFTR activation starts with the stimulation of guanylyl cyclase (GC-C) in the apical membrane by intraluminal peptides (guanylin and heat-stable *E.coli* toxin (STa) in mammals), followed by cGMP activation of a membranal cGMP-dependent protein kinase (cGK type II) and/or cGMP inhibition of a type III phosphodiesterase (PDE3), resulting in cAMP elevation and activation of cAMP-dependent protein kinase (PKA). PKA and cGKII cause net intestinal fluid secretion by a dual action: phosphorylation and activation of CFTR, and phosphorylation and inhibition of the major salt importer, the Na^+/H^+ exchanger NHE3. Our recent studies in KO mice show that the PDZ-adapter protein NHERF-1 is important for stabilization and β2-adrenergic/cAMP/cGMP activation of CFTR, whereas NHERF-2 is required to anchor cGKII in close proximity of NHE3 and to facilitate lysophosphatidic acid (LPA) induced stimulation of NHE3 and inhibition of CFTR via the LPA5 receptor. Some of these findings are presently exploited in our search for novel anti-diarrheal medicine, including the development of specific GC-C and cGKII inhibitors by high throughput screening and *in silico* modeling.

The GI phenotype of most CF patients is characterized by luminal dehydration and mucus plugs in all affected organs, resulting in pancreatic insufficiency at birth, meconium ileus (~10%), constipation, lipid malabsorption, fecal bile salt loss, and, at a later stage, biliary obstruction and fibrosis in the liver. Immunological and bioelectric studies in the intestine of CF mice ($Cftr^{tm1eur}$ F508del, congenic FVB) and homozygous F508del CF patients (resected ileum, rectal biopsies) confirmed the severely reduced intracellular processing and channel gating of F508del CFTR described previously in cultured cell models. In human distal colon, but not in CF mouse intestine, the gating defect could be restored by CFTR potentiators (e.g. VRT-532), emphasizing the specificity of CFTR-potentiator interaction. Residual transepithelial anion secretion in the ileum of $Cftr^{tm1eur}$ F508del CF mice (~20% of WT) corresponded with ~4% of correctly processed apical CFTR protein. A similar non-linearity between the amount of CFTR and anion secretion in the enterocyte was found in other “leaky” CF mouse models and in rectal biopsies from “mild CF” 3272-26A>G splice patients (~40% residual secretion, ~8% CFTR protein), indicating that only 20% of mature CFTR protein is sufficient to normalize intestinal anion transport.

Correction of GI abnormalities in CF is of prime clinical relevance considering the known impact of CF nutritional status on pulmonary function and survival. In $Cftr^{tm1eur}$ F508del CF mice, almost full correction of intestinal anion secretion could be reached *ex vivo* by low-temperature incubation (12h, 26^0C) and by short-term incubation (4-6h) at 37^0C in the presence of proteasome inhibitors (ALLN, bortezomib), in line with the exceptionally high rate of CFTR biosynthesis in the intestinal crypts (turnover rate 3-5 days). Partial correction was observed upon incubation with Vertex correctors (e.g. VRT-640), the α-glucosidase inhibitor miglustat, and the analgesic drug glafenine.

Other topics that will be addressed in this lecture include (1) the possible contribution of non-CFTR channels or electrogenic anion exchangers to intestinal Cl^- and HCO_3^- secretion. For example, the repair of STa-induced colonic HCO_3^- secretion and attenuation of mucin accumulation and intestinal obstruction in Cftr-/- mice by PPAR- γ agonists (Harmon GS et al 2010 Nat Med 16: 313) clearly involves a CFTR-independent mechanism; (2) the mechanistic basis of species-differences in the severity of obstructive organ disease in CF (intestine: pig>mouse>human; gall bladder: pig>human>mouse).

Thursday 08 April – 08:45-10:30 **Room: Esmeralda**

SYMPOSIUM 1
CFTR2010 - a space (or spatial) odyssey
Chairs: R. Ford (UK) / D. Clarke (CA)

S1.1 Mass Spectrometry Studies of CFTR Phosphorylation

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CFTR is a multidomain membrane protein which functions as a regulated chloride channel in the apical membrane of epithelial cells. Although the obligate requirement for phosphorylation of the "R domain" by protein kinase A as well as the binding and hydrolysis of ATP for channel activity is well documented, our understanding of the molecular basis for this regulation remains unclear. Certain mutagenesis studies support the utility of homology models of CFTR based on the prokaryotic ABC protein: Sav1866 in developing mechanistic models. However, these models present a static picture (of the open state) and lack information regarding conformational dynamics throughout the multiple domains of CFTR. In particular such models are limited with respect to providing insight into the regulated dynamics of the "R-domain" as this region is unique to CFTR. We are developing the tools with which to monitor protein dynamics throughout the full length CFTR protein as changes in accessibility of endogenous residues to modifying reagents, ie. sulphydryl modifying reagents and PKA-dependent phosphorylation of the "R domain". We expose the native, full length, purified and functionally reconstituted CFTR protein to these reagents and monitor site specific modification using state of the art methods in mass spectrometry.

We identified 15 of the 18 cysteine residues in the full length reconstituted and PKA phosphorylated CFTR protein as modified by the water soluble, methane thiosulphonate reagents: MTSET and MTSES in the presence and in the nominal absence of MgATP. The 3 cysteines that cannot be modified are predicted to be buried in the first membrane spanning domain. These findings support the view that the nucleotide binding domains, intracellular loops and the "R domain" are dynamic in the context of the full length protein and many regions are exposed to water at least transiently. Quantitative assessment of absolute extent of cysteine modification in both the ATP bound and unbound states of the full length protein is in progress using the methods as described below in our studies of phosphorylation.

Each of the 10 sites for phosphorylation were mapped using the high resolution Fourier transform-Orbitrap mass spectrometry and quantified by selected reaction monitoring (MRM) on a triple quadrupole instrument. Interestingly, phosphorylation was detected at ten PKA consensus sites, regardless of whether the sample was PKA treated. Notably, phosphorylation was even detected at the two monobasic PKA consensus sites, S670 and S753, which are considered to be less susceptible to PKA phosphorylation as compared to dibasic sites. Absolute quantitation of phosphopeptides prior to and following PKA activation is being assessed by spiking the sample with known quantities of stable isotope labeled "R domain peptide (collaboration with J.Forman Kay, HSC). So far, our studies of phosphorylation support the concept that the R domain is structurally dynamic with most sites accessible to and modified by PKA under resting conditions at least in Sf9 cells. These experiments will form the basis for future mass spectrometry studies of the conformational defects introduced by the major mutant; ΔF508-CFTR.

S1.2 – What can we learn about CFTR (dys)functions from 3D structures

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The high resolution, experimental 3D structures of complete ABC exporters, published since 2006, have been used to generate models of the 3D structure of the CFTR protein in different conformations. These models are useful to understand the molecular basis of the CFTR function, as well as to evaluate the impact of mutations on the CFTR 3D structure and function. The Sav1866 structure in an outward-facing conformation was first used to model the open form of the CFTR channel (1), whereas a MsbA structure in an inward-facing conformation (called “closed apo”) was afterwards considered to construct a plausible 3D model of the closed form of the channel (2). Despite the large reorganization of the membrane-spanning domains and movements of the nucleotide-binding domains, the coupling interfaces linking these domains are relatively well conserved, suggesting that these act as pivots around which the CFTR channel dynamics occurs.

We have further considered our previous models of the CFTR channel, based on the Sav1866 and MsbA 3D structures, as well as new ones constructed on the basis of the P-gp 3D structure, to highlight new structural features, which may account for specific functional features. The models especially support the hypothesis that CFTR may consist in a “broken” ABC transporter, having an “atrophied” gate at the cytoplasmic side (3). According to our model, this gate would be located at the level of the bundle formed by the four intracellular loops (ICLs). Moreover, a careful analysis of the 3D structure models revealed several potential ligand binding sites at the interface between the domains (NBD1:NBD2 interface, but also MSDs:NBDs), suggesting that these could be privileged targets for therapeutic strategies.

Supported by: Vaincre La Mucoviscidose

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(3) Gadsby DC (2009) Ion channels versus ion pumps: the principal difference, in principle. Nat Rev Mol Cell Biol 10:344–352

S1.3 – CFTR 3D Structure: New Approaches

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CFTR is a member of the ATP-binding cassette (ABC) family of proteins. Structural data for this class of membrane proteins is particularly abundant; structures for eight ABC proteins in various states have been obtained by X-ray crystallography. So far, similar progress with the full-length CFTR protein has been hampered by low expression levels, although significant progress with isolated CFTR domains has been made (ie for nucleotide-binding domains NBD1 and NBD2). Similarly, NMR has given complementary structural data on the NBDs and for the isolated regulatory (R) region.

We have employed alternative approaches to study the structure of CFTR. These approaches require much less protein than X-ray crystallography, hence are compatible with the current practical yields of purified CFTR (typically a few hundred micrograms per preparation). These are: single particle electron microscopy (SPA-EM) and electron crystallography (of 2D crystals of CFTR). The former approach has yielded much data, but so far at relatively moderate levels of resolution (see Zhang et al., J. Struct. Biol. (2009)). The latter approach requires the collection and processing of a huge amount of data, but we have recently been able to grow 2D crystals that yield higher resolution data. Processing this data such that a density map can be produced where tracing of the polypeptide chain is possible will take a few years, but we are currently approaching the point where a medium resolution map can be calculated. This should reveal information about the locations of secondary structural elements such as alpha helices.

We will present our latest data for SPA-EM and for electron crystallography of CFTR.

S1.4 - Understanding CFTR Biogenesis through the Deletion of the Regulatory Extension (RE) and Regulatory Insertion (RI)

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[2]National Institute of Health Dr. Ricardo Jorge, Centre of Human Genetics, Lisboa, Portugal

Structural knowledge of CFTR is crucial in order to gain a better understanding of how disease-associated mutations compromise the maturation and channel activity of the protein. Moreover, studies on NBD1 are of particular interest because this domain contains F508del the most prevalent CF-causing mutation, which causes defects in protein folding, plasma membrane trafficking and channel gating. Comparison of wt- and F508del-NBD1 crystal structures indicated that they do not substantially differ, except for their surface properties around the F508del residue [1,2]. Two segments may be responsible for such alteration, namely the regulatory extension (RE) and regulatory insertion (RI), both suggested to be conformationally dynamic, namely upon phosphorylation [1, 2]. The RI (Glu403-Leu435) is a ~30-residue insert between the first two ß-strands of the NBD1 ß-subdomain, and the RE (Ser654-Gly673) is at the C-terminus of NBD1, where it extends ~20 residues longer than canonical ABC domains forming a helix packing against NBD1 at the NBD1-NBD2 interface.
We hypothesized that the dynamic flexibility of these regions may also result in exposition of hydrophobic surfaces contributing to the low folding efficiency of both wt- and F508del-CFTR. Our first goal here was to study the structural/functional impact of removing the RE and the RI on full-length CFTR by assessing its *in vivo* processing. Secondly, we aimed to evaluate the effect on these variants of *in cis* mutations previously described to correct the defect of F508del-CFTR folding, namely: R1070W and G550E.
To this end, we produced ΔRE and ΔRI variants of wt- and F508del-CFTR, alone, together (ΔRE/ΔRI) and with those 2 point mutations by *in vitro* mutagenesis and used them to generate BHK stable cell lines. Results from Western blot (WB) show that wt-CFTR ΔRE, ΔRI and ΔRE/ΔRI are efficiently processed, although ΔRE/ΔRI exhibits lower levels of the mature form at steady-state. These data suggest that these variants acquire a native (folded) conformation *in vivo*, consistently with a previous study [3]. Their functional characterization by iodide efflux shows that ΔRE- and ΔRI-wt respond to agonists but not ΔRE- nor ΔRI-F508del. Moreover, removal of the RE causes a significant decrease in the function of wt-CFTR. By immunofluorescence, we show that ΔRE-wt is predominantly at the plasma membrane whereas ΔRE- and ΔRI-F508del are in the ER. Also, ΔRI-wt shows the same ER localization as F508del-CFTR with faint membrane staining. Preliminary results show that R1070W-ΔRI-F508del (but not R1070W-ΔRE-F508del) undergoes maturation. Additional studies on maturation efficiency and functional characterization of these (and of G550E) CFTR) variants are underway as well as studies with F508del-CFTR correctors.
Altogether, results from this study will help to elucidate the structural changes that account for the F508del defect and the mechanism of action of correctors.

Work supported by PIC/IC/83103/2007 grant and BioFIG - Centre for Biodiversity, Functional and Integrative Genomics (FCT, Portugal*). ACDP is a recipient of PhD fellowship SFRH/BD/17475/2004 (FCT,* Portugal*).*

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[1] Lewis H *et al* (2004) *EMBOJ* **23**:282-93.
[2] Lewis H *et al* (2005) *JBiolChem* **280**:1346-53.
[3] Gadsby *et al* (2005) *JGenPhysiol* **125**.43-55.

S1.5 - Role of the C-terminal Extremity of CFTR NBD1 in the Control of CFTR Activity

Arnaud BILLET[1], Mathilde Jollivet[1], Jean Paul Mornon[2], Isabelle Callebaut[2], Frederic Becq[1]

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[2]IMPMC, Université Pierre et Marie Curie Paris 6, UMR 7590, Paris, France

Cystic fibrosis (CF) is caused by mutations of the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). Mutations of the *CFTR* gene affect the trafficking of the protein and/or its function. It has already been shown that the fragment of NBD1 comprising the amino acids 622 to 634 is crucial for the maturation process of CFTR proteins (Chan et al., 2000). In this study we have investigated the role of the last β hairpin of NBD1 and its direct environment in the activation of CFTR channel. We performed a molecular dissection of CFTR by examining several CFTR mutants constructed by site directed mutagenesis and expressed as GFP-tagged proteins in HEK293 and BHK cells. These mutants, located in the last β hairpin of NBD1 or in contact with it, were studied downstream the β_c5 (H620Q), inside the hairpin (E621G), and within the β_c6 (S623A & S624A) or in contact with H620 (S459A in the Walker A of NBD1and F640A). Patch clamp analysis using whole cell configuration revealed a time and voltage independent Cl^- current stimulated by forskolin (Fsk) for all CFTR mutants. All the currents were blocked by 10 µM of $CFTR_{inh}$-172. The Cl^- current (I) elicited by E621G and S624A mutants was similar to wt current. We determined I at +40 mV for wt, 83.3±9.7 pA/pF; E621G, 84.2±10.1 pA/pF; S624A, 81.8±7.7 pA/pF. On the contrary the mutations introduced on both sides of the hairpin (H620Q & S623A) or in conjunction with the amino acid H620 (S459A & F640A) results in a CFTR channel with an strong increased Cl^- channel activity (I at +40 mV: H620Q, 124.6±19.4 pA/pF; S623A, 142.8±16.7 pA/pF; S459A, 192.6±15.1 pA/pF; F640A, 178.3±5.8 pA/pF). Moreover, all the mutated channels with an increase Cl^- current present a faster time course of activation. These results indicated that the orientation of the two latest β strands and the link between this hairpin and other domains, like the walker A of NBD1, could play a role in the control of CFTR channel activation. All these results suggest an important influence of the C-terminal extremity of NBD1 into the control of CFTR channel gating.

Supported by Vaincre La Mucoviscidose, Ministère de l'enseignement supérieur et de la recherche and CNRS

S1.6 - Screening for F508del-CFTR Correctors Identified by *Pharmacophore Modelling* through Western blot and NBD1 Folding Assays

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Pharmacophore modelling based on virtual screening of compounds is a ligand-based approach particularly useful when the target protein 3D structure is unavailable. Using a shape fingerprint of the low-energy conformation of known CFTR correctors we screened ~6 million commercial compounds. The resulting hits were clustered by pharmacophoric fingerprint to yield a diverse set of 250 compounds which were subjected to biological evaluation. In parallel, we built a predictive protein docking model based on recently published X-ray structures to identify putative binding sites for F508del-CFTR correctors and compared the diverse set with the sites identified by the model.

The 250 small-molecule library was screened by two parallel medium-throughput methodologies: 1) quantitative Western blot (WB) to assess rescuing of F508del-CFTR processing in CFBE cells [1] (by appearance of its mature form); and 2) wt- and F508del-NBD1 folding and stability assays, using differential scanning fluorimetry. The latter determines the compound impact in NDB1 thermodynamic and kinetic stabilities, by measuring respectively, the shift in the melting temperature (DT_m), and aggregation rate at 37°C. A T_m increase reflects higher thermodynamic stability and a decrease in aggregation rate suggests higher the kinetic stability. "Hit" compounds should increase stability, reduce aggregation propensity and promote maturation.

Sensitivity of the WB method was given by incubating cells with known F508del-CFTR correctors: VRT-325 (6.7mM;24h); C4a (10mM;6h), showing that 5-6 wells of a 96-well plate (~2-3x10^5 cells) suffice to detect F508del-CFTR maturation at 37ºC (9%;12%, respectively). In the screen, cells from 5 wells were pooled after compound incubation (10mM;24h, 37ºC and 26ºC), extracts prepared and WB performed with the 596 anti-CFTR antibody. The screen identified 4 primary "hit" compounds causing extensive rescue and/or an impressive stabilization of NBD1, respectively (% of band C rescue vs DMSO control; DT_m) TS-01-01-C9 (70%; 3.4ºC); TS-01-01-B9 (160%; 1.6ºC); TS-01-01-C5 (90%; 1.5ºC), TS-01-01-E12 (114%; 1.5ºC).

The effect of these compounds on F508del-CFTR trafficking was also assessed by a microscopy-based assay performed at 26°C in A549 cells expressing a double tagged flag/mCherry CFTR construct under an inducible (Tet-ON) promoter. The ratio of total CFTR at the membrane (assessed by an anti-Flag antibody without cell permeabilization) over the total CFTR expressed (given by the mCherry fluorescence) was determined per cell. Values were (±SEM): DMSO - 0.415 ± (0.008); Corr-4a - 0.589 (± 0.009); C9 - 0.445 (± 0.004); C5 - 0.373 (± 0.004); E12 - 0.497 (± 0.005); B9 - 0.387 (± 0.004). We could confirm by this strategy rescue of F508del-CFTR by TS-01-01-E12, which showed additive rescuing to low temperature. A secondary library of 46 compounds derived from these 4 lead primary "hits" is currently under assessment.

Pharmacophore modeling appears as a powerful method to rapidly identify new compounds correcting F508del-CFTR maturation/folding defects and substantially reduces the number of compounds screened.

Work supported by TargetScreen2 (EU-FP6-LSH-2005-037365) grant. Authors thank CFF-USA for correctors (B.Bridges, Chicago, USA) and antibody (J.Riordan, UNC, USA) and the CFTR Folding Consortium (P.Thomas, Texas, USA) for NBD1.

Reference:

[1] Bebok Z *et al* (2005) *J Phys* **569**: 601-615.

SYMPOSIUM 2 – WT and F508del CFTR trafficking – learning the rules of the road
Chairs: G. Lukacs (CA) / M. Gentzsch (US)

S2.1 - Preferential Sumoylation of ΔF508 CFTR and NBD1 Leads to Protein Degradation

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The cystic fibrosis transmembrane conductance regulator (CFTR) was the first mammalian membrane protein implicated as a substrate for endoplasmic reticulum associated degradation (ERAD), and it has served as a model for the folding or disposal of polytopic membrane proteins. Steps in ERAD usually include the recognition and removal of misfolded proteins from the ER, followed by their ubiquitylation and proteasome-dependent degradation. Due to its complex folding and domain assembly requirements, much of WT CFTR and ~100% of the common folding mutant, F508del CFTR, are degraded in most systems.

Small heat shock proteins (sHsps) bind destabilized proteins during cell stress and disease, but their physiological functions are less clear. Hsp27 is expressed in airway epithelial cells, and it selectively interacted with the common CFTR mutant, F508del, and targeted it for proteasomal degradation. This action of Hsp27 appears to result from its ability to interact physically with the SUMO E2 conjugating enzyme, Ubc9, and like Hsp27, Ubc9 selectively promoted F508del CFTR proteolysis. Similarly, the knockdown of Hsp27 or the SUMO E1 enzyme, SAE1/2, increased CFTR expression 2-3 fold. Hsp27 expression promoted the sumoylation of F508del CFTR in vivo, and disabling the SUMO pathway via SUMO E1 knockdown reduced the ability of Hsp27 to degrade mutant CFTR.

To begin to evaluate the properties of CFTR that lead to its sumoylation/degradation, we determined the modification of CFTR NBD1 by SUMO-1 in vitro. The reaction mixture included the purified components: E1, E2, SUMO-1, WT or F508del NBD1 and ATP. NBD1 sumoylation was detected either by the molecular mass shift observed upon blotting with an NBD1 antibody or from blots performed using anti-SUMO-1. SUMO modification of NBD1 increased as a function of time over a 0-120 min assay period, with significantly greater modification observed for F508del vs. WT NBD1. Reactions run at different ATP concentrations demonstrated the expected ATP dependence of NBD1 modification, and showed also that WT NBD1 sumoylation increased at low ATP whereas the F508del NBD was less sensitive to ATP in the range 0.1-2 mM. Thus, the native NBD1 conformation, as exemplified by the WT domain at high ATP, was a poor substrate for SUMO modification relative to F508del NBD1. These observations, together with the eight degree lower melting temperature of the F508del NBD1, suggest that the native NBD1 conformation is not modified by SUMO, whereas non-native conformational intermediates are preferentially sumoylated.

These findings link sHsp-mediated mutant CFTR sumoylation to protein degradation, and they raise the possibility that folding intermediates formed during CFTR biogenesis are stabilized by SUMO addition until the native conformation is obtained.

S2.2 - F508del-CFTR Increases Intracellular Ca^{2+} Signaling that Causes Enhanced Calcium-dependent Cl^- Conductance in Cystic Fibrosis

Joana Raquel Martins[1,2], Patthara Kongsuphol[2], Joana Almaça[1], Fadi Aldehni[2], Luka Clarke[1], Rainer Schreiber[2], Margarida Amaral[1,3], Karl Kunzelmann[2]

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Increase in intracellular calcium $[Ca^{2+}]_i$ activates a chloride conductance (CaCC) that has been shown to be enhanced in cystic fibrosis (CF). An ongoing controversy exists as to the reason of enhanced CaCC in CF. As shown earlier it may be due to infection of CF airways as well as accumulation of mutant cystic fibrosis transmembrane conductance regulator (F508del-CFTR) in the endoplasmic reticulum (ER), which may cause a ER stress response. In nasal epithelial cells of patients homozygous for F508del-CFTR we did not detect an increase in expression of the Ca^{2+} activated Cl^- channels TMEM16A and bestrophin 1. Also expression of plasma membrane localized TMEM16A or ER-localized bestrophin 1 was not different between CF bronchial epithelial (CFBE) cells stably expressing wtCFTR or F508del-CFTR. However, purinergic calcium signaling was enhanced in CFBE/F508del-CFTR cells. Exposure of CFBE cells to bacterial lipopolysaccharides (LPS) had only marginal effects on intracellular calcium signaling.
However, the $[Ca^{2+}]_i$ augmenting effect of F508del-CFTR was confirmed in the human airway epithelial cell line A549 after induction of expression of F508del-CFTR. We found that UTP-induced increase in $[Ca^{2+}]_i$ was significantly reduced in chloride-depleted cells. Cl^- dependence of the intracellular Ca^{2+} signaling is reminiscent of the role of bestrophin1 for intracellular Ca^{2+} signaling. As shown earlier (Barro-Soria et al, Pflügers Archive 1999), bestrophin1 is an ER-localized Cl^- channel that facilitates Ca^{2+} release from the ER by acting as a counter ion channel. Our data suggest that ER-trapped F508del-CFTR may similarly function as an ER-localized Cl^- channel, which may be the mechanism how F508del-CFTR enhances intracellular Ca^{2+} signaling. An additional reason for the increase in $[Ca^{2+}]_I$ in F508del-CFTR expressing cells may be binding of F508del-CFTR to the inositol-1,4,5-trisphosphate [IP3] receptor binding protein released with IP_3 (IRBIT). As shown previously IRBIT binds to and regulates CFTR (Yang et al, JCI 2009). Overexpression of IRBIT attenuated UTP, i.e. Ca^{2+} activated chloride conductance in *Xenopus* oocytes; and this effect that was compensated by coexpression of F508del-CFTR. Taken together these data suggest that enhanced calcium-dependent chloride secretion in CF airways is due to an increase in intracellular Ca^{2+} signaling, which may be due to an ER-chloride conductance by ER-trapped F508del-CFTR and/or binding of IRBIT to F508del-CFTR and enhanced IP_3-signaling.
This study was supported by DFG SFB699-A7 and TargetScreen2 (EU-FP6-2005-LH-037365). JRM is the recipient of a fellowship from FCT, Portugal.

S2.3 - Sequential domain folding in wild-type and ΔF508 CFTR

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To study the folding process of wild-type and∆F508 CFTR we use a range of assays, in which we produce radiolabeled protein via pulse-chase in intact cells or via in vitro translation in the presence of digitonin-permeabilized cells as a source of ER membrane. Conformational changes are followed by subjecting the protein to mild protease treatment. The resulting protease-resistant fragments represent folded (sub)domains of CFTR, the identity of which we determine using C-terminally truncated CFTR constructs, isolated domain constructs, and epitope-specific antibodies as well as by mass spectrometric analysis on purified NBD1 domain. Using these assays and reagents we examined the conformational changes CFTR's domains go through over time after synthesis, and their relationship under various conditions, including temperature and intragenic suppressor mutations.

S2.4 - - CFTR Folding in Vitro and in Vivo: Implications of Cooperative Domain Assembly

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The cystic fibrosis transmembrane conductance regulator (CFTR) architecture consists of two membrane spanning (MSD1 and 2), two nucleotide binding (NBD1 and 2) and a regulatory (R) domain. Most CF mutations lead to the channel misprocessing yet the structural basis of the folding defect remains largely unknown. To assess the contribution of domain-wise and cooperative domain folding mechanism, we determined a) the minimal domain combination that is recognized as native fold and can escape the ER associated degradation, b) the impact misfolding on conformational coupling among domains and c) the relationship between the thermostability and the biosynthetic processing of the NBD1, as well as the full-length channel. One-, two-, three- and most of the four-domain assemblies were retained at the ER. The smallest folding unit that traversed the secretory pathway was composed of CFTR lacking the NBD2 either as a linear or split polypeptide. Missense mutations in the MSD1, NBD1, MSD2 or NBD2 all caused conformational defect in multiple domains, implying interdependent domain folding. At the isolated domain level, the ΔF508 mutation destabilized the fold that was rescued by “solubilization” mutations. While second site mutations restored the isolated ΔF508-NBD1 stability close to that of the wt NBD1, they caused disporportionally smaller reversion on the ΔF508-CFTR biosynthetic processing. We propose a model that incorporates cotranslational domain-wise and posttranslational cooperative folding steps during the biogenesis of CFTR, a mechanism that provides a plausible explanation for the global misfolding caused by numerous CF mutations. The results also reinforce the notion that correction of both the ΔF508-NBD1 stability and the domain-assembly defect is required for efficient therapy in CF.

S2.5 Repair of CFTR Folding Defects Caused by Cystic Fibrosis Mutations

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Cystic fibrosis (CF) is primarily caused by deletion of Phe508 (ΔF508) in the first nucleotide-binding domain of CFTR (*1*). Repair of ΔF508-CFTR would prevent CFΔ F508-CFTR has three major defects that need to be repaired: 1) The mutant is defective in folding and delivery to the cell surface; 2) It has low channel activity compared to the wild-type protein; and 3) The mutant is unstable at the cell surface. In previous studies on the equivalent mutant (Δ Y490) in the structurally similar P-glycoprotein (P-gp) drug pump, we found that defects in folding and activity could be specifically and efficiently repaired when the protein was expressed in the presence of compounds that bound directly to the protein (*2*). Our goal has been to learn how processing mutations inhibit folding and how direct binding of compounds repair these defects.

In cross-linking studies, both the CFTR and P-gp deletion mutants appeared to be trapped as folding intermediates with incomplete domain-domain interactions (*3*). Defective domain-domain interactions in ΔF508-CFTR could be repaired by carrying out expression in the presence of correctors to promote maturation of the mutant (*4*) in the endoplasmic reticulum (*5*). Multiple correctors had an additive effect on maturation (*6*). If correctors promote folding of Δ F508-CFTR through direct interactions with the protein, then it should be possible to mimic these effects with suppressor mutations. Changing Val510 in ΔF508-CFTR to an acidic residue promoted maturation of the protein (*7*) and caused a 4-fold increase in stability at the cell surface (unpublished observation). These results suggest that specific interaction of correctors with Δ F508 could promote maturation and increase its stability at the cell surface. Specific rescue would reduce potential side effects because they would be less likely to alter expression or activity of other proteins.

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Supported by Canadian Institutes of Health Research (Grant 62832). DMC is the recipient of the Canada Research Chair in Membrane Biology.

S2.6 - Mechanisms of F508del-CFTR Rescuing by Genetic Revertants, Low Temperature and Small Molecules

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CFTR bearing F508del, the most common CF-causing mutation, is retained intracellularly at the endoplasmic reticulum (ER) due to ineffective folding and sent to premature degradation through the ubiquitin proteasome pathway (UPP). Accordingly, most mutant protein fails to reach its proper location at the plasma membrane. Several approaches have been used in the recent years to rescue the misfolding and trafficking defects of the F508del-CFTR mutant and to understand the underlying mechanisms. These strategies either improve folding or circumvent the ER quality control (ERQC) that targets F508del-CFTR to UPP, or both. They include: 1) low temperature incubation (26ºC); 2) presence of genetic revertants (second-site mutations that either remove ERQC retention signals or stabilize its folded conformation); and 3) small molecules (interacting directly with F508del-CFTR to favour correct folding and/or indirectly to promote traffic). Understanding how F508del-CFTR is rescued to the cell surface by distinct agents enables us to mimic such effects by small molecules. Moreover, identifying distinct rescuing mechanisms, will possibly allow usage of different therapeutic molecules for enhanced results.
Our aim is to assess the synergistic/additive effects of F508del-CFTR correctors with those of several genetic revertants of F508del-CFTR and of low temperature incubation to learn more about their mechanism of action (MoA). As an example, we tested here the VRT-325 small molecule.
To this end, the following single F508del-CFTR revertants (stably expressed in BHK cells) were tested: I539T; G550E; R553M; R553Q; R555K; R1070W. Also the following multiple revertants: 4RK; G550E/R553Q; R553M/R555K; R553Q/R555M; 4RK/G550E; F494N/Q637R; and F429S/F494N/Q637R. Western Blot was performed after incubating these cell lines at 26ºC for 48h and/or treatment with VRT-325 (6.7µM; 24h). Densitometry was used to calculate for each variant the percentage of band C vs. the total of CFTR expressed. Data were normalized to band C percentage in BHK cells expressing wt-CFTR. Results so far regarding efficacy of F508del-CFTR rescuing show that: i) R553M is the most effective single genetic revertant; ii) R553Q/R555K is the most effective double revertant combination; iii) VRT-325 is less efficient than low temperature. Regarding additivity of different rescuing agents; iv) low temperature additivity is evident for all the genetic revertants tested except R553M and is most evident for G550E; v) VRT-325 exhibits highest additivity with the G550E revertant; vi) the only genetic revertants exhibiting additivity with both low temperature and VRT-325 are R1070W, R553M/R555K and G550E/R553Q; vii) 4RK/ G550E restores F508del-CFTR processing to wt-CFTR levels. Results iv) and v) suggest that rescuing by these agents takes place by distinct MoA.
Altogether, these data contribute to further elucidate the MoA of F508del-CFTR correctors, as exemplified here for VRT-325, and its relationship with specific residues critical for CFTR folding.

Work supported by PIC/IC/83103/2007 grant and BioFig pluriannual funding (FCT, Portugal). MS is recipient of SFRH/BD/35936/2007 fellowship (FCT, Portugal). Authors thank CFF-USA for correctors (B.Bridges, Chicago, USA) and antibody (J.Riordan, UNC, USA) as well as P.Haggie (UCSF, CA USA) for the YFP plasmid.

Thursday 8 April – 14:30-16:15 **Room: Esmeralda**

SYMPOSIUM 3 – Ion transport networks in epithelia
Chairs: K. Kunzelmann (DE) / B. Stanton (US)

S3.1 - Physiology of Anoctamins

Karl Kunzelmann, Inna Uliyakina, Yuemin Tian, Patthara Kongsuphol, Jiraporn Ousingsawat, Rainer Schreiber

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TMEM16A and bestrophin 1 are Ca^{2+} activated chloride channels. In epithelial cells, bestrophin 1 controls receptor mediated Ca^{2+} signaling by facilitating Ca^{2+} release from the endoplasmic reticulum, thereby indirectly activating membrane localized TMEM16A currents. In contrast to bestrophin 1, the Ca^{2+} activated Cl^- channel TMEM16A (anoctamin 1, ANO1) shows most of the biophysical and pharmacological properties that have been attributed to Ca^{2+} dependent Cl^- channels in various tissues. Moreover, TMEM16 proteins appear to be a crucial component of epithelial volume regulated Cl^- channels and may also have a function during proliferation and apoptotic cell death. The calcium-activated chloride channel anoctamin1 (ANO1; TMEM16A) is fundamental for the function of epithelial organs. Mice lacking ANO1 expression exhibit transport defects and a pathology similar to cystic fibrosis. They also show an impressive general defect of epithelial electrolyte transport. Because of its role for Ca^{2+} dependent Cl^- secretion in human airways, it is likely to become a prime target for the therapy of cystic fibrosis lung disease, caused by defective cAMP-dependent Cl^- secretion. When we analyzed expression of all ten anoctamins (ANO1- ANO10) in a broad range of murine tissues, we detected predominant expression of ANO1, 6, 7, 8, 9, 10 in epithelial tissues, while ANO2, 3, 4, 5 are common in neuronal and muscle tissues. In Fisher Rat Thyroid (FTR) cells, most ANO proteins localized to the plasma membrane but only ANO1, 2, 6 and 7 produced a Ca^{2+} activated Cl^- conductance. Patch clamping of ANO-expressing FRT cells indicated that apart from ANO1 also ANO6 and 10 produced chloride currents, albeit with very different Ca^{2+} sensitivity and activation time. We conclude that each tissue expresses a set of anoctamins that form cell and tissue specific Ca^{2+} dependent Cl^- channels.

S3.2 - Slc26 anion transporters involvement in acid-activated duodenal HCO_3^- secretion

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Backround: HCO_3^- secretion protects the proximal duodenum against damage by gastric acid. Duodenocytes express CFTR as well as at least three members of the SLC26 family (Slc26a3, 6 and 9) in the apical membrane.
Aim and methods: To establish the importance of the different apical anion transporters during basal, acid- and forskolin (FSK) - stimulated duodenal HCO_3^- secretion *in vivo*, CFTR-, Scl26a3, 6 and 9-deficient mice and WT littermates were anesthetized, the proximal duodenum was luminally perfused with saline, 10^{-4} M FSK, or pH 2.2 for 5 min, and HCO_3^- was continuously titrated in the perfusate during controlled systemic acid/base parameters. The relative crypt and villous expression of the transporters was measured by immunohistochemistry, laser micro-dissection and qPCR.
Results: Basal duodenal HCO_3^- secretion was slightly reduced in the *Slc26a6*$^{-/-}$, and more strongly reduced in the *Slc26a3*$^{-/-}$, *Slc26a9,* and CFTR-deficient mice compared to WT littermates. FSK-stimulated secretory response was normal in *Slc26a6*$^{-/-}$, *Slc26a3*$^{-/-}$, *Slc26a9*$^{-/-}$ mice, and was virtually abolished in CFTR-deficient mice. Surprisingly, acid-activated HCO_3^- secretion was unaltered in the *Slc26a6*$^{-/-}$ duodenum, but strongly reduced in *Slc26a3*$^{-/-}$ and *Slc26a9*$^{-/-}$ duodenum, and abolished in the CFTR-deficient duodenum. Laser micro-dissection and immunohistochemistry revealed a villous-predominant expression of Slc26a6 and Slc26a3 and crypt-predominant expression of CFTR and Slc26a9.
Conclusions: Genetic deletion of intestinal Slc26 anion transporters reveals their differential involvement in basal, acid-stimulated and FSK-stimulated HCO_3^- secretion in murine duodenum. Most likely, different signalling in acid- vs FSK-stimulated HCO_3^- secretion explains this differential involvement, rather than the differential crypt-villus expression pattern.

S3.3 - Interaction of ENaC and CFTR

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In CF airway epithelia, deficient CFTR channel activity and the absence of CFTR-dependent inhibition of the epithelial sodium channel (ENaC) result in dehydration of airway surfaces. While it has been established that mutations in CFTR result in defective regulation of ENaC and consequent sodium hyperabsorption in CF airways, the detailed mechanism by which CFTR mediates inhibition of ENaC remains unclear. In our studies we have applied electrophysiological measurements, biochemical techniques and confocal immunofluorescence microscopy to illuminate the regulation of ENaC by CFTR.

Co-immunoprecipitation experiments confirmed that a variety of different CFTR fragments physically interact with ENaC, however, not all of these fragments were capable of modifying ENaC function as assessed by electrophysiological measurements on *Xenopus laevis* oocytes. To confirm the physiological relevance of ENaC-CFTR interactions, we have made use of highly specific antibodies to assess association of these ion channels in native epithelia.

Furthermore, we found that individual ENaC subunits (α, β, γ), when overexpressed in BHK-21 cells, reside exclusively at the Endoplasmic Reticulum, however, co-expression of all 3 subunits resulted in surface expression and co-localization with CFTR at the plasma membrane. Insertion of an extracellular tag into the γ-subunit of ENaC and labeling with an antibody on intact cells allowed confirmation of the surface localization and also permitted monitoring of intracellular trafficking of ENaC. Similar to CFTR, ENaC is rapidly internalized from the plasma membrane and transfers to endocytic compartments. Currently, we are investigating whether CFTR regulates ENaC activity at the cell surface by modulation of its processing and intracellular trafficking.

S3.4 - Regulation of ENaC by the Stress Response Protein SERP1

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Cystic Fibrosis (CF) is characterized by enhanced Na^+ absorption in the airways, which contributes to reduced airway surface liquid (ASL) and compromised mucociliary clearance. Therapeutic strategies have been developed to overcome enhanced Na+ absorption by directly inhibiting the epithelial Na+ channel (ENaC). Current results from those strategies demonstrate only limited success, which prompted us to search for alternative strategies. In the present study we identified SERP1 (stress-associated ER protein 1, also known as RAMP4, ribosome-associated membrane protein 4) as a novel binding partner of ENaC in a split-ubiquitin screening using ß-ENaC as bait. Expression of SERP1 was previously shown to be enhanced during cellular stress, causing accumulation of unfolded proteins in the endoplasmic reticulum (ER). SERP1 is homologous to yeast suppressor of SecY 6 protein (YSY6), a suppressor of a secretory pathway mutant, which suggests a role in pathways controlling membrane protein biogenesis at the ER level. SERP1/RAMP4 also controls biogenesis by interacting with the molecular chaperone calnexin.
Here, we examined the role of SERP1 in regulating ENaC-expression. Using amiloride-induced change in FMP fluorescence as a read-out for the amount of amiloride sensitive epithelial Na+ conductance (FMPAmil), we found that treatment of both H441 and A549 cells with 3 different SERP1 siRNAs significantly enhanced FMPAmil. This enhancement, however, seems to be attenuated (A549 cells) in the presence of a cocktail of protease inhibitors, but not by the endocytosis inhibitor dynasore. Curiously, steady-state expression of ßEnaC is reduced. In contrast, overexpression of SERP1 largely reduced FMPAmil. Amiloride-sensitive transport was also measured in Ussing chamber experiments on H441 cells grown on permeable supports. Treatment of the cells by SERP1-siRNA increased the amiloride short-circuit current. SERP1 was found to co-localize in the ER with all 3 ENaC subunits as well as with calnexin. Taken together SERP1 appears to be a novel regulator of ENaC expression and a potential new target for drug therapy of CF.

Work supported by TargetScreen2 (EU-FP6-LSH-2005-037365) grant; SFB699 A6. DF, JA and SL are recipients of PhD fellowships SFRH/BD/43313/2008, SFRH/BD/29134/2006 and SFRH/BD/47445/2008, respectively (FCT, Portugal)

S3.5 - Development of a Multi-Cellular Co-Culture Model of Normal and Cystic Fibrosis Human Airways *in vitro*

Anne Bielemeier[1], Lindsay J Marshall[1]

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Introduction: Understanding the disease pathogenesis of CF currently relies on animal and/or single cell culture models. Whilst these approaches partially enable the study of CF, the models have inherent limitations.

Objectives: We intend to overcome some of these limitations by developing a multi-cellular co-culture model of human airways *in vitro*. Our co-culture model for non-CF airways uses human pulmonary fibroblasts (HPF) and human bronchial epithelial cells (Calu-3) grown at air liquid interface (ALI). This model is adapted to mimic CF by replacement of Calu-3 with IB3-1 (the CF cell line bearing delta F508/W1282X).

Methods:

Cell Viability: Optimal conditions for co-culture growth were defined by light microscopy, flow cytometry and a fluorescence-based assay after exposure to fresh and conditioned medium.

Cell Morphology: Cytokeratin (CK) antibodies were used to detect the specific epithelial cell markers, CK5 and CK8 in single and co-cultures. The presence and location of fibroblasts were confirmed with 1B10, an antibody specific for fibroblast surface antigens. Zonulae occludens-1 (ZO-1) staining was used as evidence of tight junction formation.

Cell Physiology: Differentiation to a "tight" epithelial cell barrier was monitored by measuring transepithelial electrical resistance (TER) over time at ALI. Apical fluid secreted by co-cultures was examined for the presence of mucin using a MUC5AC antibody.

Results and Discussion:

Cell Viability: Suitable culture medium has been defined for both co-culture models, demonstrated by maintenance of typical morphology, viability and growth characteristics.

Cell Morphology: CK staining indicates basal and differentiated epithelial cell populations after ALI has been established. ZO-1 staining indicates that tight junctions are present at the apical side of co-cultures, confirming that a polarised cell layer is formed.

Cell Physiology: Increasing TER indicates an increase in the barrier function of epithelial cell cultures and co-cultures but not for HPF in mono-culture. Mucin is secreted apically and preliminary results indicate mucus hypersecretion in CF models compared to non-CF.

These results are an invaluable starting point for developing an *in vitro* model employing multiple, human cell types for further investigation of CF. We envisage that our models will be useful for comparing the response of CF and non-CF airways to infection and inflammation.

We would like to thank the Humane Research Trust for financial support.

S3.6 - Spleen Tyrosine Kinase (SYK) Phosphorylates CFTR NBD1 and Interacts in vivo with CFTR

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Regulation of CFTR intracellular trafficking as well as channel activation is the result of a complex network of CFTR interacting proteins, that include molecular chaperones, glycosidases, the basal trafficking machinery and other factors, among which protein kinases and phosphatases are particularly relevant. Regulation of CFTR biogenesis/traffic by phosphorylation and dephosphorylation is still poorly understood. Spleen Tyrosine Kinase (SYK) is a non-receptor tyrosine kinase, described to have a role in signal transduction, and its consensus phosphorylation site consists of a tyrosine followed two negatively charged residues. In CFTR sequence, this consensus appears once at Tyr512 (i.e., very close to residue F508, deleted in most CF patients).

Our aim here was to identify the putative role of SYK on CFTR biogenesis and trafficking.

Firstly, by qRT-PCR analysis, we demonstrate that SYK is endogenously expressed in epithelial respiratory cells (Calu-3 and virally transduced wt-or F508del-CFBE), thus arguing for the physiological significance of this kinase in human airway epithelial cells. Next, data from CFTR co-immunoprecipitation followed by western blot (WB) for SYK (and vice-versa) showed that SYK interacts *in vivo* with CFTR. Then, *in vitro* phosphorylation, using immunoprecipitated SYK and purified CFTR-NBD1 or recombinant NBD1-Y512F, revealed that SYK undergoes auto-phosphorylation but that it also phosphorylates CFTR-NBD1 but not NBD1-Y512F. Furthermore, data also show that phosphorylation is abolished when dead-kinase SYK is used in this assay.

To further elucidate the biochemical and functional consequences of mutating Tyr512, we generated cell lines stably expressing CFTR mutants, where Y512 was substituted by either a neutral residue (Y512A and Y512F, mimicking the bulky side group of tyrosine) or by an acidic residue (Y512D, Y512E) both in the wt- and F508del-CFTR backgrounds. Pulse-chase experiments followed by CFTR immunoprecipitation and WB in these cells lines show that Y512A/D-CFTR have both decreased steady-state levels and efficiency of processing vs those of wt-CFTR, whereas Y512F-CFTR does not evidence significant differences neither in immature form turnover nor in its maturation efficiency. CFTR function of these mutants was also determined using the iodide efflux assay. Results show that cells expressing Y512A-CFTR and Y512D-CFTR have, respectively ~15% and ~60% reduction in the IBMX/Fsk-stimulated iodide efflux vs those expressing wt-CFTR.

Altogether, our data suggest phosphorylation of wt-CFTR by SYK plays a positive role in its stability and maturation and that this effect seems to be mediated by the consensus site for SYK at Y512-CFTR.

Work supported by TargetScreen2 (EU- FP6-LSH-2005-037365) grant and pluriannual funding of BioFig (FCT, Portugal). SL is recipient of PhD fellowship SFRH/BD/47445/2008 (FCT, Portugal).

Thursday 08 April – 16:45-18:15 **Room: Esmeralda**

SPECIAL GROUP DISCUSSION I
Modulator insights into wt and F508del CFTR behavior
Moderators: L. Galietta (IT) / I. Braakman (NL)

Mutant CFTR is a druggable target. In particular, the gating defect associated with class III mutations can be overcome with drug-like compounds called potentiators. These compounds, also identified by high-throughput screening of large chemical libraries, enhance the activity of mutant CFTR channels to normal levels. The trafficking defect caused by the deltaF508 mutation can be also ameliorated by small molecules called correctors. However, deltaF508 correctors are typically more difficult to identify than potentiators and their efficacy is also smaller. Furthermore, it is not clear whether correctors act by binding directly to CFTR or by modulating the activity and/or expression of other cell proteins.

This Discussion Group Session will address the following issues:

1) Do the correctors and potentiators work in any cell? Are the molecular mechanisms related to deltaF508 mistrafficking universal or cell type dependent?

2) Are the cell models used to study deltaF508 representative of the defect in vivo?

3) Why do all correctors rescue deltaF508 only to a maximum of 15%? Does this tell us something about the defect or about the rescue?

4) How do we find out what is needed for increased rescue?

Thursday 08 April – 16:45-18:15 **Room: Rubi B**

SPECIAL GROUP DISCUSSION II
CF biomarkers now and on the horizon
Moderators: C. De Boeck (BE) / J.P. Clancy (US)

Currently available CF therapies target symptoms that result from CFTR dysfunction, and their systematic application has lead to steady improvements in CF patient longevity and quality of life. Novel strategies that target mutant CFTR proteins (ie: CFTR modulators) and restore CFTR function are currently in a number of clinical trials, and their use requires the development of novel CFTR biomarkers to detect biologic effects and to link their mechanism of action to clinical outcome measures. Currently, there are only two CFTR biomarkers that have frequently been used in multi-center clinical trials of CFTR modulators, including sweat chloride [Cl-] measurements and the nasal potential difference measurement (NPDs).

The sweat [Cl-] is an indirect measure of CFTR activity in that it relies on retained activity of the epithelial sodium channel (ENaC) for Cl- transport through CFTR. It is an attractive biomarker, however, due to it's lack of CF tissue pathology and thus potentially high sensitivity to detect CFTR effects.

The NPD is capable of isolating CFTR-dependent Cl- transport independent of ENaC and CFTR regulation is similar compared with the lower airway, but typically demonstrates a lower signal/noise ratio than sweat [Cl-] measures, and is technically more demanding for study subjects and operators.

Both of these CFTR biomarkers are undergoing optimization for use in clinical trials, including the development of standard operating procedures (SOPs) for assay conduct, hands-on training sessions, development of sequential qualification steps for operators, and centralized over-read/interpretation of data. The results from recently conducted Phase II trials of CFTR modulators (eg: PTC124, VX-770, VX-809) indicate that both assays are capable of detecting biologic effects of modulators *in vivo*, but their capacity to detect bioactivity has varied across studies depending on the nature of the modulating strategy, study sites, and the specifics of assay conduct. In addition, the relationship between restored CFTR activity as measured by sweat [Cl-] and the NPD compared with clinical outcome measures remains unclear.

In this Special Group Discussion, recent developments in the use of these assays in CFTR modulator trials will be discussed, identifying areas for research and optimization. In addition, experience with new and novel CFTR biomarkers will be presented and discussed by SGD participants, including GI outcome measures (Intestinal Current Measurements – ICM, biochemical detection of CFTR, and real time RT-PCR quantification of CFTR transcript levels in rectal biopsies), detection and localization of CFTR by immunostaining in patient samples, emerging imaging modalities, and potential markers of CFTR expression and activity in blood samples. It is clear that continued identification, characterization, and standardization of new CFTR biomarkers is needed. Defining the relationships between CFTR biomarkers and clinical outcome measures will enhance the conduct of CFTR modulator clinical trials, and accelerate bringing new therapies to CF patients.

Friday 09 April – 08:45-10:30 **Room: Esmeralda**

SYMPOSIUM 4 – Mucus Biogenesis and disruption in CF
Chairs: C. W. Davis (US) / M. Gray (UK)

S4.1 – Airway Goblet Cells: Mucin Synthesis and Secretion

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The polymeric mucins, MUC5AC and MUC5B, synthesized and secreted from goblet cells and submucosal glands in the airways epithelium are synthesized in the endoplasmic reticulum as dimers, C-terminally bonded via cysteine knot domains. In the Golgi apparatus the mucin dimers are polymerized via N-terminal D domains, heavily O-glycosylated, and released from the trans-Golgi network as transport vesicles that coalesce into immature mucin secretory granules (immature SMGs). Following maturation in goblet cells, the ~1 μm mature SMGs are stored until ATP or UTP, activating the apically located $P2Y_2$-R/Gq/PLC signalling complex, triggers their Ca^{2+}-dependent exocytic release. Treatment of airway epithelium with maximal doses of agonist elicits a full emptying of mucin stores in ~15 min; recovery of mucin stores begins 4 hr later, with maximal mucin synthesis between 8 – 16 hr. Full recovery of mucin stores is achieved in 36 – 48 hr. Exocytosis of SMGs appears to utilize the same families of SNARE proteins that underlie neurotransmission and endocrine and exocrine secretion, SNAP25, Vamp, and Syntaxin, with SNARE assembly and priming regulated by Munc18, Rab3 and Munc13, complexin, and the Ca^{2+} sensor, synaptotagmin. Interestingly, recent and emerging data from the airways of genetically manipulated mice suggest that there are at least two pathways of regulation, one of which may relate to an important, but under appreciated baseline mode of secretion. Clara cells in tracheas of WT mice under control conditions lack PAS+ mucin stores; however, sensitizing and challenging mice with ovalbumin (OVA) induces mucous metaplasia, in which Clara cells become engorged with PAS+ mucin stores. Clara cells of non-OVA treated mice deficient for $P2Y_2$-R, nPKCε, and synaptotagmin 2 (Syt2) are similar to WT controls in lacking PAS+ staining, but mucin secretion in the same deficient mice treated with OVA is substantially inhibited. Munc13-2 null mice exhibit a different pattern: non-OVA treated Clara cells are PAS+, whereas these same cells, as well as Clara cells in mice treated with OVA, exhibit no inhibition of agonist-stimulated mucin secretion. Likely, the other Munc13 isoform expressed in airways epithelium, Munc13-4, compensates for the lack of Munc13-2 in agonist-induced mucin secretion. Munc13-2 appears essential for release of mucins at baseline, with PAS+ mucin stores accumulating in its absence due to the loss of a basal secretory pathway. Hence, we speculate that Munc13-4 and Syt2 define the agonist-regulated pathway for mucin secretion, and Munc13-2 and an unidentified Syt underlie the pathway for basal secretion.

S4.2 - How does mucin biogenesis contribute to mucus accumulation in CF?

Sally Williamson[1], Luke Bonser[1], C. William Davis[2], John K. Sheehan[2] and David J Thornton[1]

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The airways mucus gel performs a critical function in the innate defense of the respiratory tract against environmental challenges (1), and the physical properties of this mucin-rich layer are vital for normal mucociliary transport. In CF, accumulation of mucus which can tether to the airway surface is an important pathologic feature; resulting in an environment within which bacteria can flourish and the chronic inflammatory reactions that follow lead to airway damage.

In normal physiology, the polymeric mucins MUC5AC and MUC5B provide the organising framework of the airways mucus gel and are major contributors to its rheological properties (2). Entanglements, along with specific non-covalent interactions, of these very large, disulphide-linked polymers are key elements in mucus gel formation (3). The macromolecular organisation of MUC5AC and MUC5B are likely to profoundly affect mucus properties. No studies have identified changes in mucin macromolecular properties that fully explain the sub-optimal mucus transport properties. However, there are some clues, in particular the MUC5B mucin that was isolated from a mucus gel-plug following death due to *status asthmaticus* (4). These mucins exhibited an unusual branched, cross-linked morphology in the electron microscope, rather than the more usual linear thread-like appearance of 'normal' polymeric mucins isolated from sputum. We hypothesised that this apparent change in macromolecular architecture might, explain the viscid nature of the mucus gel in this situation. Importantly, Sheehan and co-workers have proposed that this cross-linked form of MUC5B is an incorrectly processed intracellular stored form of the mucin that normally undergoes proteolytic modification to allow efficient unfolding of the mucin polymer, and that it is the disruption of this process that may lead to abnormal mucus formation in CF (5).

Further insight into the potential functional role of proteolytic processing of the mucins comes from the literature on vWF. This polymeric glycoprotein shares similar domain organisation to MUC5AC and MUC5B and like the polymeric mucins vWF is stored in membrane granules within cells prior to regulated secretion. N-terminal cleavage of vWF generates a propeptide that is suggested to be important for condensation of this polymeric glycoprotein within the Wiebel-Palade storage bodies, where it acts to constrain the molecule in a 'coiled' state. After secretion, the pH-dependent dissociation of the propeptide allows unfurling of the polymer into the extracellular environment (6).

In this presentation data will be presented on the proteolytic processing and macromolecular form of the MUC5B mucin. These studies have been performed using MUC5B produced by primary bronchial epithelial cells in air-liquid interface culture (normal and CF cells) and MUC5B collected from freshly secreted saliva. Proteolytic processing of intra- and extracellular mucins was analysed using a combination of MUC5B mucin-domain specific antisera and tandem mass spectrometry of the purified mucin. These studies have revealed no evidence that MUC5B undergoes similar proteolytic cleavage to vWF. Using sucrose gradient centrifugation on MUC5B secreted from primary bronchial cells in culture (and from saliva), we have found that the macromolecular form of the mucin can vary depending on the time post-secretion and the extracellular environment.

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S4.3 – A new Assay for Modulation of Ion Transport and Mucus Clearance in Cells and Tissues: Video-Rate Ultrahigh-Resolution 3D Optical Imaging by Spectral Domain Optical Coherence Tomography

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Rationale: The study of mucociliary clearance in vitro and in vivo would be advanced by the development of a high-speed imaging technology for visualizing the respiratory mucosa at the cellular level, and could yield new insights into disease pathogenesis. Spectral domain optical coherence tomography (SD-OCT) is an emerging technique capable of providing reflectance images with subcellular resolution at video rates.

Methods: We have developed an SD-OCT system with an axial resolution of 1 μm and a transverse resolution of 2 μm. Cross-sectional images were acquired at a speed of 32 frames per second with 512 axial lines (reflectance as a function of depth) per image. We additionally obtained three-dimensional images by scanning the sample in two dimensions. Three-dimensional images of formalin-fixed, sectioned porcine and human (CF and non-CF) bronchial tissue segments were obtained immediately following prosection ex vivo. Fresh human bronchial tissues were also imaged while bathed in DMEM. Living fully-differentiated primary human airway epithelial cells (HAE) derived from CF and non-CF individuals were studied on air-liquid interface cultures in response to the CFTR potentiator VX-770 (10 μM basolateral addition x 24 hrs).

Results: In fixed tissues, a total of 512 cross-sectional images were acquired and digitally stored within 16 sec. Cilia were easily identified in the image and individual epithelial cells were also visible, including cellular morphology. Three-dimensional images of intact tissues showed gland ducts containing mucus. Imaging of living human tissues ex vivo demonstrated significant amounts of adherent mucus on the surface of CF tissues not present in normal and disease controls. Cilia beat frequency (CBF) was estimated at 3.5 Hz in CF and 4.3 Hz in control tissues ($P<0.05$, $n=5$). Mucus extrusion from glands was also observed. In HAE, airway surface liquid depth, CBF, and mucus transport rates were readily quantified. In CF epithelia, depleted ASL depth (4.1 μm vs. 16.4 μm, $P<0.001$, $n=20$) and reduced CBF (1.8 Hz vs. 4.6 Hz, $P<0.05$, $n=11$) was observed in comparison to WT HAE monolayers, each after 5 days of mucus accumulation. Delayed mucus transport was also observed in CF epithelia (0.1 mm/min vs. 3.7 mm/min, $P<0.01$). WT HAE cells demonstrated a robust response to VX-770, with an improvement in ASL depth (16.8 μm vs. 10.4 μm, $P<0.001$, $n=10$) and mucus transport (23.0 mm/min vs. 3.7 mm/min, $P<0.05$), and a trend towards increased CBF (5.5 Hz vs. 4.9 Hz). Results were confirmed by conventional measures of anion transport, ASL depth, and mucus transport rates.

Conclusions: Results demonstrate the potential of SD-OCT to provide video–rate subcellular imaging of fixed and living pulmonary epithelia without administration of contrast medium, and is highly sensitive to the effects of the CFTR potentiator VX-770 in living cells. The future development of a probe for in vivo monitoring of mucociliary transport, CBF, gland function, and ASL depth in vivo could provide new avenues for improving our understanding of respiratory mucosal pathophysiology in real-time, and enable longitudinal assessment of the response to novel CFTR modulators and other agents that restore normal ion transport.

S4.4 - Gap Junctions Contribute to Airway Surface Liquid Homeostasis in Human Airway Epithelial Cells

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The airway surface liquid (ASL) plays a critical role in lung defense and is required to ensure hydrated and clearable mucus. It is determined by active transepithelial salt transport, mainly involving cystic fibrosis transmembrane conductance regulator (CFTR) activation. CFTR activity is regulated by protease-activated receptors (PAR) at the basolateral membranes and adenosine receptors (ADO-Rs) at the apical membrane; both pathways involved the release of prostaglandin E2 (PGE2) and stimulation of their basolateral receptors (EP-Rs). Thus coordination of the signaling generated in airway cells by these G-protein coupled receptors (GPCRs) is critical for appropriate regulation of ASL height and efficient mucociliary clearance. In this context, gap junction (GJ) channels may contribute to the coordination of intercellular signaling since they provide direct aqueous pores between neighboring cells. Therefore GJs, which are formed by connexin proteins, allow intercellular spread of ions, nucleotides and second messengers for coordinating the tissue activity. In the submucosal gland Calu-3 cell line, we found a parallel regulation of CFTR and GJs in response to GPCRs stimulation. Indeed, activation and inhibition of ADO and PGE2 pathways enhanced and decreased, respectively, the extent of GJ intercellular communication. Moreover, inhibition of GJs prevented CFTR currents induced by PAR in Ussing chambers. Then, to investigate the consequences of GJ-CFTR interaction on fluid secretion in Calu-3 cells, we monitored ASL volume by confocal microscopy and images 3D reconstruction. Stimulation of Calu-3 cells with PGE2 increased ASL volume. This effect was dependent on CFTR activity, because PGE2 failed to enhance ASL volume in genetically modified Calu-3 cell line lacking CFTR. Interestingly, the PGE2-induced ASL volume increase was abolished in the presence of the GJ blocker, indicating that GJs mediate fluid secretion in Calu-3 cells via CFTR. Finally, to confirm the importance of GJs on ASL volume regulation in a cell system closer to the human airway physiology, we repeated the experiment on primary cultures of well polarized human airway epithelial cells (HAECs). HAEC cultures showed active Na^+-dependent absorption of ASL, which was blocked in the presence of amiloride. In agreement with the results obtained on Calu-3 cells, PGE2 induced an increase of ASL volume, which was prevented with the GJ blocker. Thus, our results show that GJs coordinate a signaling network, comprising CFTR, ADO-Rs, PARs and EP-Rs, which is required for ASL volume homeostasis in airway epithelial cells.

Supported by VLM, FNRS, FFC, Schmidheiny and Novartis Consumer Health.

S4.5 - The Switch of Intestinal Slc26 Exchangers from Anion Absorptive to HCO_3^- Secretory Mode is Dependent on CFTR Anion Channel Function

Anurag Kumar Singh[1], Brigitte Riederer[1], Mingmin Chen[1], Fang Xiao[1], Regina Engelhardt[1], Olof Nylander[2], Manoocher Soleimani[3], Ursula Seidler[1]

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CFTR has been recognized to function both as an anion channel and a key regulator of Slc26 anion transporters in heterologous expression systems. Whether this regulatory relationship between CFTR and Slc26 transporters is seen in native intestine, and whether this effect is coupled to CFTR transport function or other features of this protein has not been studied. The duodenum of anesthetized CFTR-, NHE3-, Slc26a6-, and Scl26a3-deficient mice and WT littermates were perfused and duodenal HCO_3^- secretion (DBS) and fluid absorptive or secretory rates were measured. The selective NHE3 inhibitor S1611 or genetic ablation of NHE3 significantly reduced fluid absorptive rates and increased DBS. Slc26a6 (PAT1) or Slc26a3 (DRA) ablation reduced the S1611-induced DBS increase and reduced fluid absorptive rates, suggesting that the effect of S1611 or NHE3 ablation on HCO_3^- secretion may be an unmasking of Slc26a6 and Slc26a3-mediated Cl^-/HCO_3^- exchange activity. In the absence of CFTR expression or after application of the CFTR(inh)-172, fluid absorptive rates were similar to WT, but S1611 induced virtually no increase in DBS, demonstrating that CFTR transport activity, and not just its presence, is required for Slc26-mediated duodenal HCO_3^- secretion. A functionally active CFTR is an absolute requirement for Slc26-mediated duodenal HCO_3^- secretion, but not for Slc26-mediated fluid absorption, in which these transporters operate in conjunction with the Na^+/H^+ exchanger NHE3. This suggests that Slc26a6 and Slc26a3 need proton recycling via NHE3 to operate in the Cl^- absorptive mode, and Cl^- exit via CFTR to operate in the HCO_3^- secretory mode.

S4.6 - Proteomics Characterization of Nasal Epithelial Cells: Their Use for Biomarkers Discovery in CF

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Background: CF is a monogenic multiorgan disorder but progressive lung disease accounts for 95% of morbidity and mortality among CF patients. Nasal respiratory epithelium is representative of airway system and epithelial cells account for approximately 80-95% of nasal brushing (NB) cellular content. Since nasal epithelium reflects characteristic ionic transport abnormalities of lower airways in CF dysfunction, characterization and comparison of NB cells proteome obtained from CF patients with mild or severe lung disease and healthy volunteers by a high-throughput proteomics technology is of great importance.

Experimental: Epithelial cells were collected by a brushing procedure[1] from nasal mucosa of non-CF healthy individuals (n=27), carriers of a single mutated *CFTR* allele (n=18) and CF patients with 2 identified mutations presenting Mild (n=10) or Severe (n=17) lung disease. Cytological characterization was achieved by Leishman staining and light microscopy and samples were pooled according to above criteria. Pooled NB samples were fractionated into soluble- or membrane-enriched fractions by differential centrifugation and global proteome profiling was achieved by using complementary gel-based and gel-free mass spectrometry (MS) proteomics: two-Dimensional Difference In Gel Electrophoresis (2D-DIGE) coupled to Matrix Assisted Laser Desorption/Ionization-Time Of Flight MS (MALDI-TOF/TOF) and 2D-Liquid Chromatography coupled to tandem MS (2D-LC-MS/MS) using a label-free strategy. Differentially expressed proteins were analyzed concerning their function, biological processes and cellular localization using dedicated software packages.

Results: Overall, 719 and 2525 proteins were identified by at least 2 peptides in soluble and membrane enriched fractions by LC-MS/MS, respectively. Of the identified proteins, 306 were present in both fractions. Proteins with positive identification in all groups under study were used to characterize this biological sample using Ingenuity Pathway Analysis (IPA) retrieving processes such as oxidative phosphorylation, cellular organization and locomotion or molecular transport. Epithelium-specific proteins such as KRT5, 8, 19, MUC-5AC, SCCA1 were identified as previously studied by our group[2]. Biological processes in which the differentially expressed proteins participate in are related to glycolysis/gluconeogenesis, glutathione metabolism, protein folding or pentose phosphate pathway for the soluble fraction and oxidative phosphorylation, metabolism of xenobiotics by Cyt. P450, retinol metabolism and cell junction signaling for the membrane fraction. Differentially expressed proteins are involved in cytoskeleton organization (b-actin), chronic inflammation (PLUNC, cathepsins or lysozyme C) or acting like molecular chaperones (GRP75 and HSP90AB1).

Proteins such as CBR1 and ALDH1A1 previously found by our group as associated with abnormal responses of CF mutant mice to injury [3] were also identified as desregulated in CF NB. The protein profiling of NB soluble fraction by 2D-DIGE was analyzed by dedicated software Progenesis SameSpots™ and the differentially expressed spots are currently under identification by MALDI-TOF/TOF MS.

Conclusions: Cell fractionation together with gel-based and gel-free proteomics approaches provided in-depth characterization of NB proteome that could be potentially involved in CF pathophysiology, allowing identification of candidate biomarkers for clinically manifested CF and distinguish it from other respiratory diseases.

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Friday 9 April – 11:00-12:50 **Room: Esmeralda**

SYMPOSIUM 5 – Inflammatory signalling in CF lung disease
Chairs: G. Cabrini (IT) & A. Mehta (UK)

S5.1 - Inflammatory signalling in CF lung disease

N.G. McElvaney

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Cystic fibrosis (CF) lung inflammation is characterised by interactions between inflammatory cells, mainly neutrophils, bacteria and their products and airway epithelium. This complex interaction gives rise to the severe cycle of inflammation seen on the CF airway epithelial surface which accounts for much of the morbidity and mortality associated with this condition. All three components of this inflammation are deregulated to some degree in CF. The CF neutrophil behaves in an abnormal fashion secreting increased quanta of proteases particularly neutrophil elastase (NE) which can cause direct damage to airway epithelium, increase mucus production, decrease ciliary clearance and inactivate host defence mechanisms such as immunoglobulins, complement and complement receptors. In addition NE and other proteases, which it up regulates, can inactivate many important locally produced antimicrobials and antiproteases such as secretory leukoprotease inhibitor (SLPI), elafin, lactoferrin, LL37 and defensins. Add to this the presence of bacteria and their secreted components such as Pseudomonas proteases, the proteases produced by anaerobes and the toxins produced by aspergillus and there is a potent mix of potentially toxic material on the airway epithelial surface. In addition many of these bacteria also possess ligands which can signal through the Toll like receptor pathways and other airway receptors to produce an enhanced inflammatory signal. The airway epithelial surface itself in CF is not a mere passive bystander but an active participant in inflammation with an ability to produce a wide variety of cytokines and antimicrobials all of which have a part to play in resolving and enhancing inflammation. In this presentation we will discuss the various inflammatory pathways activated in the CF lung and delineate the role of inflammatory cells, bacteria and airway epithelium in these processes. We will evaluate how these processes may be modulated with a view towards better understanding of the condition and the potential for new therapeutic interventions.

S5.2 –CFTR Intractome and Inflammation

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Abstract details are not authorized for inclusion

S5.3 - Pharmacological modulation of chemotactic signalling in respiratory models

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The hallmark in CF airway pathology is a characteristic neutrophil-dominated inflammation, with elevated concentrations of pro-inflammatory cytokines and chemokines, mainly interleukin-8 (IL-8). Excessive inflammation strongly contributes to progressive tissue damage, which is inefficiently managed by broad-spectrum anti-inflammatory drugs such as corticosteroids and ibuprofen.

CF bronchial epithelial cells are considered critical participants of the inflammatory response since they express mutated CFTR protein, which has been proposed to promote a constitutive inflammatory response. In parallel, once infection is established, bronchial epithelial cells sense bacterial components through Pattern Recognition Receptors and transduce the proinflammatory signals, activating a redundant series of kinases and adapters, ultimately leading to the activation of nuclear trascription factors and expression of pro-inflammatory genes.

In order to find innovative drugs specifically tailored to reduce the excessive inflammation in CF lung without reducing the anti-infective defences, we promoted a cooperation network to tackle the complex interactions of the *P.aeruginosa*-dependent inflammatory response in bronchial epithelial cells.

Results will be therefore presented as brief examples on:

a. anti-inflammatory properties of furocoumarin molecules by *in silico*, *in vitro* (bronchial epithelial cells) and *in vivo* (murine lung) models;
b. anti-inflammatory properties of molecules modulating plasma membrane sphingolipids, as potential therapeutic targets;
c. role of intracellular calcium signalling on IL-8 gene expression in bronchial epithelial cells *in vitro*;
d. mapping IL-8 transcription machinery by transcription factor *decoy* strategy.

Overall, these studies wish to suggest strategies to highlight novel therapeutic targets and drugs for lung inflammation in cystic fibrosis.

Supported by the Italian Cystic Fibrosis Research Foundation with different projects

S5.4 - NF-κB Inflammatory Signalling is Suppressed by CFTR in a Number of Cell Models

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Introduction: Little is understood on how defects in the CFTR gene can cause persistent chronic pulmonary infection and inflammation. Numerous studies have shown that inflammatory signalling through the NF-κB pathway is increased in CF lungs and that this is linked to the production of pro-inflammatory cytokines such as interleukin 8 (IL-8)**.** However, there is little consensus on the mechanism(s) which link CFTR and its inheritable mutant forms to chronic lung inflammation. Recent work both in our lab and by Vij et al suggests that wild type CFTR can suppress inflammation (Vij, Mazur et al. 2009). Here we demonstrate that this idea holds true in a variety of cell models.

Methods: We used 2 different cell lines (NCI-H441 cells, a human lung adenocarcinoma cell line of bronchiolar (Clara) cell lineage and H57 cells, a HeLa cell derived cell line which stably over expresses the NF-κB luciferase construct) to examine the role of wild-type CFTR on inflammation. For luciferase assay cells were seeded in 12-well plates. The 4-κB-luciferase reporter plasmid contains four NF-κB binding elements 3' (TGGGGACTTTCCGC) 5' upstream of the thymidine kinase minimal promoter region that drive expression of the firefly luciferase reporter gene. H441cells were transfected with 400 ng of 4-κB-luciferase reporter plasmid using lipofectamine 2000 reagent. H57 cells stably express the 3Enhancer-κB-conA-Luc plasmid. Both cell lines were transfected with increasing amounts of CFTR up to 400 ng. To rule out a DNA dose effect, transfections were supplemented with empty vector plasmid to keep total DNA constant (for example, the 100 ng transfection of CFTR contained 300 ng of empty vector). 24h post transfection cells were incubated for 4 hours with fresh media or stimulated with 10 ng/ml TNFα. Supernatants were retained for IL-8 Elisa assays and cells lysed for luciferase assays.

Results: Our results show that in H441 cell, the level of CFTR expression correlates inversely with endogenous and stimulated NF-κB activity and IL-8 release. We also find the basal NF-κB activity in the HeLa derived H57 cell line is suppressed by increasing amounts of CFTR. We find that transfection of $2x10^5$ H441 cell or $1x10^5$ H57 cells with 400ng of CFTR in a well of a 12 well plate results in approximately 40% reduction in NF-κB activity.

Conclusions: These data together with that of Vij et al in HEK293 cells suggest that this reduction in inflammation following wild type CFTR transfection suggests that wild-type CFTR induced inflammation suppression is a broad phenomenon. These data indicate that CFTR has anti inflammatory properties and that the hyper-inflammation found in CF may be due to a disruption of the suppressive signalling link between CFTR and NF-κB and that one cause of the inflammation observed in CF is due lack of functioning CFTR at the cell membrane. We are focussing on the signalling link in current work.

This work was funded by the CF Trust PJ549

Reference: Vij, N., S. Mazur, et al. (2009). "CFTR is a negative regulator of NFkappaB mediated innate immune response." PLoS ONE **4**(2): e4664.

S5.5 - Modulation of Sphingolipids Metabolism Reduces the Neutrophil Chemotaxis in Human and Murine Respiratory Models *in vitro* and *in vivo*

Maria Cristina Dechecchi[1], Paola Mazzi[2], Moira Paroni[3], Federica Cioffi[4], Elena Nicolis[1], Anna Tamanini[1], Valentino Bezzerri[1], Federica Quiri[1], Ilaria Lampronti[5], Irene Mancini[5], Monica Borgatti[5], Alessandra Bragonzi[3], Roberto Gambari[5], Maria Teresa Scupoli[4], Giorgio Berton[2], Giulio Cabrini[1]

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Investigation on novel targets for the treatment of CF lung inflammation is a major priority, considering that at present no fully satisfactory anti-inflammatory treatment is available. Growing evidence suggests sphingolipids (SLs) as novel targets for the treatment of pulmonary disorders, including CF (Uhlig, 2008). The accumulation of the SL ceramide has been identified as one of the key regulators of inflammation and infection in CF airways in different CFTR-/- mouse models (Teichgraber, 2008). Miglustat, an inhibitor of the synthesis of glycoshingolipids (GSLs), used for treating type I Gaucher disease and others GSLs storage disease, produces an anti-inflammatory effect in bronchial epithelial cells (Dechecchi, 2008). In this study we tested the effect of miglustat in murine models of lung infection and inflammation. C57BL/6 mice were challenged *with* aqueous solution of miglustat (2 mg/dose) by intraesophageal gavage at 72, 48 and 24 hours before intranasal instillation of LPS or a treatment schedule of 400mg/kg one hour before the i.t. inoculum with *P. aeruginosa* strain PAO1. Bronchoalveolar lavage fluid (BALF) was collected 4 h post LPS challenge or PAO1 infection and the inflammatory response in terms of leukocyte recruitment and MPO activity in the airways was analyzed. Miglustat significantly reduces the amount of neutrophils recruited in BALF and MPO activity both in LPS stimulated and PAO1 infected mice. To support the hypothesis that the anti-inflammatory effect of miglustat is related to changes in SLs metabolism, CF bronchial cells were treated with amitriptyline, an inhibitor of two key enzymes of the SLs metabolism: acid sphingomyelinase and acid ceramidase. Amitriptyline (10 microM), added to IB3-1 and Cufi-1 cells 4 hours before infection with *P.aeruginosa*, significantly inhibits the expression of IL-8 mRNA by about 50 %, in agreement with the anti-inflammatory effect obtained *in vivo* in CF mice (Teichgraber, 2008; Becker, 2009). To demonstrate that miglustat and amitriptyline affect the immune response through changes in cellular ceramide levels, the analysis of ceramide, expressed on the cellular membrane of cells infected by *P. aeruginosa,* was measured by FACS analysis with anti-ceramide antibody MAS 0020 (Glycobiotech). Ceramide staining in IB3-1 and CuFi-1 cells increases upon infection with *P.aeruginosa.* Both miglustat and amitriptyline significantly reduce the increase of ceramide expression induced by *P.aeruginosa.* Measurement of apoptotic cells demonstrate that miglustat does not produce any significant effect on apoptosis. Collectively these results strongly suggest that miglustat, tested at concentrations much lower than those necessary to correct lysosomal storage diseases, could be a promising molecule to correct the excessive neutrophil chemotaxis observed in the lung of patients affected by CF, without producing significant pro-apoptotic effects and strengthen the hypothesis that SLs metabolism represents a target to restore a normal inflammatory response in CF epithelium.

***Supported by**: Italian Cystic Fibrosis Research Foundation (#12/2008 and CFaCore) with the contribution of "Delegazione FFC di Vicenza con le scuole vicentine*".

S5.6 - IL-8 Production by Calu-3 Cells Depressed for CFTR and in CF Human Airway Epithelial Cells

Tecal Dudez[1], Davide Losa[1], Mike A Gray[2], A W Cuthbert[3], Scott O' Grady[4], Marc Chanson[1]

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A pro-inflammatory state of the human airway epithelium has been proposed to explain the exaggerated recruitment of neutrophils to the airways of patients with cystic fibrosis (CF). From early clinical observations to the latest research, a polemic issue has been whether mutant CFTR, or presence of a dysfunctional CFTR, triggers an enhanced production of the chemokine IL-8 by airway epithelial cells. As an attempt to address this question, we have compared IL-8 release in Calu-3 cells where CFTR was knocked-down by siRNA (kdCFTR cells) or knocked-out by shRNA (SH3 cells). The production of the cytokine was evaluated in parallel experiments in parental Calu-3 cells and in Calu-3 cells expressing an altered shRNA CFTR sequence (Alter cells). We also measured IL-8 release by well polarized cultures of human airway epithelial cell (HAECs) from non-CF and CF origin. We have first verified for CFTR expression and function in the Calu-3 cell lines. CFTR was virtually undetectable by Western blot in SH3 cells and short-circuit currents (Isc) were absent in Ussing chambers (1.7 ± 0.5 mA/cm^2, n=7, as compared to 18.7 ± 6.3 mA/cm^2, n=7 in Alter cells). CFTR was still expressed in kdCFTR cells but Isc currents were strongly reduced (3.7 ± 1 mA/cm^2, n=9, as compared to 16 ± 4 mA/cm^2, n=18 in Calu-3 cells). IL-8 was measured in the Calu-3 cell lines grown on plastic dishes in the absence or presence of TNF-a. However, no difference in basal or stimulated IL-8 release was observed between the different cell lines, whatever expression of CFTR was reduced or abolished. These experiments were repeated on well polarized Calu-3 cells grown on Transwell filters, a culture condition which again did not affect the production of the chemokine between the cell lines. We next evaluated IL-8 release by HAEC cultures on Transwell filters. HAECs could be amplified up to 3 passages, and for each passage differentiated at the air-liquid interface into a mucociliated pseudostratified epithelium within 30 days. The production of IL-8, which is highly elevated in CF HAEC cultures, decreased with time of differentiation to the level of non-CF cultures. At this time of the culture, we then measured the release of IL-8 induced by TNF-a as a function of cell passage. Although CF HAECs showed enhanced basal and stimulated release of IL-8 at early passage, this difference vanished for higher passages. Finally, secretions of 7- and 30-days old cultures generated from passage 2 HAECs were subjected to a cytokine array. Seven days CF HAEC cultures showed increased production of GM-CSF, GRO-a, IL-5, IL-6, IL-7 and MCP-1 but decreased production of TIMP-1 as compared to non-CF cultures. This difference, with the exception of IL-6, disappeared in 30 days HAEC cultures. Thus, our results revealed major differences in cytokine production in non-differentiated CF HAEC as compared to non-CF cells; this difference was abolished in well polarized HAECs. They also indicate that there is no intrinsic CFTR-dependent anomaly of cytokine secretion by CF HAECs or CFTR-depressed Calu-3 cell lines.

Supported by "VLM" and FNRS

Friday 9 April – 18:00-19:30 **Room: Rubi B**

SPECIAL GROUP DISCUSSION – III
Alternate restorative strategies for CF
Moderators: F. Van Goor (US) & M. Mall (DE)

Although tremendous progress has been made in the development of small molecule compounds that correct and/or potentiate mutant CFTR function, it is currently not known whether this pharmacological approach will improve CFTR function sufficiently to result in an effective therapy of CF organ disease. Therefore, the aim of this session is to discuss alternative strategies to restore/circumvent CFTR malfunction on the cellular and whole organ level in the airways as well as in other organ systems.

Specifically, this Special Group Discussion will focus on the following issues:

1. Alternative pharmacological strategies to restore the ion and fluid transport defects in CF.
   - What is the current progress in the pre-clinical and clinical development of ENaC blockers and alternative Cl^- channel modulators?
   - How would combinations of CFTR-modulators and alternative ion transport strategies impact clinical efficacy?

2. Current targets and approaches for regenerative cell-based therapies, such as embryonic stem cells (ES) and inducible pluripotent stem cells (iPS)

3. Will alternative ion transport pathways and restorative cell-based therapies compensate for the pleiotropic consequences of mutations in the *CFTR* gene?

4. Based on the current knowledge, what should be future directions of basic and clinical research?

Friday 9 April – 18:00-19:30 **Room: Esmeralda**

SPECIAL GROUP DISCUSSION – IV
Inflammation in CF – Getting to the routes by digging out the roots
Moderators: A. Mehta (UK) & M. Chanson (CH)

Clinicians find it difficult to manage CF inflammation- either infection-induced deterioration of the lung, or decline in the absence of cultured pathogens. Clinical practice might well differ if the excessive inflammatory response, which is a hallmark of CF, might be partly independent of infection. Hence the inflammation discussion will begin with a clinical view from Stuart Elborn.

Hypothesis: A hyper-inflammatory state has been postulated to play a role in the pathogenesis of CF airway disease. A polemic issue has been whether it is the absence of wt-CFTR, or presence of a dysfunctional CFTR that triggers dysregulated inflammatory pathways affecting the production of proteo-lipid (chemokines, cytokines eiocosanoids) mediators. Since the origins and mechanisms underpinning CF inflammation are disputed. A major aim of this discussion is to clarify the degree to which inflammation occurs as a result of the following processes:

1) Is localization of CFTR in the plasma membrane required for basal or stimulated inflammatory mediator production?
2) Does CFTR absence or dysfunction trigger other defects in the production of inflammatory mediators?
3) What are the potential hubs (eicosanoid pathway, NF-□B pathway, oxidative stress/mitochondrial dysfunction) in the CFTR interactome?
4) Are available animal and cell models of CF capable of yielding clues to the above?

Objective 1: Getting the roots right.

ROOT 1, because both wild type (say 75%) and F508del CFTR (say 90%) are degraded, how much excess inflammation occurs when CFTR is missing? One consequence is the question whether the missing/disturbed regulation occurs because the residual (undegraded) 25% of wild type is the element that is missing in F508del cells to leave only 5% of mutant CFTR in cell.

ROOT 2, is CF inflammation a knock-in disease where the residual F508del (say 5% left over) has one or more positive function(s) of a magnitude sufficient to induce disease? The assumption here is that F508del in this setting exerts its heterozygote advantage even when allied to a wild type CFTR and exhibits its disease propensity when allied to another copy of the same defect in half the CF patients in Northern Europe. Clearly, all roots cause processes knock-out and knock-in could be operant together.

Objective 2: Getting the routes right.

This session will promote an open discussion to foster future research on the inflammatory response of CF airway epithelial cells. For example:

- Are epithelial cells the only target of CFTR dysfunction(s) and source(s) of inflammation?

What about CFTR in leukocytes, endothelial cells and immune cells. The assumption is that altered responses of these cells, together with defective CFTR chloride channel function in epithelial cells, may contribute to various degrees to the CF airway phenotype.

Saturday 18 April – 09:00-10:30 **Room: Esmeralda**

SYMPOSIUM 6 – Understanding CF microbial targets and the CF pulmonary micobiome
Chairs: S. Elborn (UK) & J.P. Clancy (US)

S6.1 - Tobramycin and FDA-approved iron chelators eliminate *P. aeruginosa* biofilms on cystic fibrosis airway epithelial cells

Bruce A. Stanton, Sophie Moreau-Marquis and George O'Toole

Dartmouth Medical School, Hanover, NH USA

The ability of *P. aeruginosa* to form antibiotic-resistant biofilms is thought to account for the inability of current therapies to resolve bacterial infections in the lungs of patients with cystic fibrosis (CF). However, there is no information on the antibiotic resistance of *P. aeruginosa* biofilms grown on human airway epithelial cells or on the effects of airway cells on biofilm formation by *P. aeruginosa*. Thus we developed a co-culture model to examine biofilm formation by *P. aeruginosa* growing on polarized human CF airway epithelial cells. CF airway cells increased the resistance of *P. aeruginosa* to tobramycin (Tb) by >25-fold compared with *P. aeruginosa* grown on abiotic surfaces. Thus, the concentration of Tb required to kill *P. aeruginosa* biofilms on airway cells is 10-fold higher than the concentration in the lungs of CF patients. In addition, CF airway cells expressing ΔF508 -CFTR significantly enhanced *P. aeruginosa* biofilm formation, and rescue of cells expressing ΔF508 -CFTR with wild-type CFTR reduced biofilm formation. Iron (Fe) content of the airway surface liquid (ASL) in CF is elevated compared to the ASL in WT-CFTR cells, and Fe is known to enhance *P. aeruginosa* growth. Thus we investigated whether enhanced biofilm formation by *P. aeruginosa* on ΔF508-CFTR cells was due to increased Fe release by airway cells. We found that airway cells expressing ΔF508 -CFTR released more Fe than cells rescued with WT-CFTR. Moreover, Fe chelation with conalbumin reduced biofilm formation on CF airway cells, whereas Fe supplementation enhanced biofilm formation on airway cells expressing WT-CFTR. These data demonstrate that human airway epithelial cells promote the formation of *P. aeruginosa* biofilms with a dramatically increased antibiotic resistance, in part, by increasing Fe release into the ASL. Given the positive role for iron in biofilm development, we investigated whether the FDA-approved iron chelators deferoxamine and deferasirox would enhance the ability of tobramycin, the primary antibiotic used to treat CF lung infections, to eliminate *P. aeruginosa* biofilms. The combination of tobramycin with deferoxamine or deferasirox reduced established biofilm biomass by approximately 90% and reduced viable *P. aeruginosa* by 7-log units. Neither chelators, nor tobramycin, nor deferoxamine, nor deferasirox alone had such a marked effect. The combination of tobramycin and FDA-approved iron chelators also prevented the formation of biofilms on CF airway cells. These data suggest that the combined use of tobramycin and iron chelators may be an effective therapy to treat patients with CF and other lung disease characterized by antibiotic-resistant *P. aeruginosa* biofilms.

S6.2 - Clonal variation of *Pseudomonas aeruginosa* isolates from CF airways

Nina Cramer[1], Jens Klockgether[1], Colin Davenport[1], Antje Munder[1], Christian Herrmann[1], Susanne Herrmann[1], Alessandra Bragonzi[2], Lutz Wiehlmann[1], Burkhard Tümmler[1]

[1]Cystic Fibrosis Research Group, Clinic for Pediatric Pneumology and Neonatology, Medizinische Hochschule Hannover, Germany;
[2]Cystic Fibrosis Unit, San Raffaele Scientific Institute, Milano, Italy

Pseudomonas aeruginosa is the most common bacterial pathogen in the CF patient community. Genotyping of informative markers of core and accessory genome in > 1,000 *P. aeruginosa* isolates from 38 CF centres in 13 European countries by a custom-made microarray revealed that the dominant clonal complexes of the global *P. aeruginosa* population were also the most abundant clones among the CF isolates. The spectrum of CF isolates covers a large part of the current *P. aeruginosa* population. A CF-diagnostic signature was seen at hotspots of microevolution in the *P. aeruginosa* genome. Analysis of published and in-house generated genome sequences revealed 0.3 – 1% interclonal and ≤ 0.01% intraclonal sequence diversity of the core genome.

Sequential *P. aeruginosa* isolates have been collected in half-year intervals from all 35 CF patients regularly seen at the CF clinic Hannover who had become colonized with *P. aeruginosa* in their lower airways between 1983 and 1991. Of 49 detected clonal complexes, five clones that are also common in the inanimate environment were identified in 40% of the sequential CF isolates. Co-colonisation of different clones was frequent during the first four years of colonization. Sixty percent of the patients were still harbouring the initially acquired clone after 20 years of colonization.

Genotypic and phenotypic features have been studied in subsets of the strain collection by us or collaborators part of which has been published (J Infect Dis 1994;170:1616-1621; J Mol Biol 1997;271:386-404; Mol Microbiol 2003;47:145-158; Infect Immun 2005;73:1695-1703; Microbiology 2006;152:3261-3269; 2007;153:1445-1454; Am J Respir Crit Care Med 2009;180:138-145). Major typical findings: *P. aeruginosa* in CF lungs is prone to gross changes of the chromosomal frame, i.e. insertion, deletion, inversion and transposition. Strains become LPS- and pyocin-deficient and lose their susceptibility to phages. During chronic colonization large proportions of strains become immotile by the loss of pili and/or flagella, are quorum-sensing deficient and reduce or even abolish the production of type II and III secretion effector proteins. Intraclonal diversification was most pronounced in antimicrobial susceptibility, binding capacity to human epithelial cells and mucins and in colony morphotype, the hallmarks being the emergence of small colony variants and mucoid colonies. Mucoid conversion was observed in most patients' lungs during the first five years of chronic colonization, but after ten years numerous isolates with a *mucA* loss-of-function mutation had reverted to a non-mucoid morphotype probably by secondary site mutation(s). These losses of cellular appendages and virulence factors were however not accompanied by a global loss of bacterial fitness in vitro or in the capacity to establish chronic infection in the murine respiratory tract. Recent comparative ultradeep genome resequencing of first, mid-term and late isolates uncovered purifying and positive selection at numerous loci in the mid-term and/or late clonal variant, the majority of which unrelated to known pathogenicity traits. The large proportion of de novo mutations in metabolic and regulatory genes calls for a systems biology approach to understand the microevolution of *P. aeruginosa* in CF lungs in more depth.

S6.3 – Anerobes and the sputum microbiome in CF

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Queen's University Belfast, N Ireland

Cystic fibrosis (CF) lung disease reflects a failure of airways host defense against bacterial infection. The pathogenesis of CF lung disease is complex and reflects abnormalities in multiple host defense mechanisms. In the CF lung, the O_2 tension within infected mucus plaques/secretions is markedly low, i.e., < 2 torr, reflecting the accelerated O_2 consumption of hyperactive CF airway epithelia and the consumption of O_2 by aerobic bacteria embedded in mucus. This observation has provoked a dramatic change in the view of the microbiology of the CF lung, with intense efforts underway to describe the mode of metabolism and pathogenesis of *P. aeruginosa* under anaerobic conditions. This observation also spurred the search for infection in CF airway mucus by anaerobes. We have demonstrated the presence of strict anaerobes present in CF secretions by anaerobic culture and molecular detection methods, that these anaerobes are unlikely to be oral contaminants, and that many CF patients have numbers of anaerobes in sputum equal to or greater than *P. aeruginosa*. Molecular data from T-RFLP and deep sequencing suggest that not only were there anaerobes in CF sputum, but they exhibit far greater microbial diversity than previously appreciated.

Because anaerobic conditions exist in the lungs of CF patients with persistent respiratory infection (1), we hypothesized that anaerobic bacteria, not detected by routine aerobic culture methods, could be present as polymicrobial or "mixed" CF pulmonary infections. Sputum samples from 50 clinically stable adult CF patients were cultured under strict anaerobic conditions. A range of potentially pathogenic anaerobic species (e.g. *Prevotella, Veillonella*) were detected in high numbers (up to 9×10^7 cfu/g of sputum) from 42/66 (64%) samples (15). Anaerobes were cultured from 27/39 (69%) samples from which *P. aeruginosa* was cultured vs. 11/27 (41%) samples from which *P. aeruginosa* was not cultured. Colonisation with *P. aeruginosa*, therefore, significantly increased the likelihood that anaerobic bacteria were present in sputum (Chi-squared test, $P < 0.01$). In a subsequent study, 26 CF patients with an acute pulmonary exacerbation were studied before and after intravenous antibiotic therapy. A similar spectrum of anaerobic species, in comparable numbers to the study cited above, was detected from all patients prior to commencing antibiotic therapy, with the aerobic pathogens (*P. aeruginosa* or *B. cepacia* complex) detected in similar numbers. Anaerobic bacteria were also detected in samples from 23/26 patients at the end of antibiotic treatment, and these anaerobes were present in lower numbers than before antibiotic treatment. Other groups have found similar results and the predominant species detected by culture in our studies had also been detected in a high proportion of patients with CF by molecular analysis in adult patients Interestingly we and others have detected anaerobes in bronchoalveolar lavage fluid from infants and children with CF, albeit in lower frequency, These were anaerobes typically identified in adult CF sputum (e.g. *Prevotella*), suggesting that anaerobic infection may occur early in CF pathogenesis.

S6.4 - *Pseudomonas aeruginosa* Infection Drives Functionally Unopposed Matrix Metalloproteinase Activity In Lung Parenchymal And Inflammatory Cells

Joanne F Moffett[1], Danny F Mc Auley[1], Joseph S Elborn[1], Cecilia M O'Kane[1]

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Introduction: Cystic fibrosis (CF) lung disease is characterised by bronchiectasis; progressive destruction and dilatation of the airways. This abnormal remodelling of the airways occurs in the setting of chronic infection, particularly with *Pseudomonas aeruginosa (PA*), and is associated with loss of elastin and cartilage, with progressive submucosal fibrosis. Matrix Metalloproteinases (MMPs) are a group of enzymes which have the ability to degrade all known components of the extracellular matrix, and may therefore contribute to the abnormal matrix turnover that occurs in CF. Net MMP activity is dependent on the molar ratio of MMPs:Tissue Inhibitor of Metalloproteinases (TIMPs).
Hypothesis: Infection with PA drives MMP secretion by both inflammatory cells and parenchymal cells in CF, resulting in subsequent airway remodelling.
Methods: Human bronchial epithelial cells (HBE), cystic fibrosis bronchial epithelial (CFBE) cells and peripheral blood monocytes were stimulated for 4 hours with increasing doses of PAO-1-the well-characterised laboratory strain of PA, or conditioned media from PA infected monocytes (coMPA) for 72 hours and supernatants collected. Recombinant human TIMP-1 (the major inhibitor of MMP-9) was incubated directly with PA to investigate whether PA could directly destroy this protein. MMP-9 was measured by gelatin zymography and ELISA, and TIMP-1 was measured by ELISA. Intracellular signalling was investigated with Western blotting.
Results: PA infection caused a dose dependant reduction in TIMP-1 by HBE cells (1.6+/-0.19ng/ml for MOI (Multiplicity of Infection)-100 vs 2.5+/- 0.19ng/ml in controls $p< 0.033$), and monocytes (124.3 +/- 10.95pg/ml for MOI 100 vs 384.1+/- 39.90 pg/ml in controls $p< 0.001$). Given the rapid reduction in TIMP-1 we investigated whether PA could directly destroy TIMP-1. After 4 hours of incubation with PA, TIMP-1 was no longer detectable by ELISA ($p< 0.001$). CoMPA significantly increased MMP-9 induction from both HBE cells (10.29+/-0.6ng/ml coMPA vs 5.2+/-0.3ng/ml for controls $p< 0.001$) and CFBE cells (16.6+/-2ng/ml coMPA vs 6.0+/-0.85ng/ml for controls $p< 0.001$). TIMP-1 was significantly reduced in CFBE following coMPA stimulation ($p=0.001$) and reduced in the supernatants of HBE cells, however this failed to reach significance. Our data also suggests that the secretion of MMP-9 in response to PA infection is regulated by p38, JNK and ERK mitogen activated protein kinases.
Conclusion: Direct PA infection and coMPA stimulation both induced significant production of MMP-9 by lung epithelial and monocytic cells,and is accompanied by a rapid loss of TIMP-1; its major inhibitor. These data suggest that PA infection can induce functionally unopposed MMP-9 activity both in the lung parenchyma, and from newly recruited inflammatory monocytes,and may represent a mechanism for the excessive tissue damage observed in CF.

S6.5 - Trappin-2, an Antimicrobial Peptide, Induces a Flagellin/TLR5-Dependent Modulation of Alveolar Macrophage & Epithelial Cell Activity

Delphyne Descamps[1], Mathieu Le Gars[1], Viviane Balloy[1], Reuben Ramphal[2], Michel Chignard[1], Jean-Michel Sallenave[1,3]

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Introduction: Epithelial-derived endogenous antimicrobial molecules such as defensins, SLPI and trappin-2 have the potential to positively affect the phenotype of phagocytic myeloid cells (macrophages, neutrophils, dendritic cells), by modifying their differentiation, chemotactic properties and activation in different lung pathologies. Recently, it has been shown that trappin-2 (T2, R&D System) can improve the clearance of *Pseudomonas aeruginosa* (*P.a*) by alveolar macrophages (AM) in vitro and in vivo (Wilkinson *et al*. Am. J. Pathol, 2009, 174:1338-46). The aim of the present study is to study T2 opsonizing effect on the clearance of *P.a* by AM and a potential modulation on cytokine productions by AM and lung epithelial cells stimulated with *P.a*.

Methods:

Measurement of the early clearance of *P.a* by AM

PAK (a wild-type P.a. strain), PAK∆*fliC* (a flagellum-deficient PAK) or PAKL88 (a PAK strain with a TLR5 binding site deficient flagellum) were pre-incubated or not with T2 (100 nM, a sub-lytic concentration) for 30 min at 37°C. These suspensions were then added to murine AM (MH-S cell line) at a multiplicity of infection of 0.1. Co-cultures were then incubated for 2 h before lysis with 0.1% Triton X-100. Serial dilutions were subsequently plated on LB agar to determine remaining bacterial CFU.

Modulation of AM & epithelial cell activation by *P.a* or purified *P.a* flagellins

AM, Beas2B or A549 cells were stimulated with *P.a* or flagellins pre-incubated with PBS or T2. TNF-α or IL-8 secretions were then measured by ELISA.

Results: We confirm that T2 enhances the early clearance of wild-type *P.a* by AM but not that of PAK∆*fliC* or PAKL88. In addition, T2-pre-incubated PAK but not PAK∆*fliC* increases AM or lung epithelial cells TNF-α and IL-8 secretions. Moreover, Beas2B epithelial cells stimulated with PAK-flagellin pre-incubated with T2 secreted higher levels of IL-8, when compared with cells incubated with flagellin alone.

Conclusion: Our data suggest that T2, by 'opsonising' *P.a* and by increasing cytokine production of AM or lung epithelial cells may constitute an important modulator of innate immune responses. Moreover, T2 activity requires *P.a* flagellin-TLR5 interaction. We are currently assessing whether T2 could improve the potential defective bacterial clearance of *P.a* observed in cystic fibrosis.

S6.6 - The Effect of Budesonide in a Refined Rat Model of Respiratory Infection with *Pseudomonas Aeruginosa*

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Striking a balance between effective suppression of excessive inflammation whilst maintaining host defense remains a key challenge in the treatment of cystic fibrosis. We evaluated whether therapeutic budesonide treatment could attenuate inflammation without modulating bacterial load in a refined rat model of respiratory infection with *Pseudomonas aeruginosa* (P.a.). *P.a.* PAO1V strain (University of Colorado) was embedded in agar beads in the presence of 0.01% v/v of the surfactant SPAN® 80. Under isofluorane anaesthesia, male Sprague Dawley rats (250-300g; n=16/group) were inoculated (i.t.) with agar beads containing 10^5 colony forming units (cfu) of P.a. Sham groups were given sterile beads. Rats were dosed with 3 mg/kg (p.o.) budesonide or vehicle 16 hr post inoculation, subsequently dosed twice a day and culled on days 2 or 5. Cfu counts were performed on lung tissues; cell counts were performed on bronchoalveolar lavage fluid (BALF). Data are expressed as mean ± S.E.M. and analysed using a Kruskal-Wallis test with a Dunn's post test and a Mann Whitney test where appropriate. P.a. infection significantly elevated neutrophils 2 and 5 days post infection (**Table 1**, P< 0.05). Budesonide augmented the P.a.-induced BALF neutrophils 2 (P< 0.05) and 5 days (P< 0.01, **Table 1**) post infection. Budesonide significantly increased bacterial load at 2 days (**Table 1**, P< 0.05, n=8) but not at 5 days post inoculation. In conclusion budesonide enhanced inflammatory cell infiltrate and increased bacterial load. As patients may be intermittently treated with antibiotics these findings may warrant further evaluation. Future studies will focus on evaluating the effect of a combination of budesonide and antibiotics.

	Day 2				Day 5			
	Sham Vehicle	Sham Budesonide	P.a. Vehicle	P.a. Budesonide	Sham Vehicle	Sham Budesonide	P.a. Vehicle	P.a. Budesonide
Log cfu	1.5±0.0	1.5±0.0	4.4±0.3	5.4±0.2$	1.5±0.0	1.5±0.0	5.2±0.2	5.6±0.1
Total cells	4.0±0.6	4.0±0.6	17.0±7.0	41.0±7.0$	6.0±1.0	7.0±1.0	20.0±3.0*	28.0±3.0$
Neutrophils	0.3±0.1	0.3±0.1	12.0±4.0*	29.0±6.0$	0.1±0.03	0.3±0.1	3.0±1.0*	13.0±3.0$$

[Table 1: Lung Tissue cfu and BALF cells (x105).]

* P< 0.05 versus sham; $ P< 0.05; $$ P< 0.01 versus P.a. vehicle

SYMPOSIUM 7 – Modelling of CFTR defects
Chairs: J. Engelhardt (US) & M. Drumm (US)

S7.1 - Preclinical evaluation of therapeutic effects of ENaC blockers on cystic fibrosis-like lung disease in mice

Marcus A. Mall

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Increased ENaC-mediated airway Na^+ absorption, airway surface liquid (ASL) depletion and reduced mucus clearance are characteristic abnormalities in the pathogenesis of cystic fibrosis (CF) lung disease and cause CF-like lung disease with spontaneous pulmonary mortality, airway mucus obstruction, goblet cell metaplasia and chronic airway inflammation in βENaC-overexpressing (βENaC-Tg) mice. We used this mouse model to test if inhibition of increased airway Na^+ absorption by the classic ENaC blocker amiloride and novel long acting analogs has therapeutic benefits on CF-like lung disease *in vivo*. We compared effects of preventive versus late therapeutic intervention by treating newborn or adult βENaC-Tg and wild-type mice by intrapulmonary administration of ENaC blockers or vehicle alone t.i.d. for 2 weeks. Subsequently, mice were euthanized, bronchoalveolar lavage (BAL) performed to determine effects on airway inflammation, and lungs processed for histology and morphometry to assess effects on airway mucus obstruction. In these studies, we demonstrated that preventive treatment (i.e. from the first day of life) with the short-acting reference compound amiloride resulted in a significant reduction in pulmonary mortality, airway mucus content and BAL inflammatory cells compared to vehicle treated βENaC-Tg mice. However, similar to previous clinical trial in CF patients, amiloride therapy had no benefits on airway mucus obstruction or airway inflammation if treatment was started in older βENaC-Tg mice with established lung disease. Based on these findings, we hypothesized that the ineffectiveness of late amiloride therapy may either be due to the low potency and rapid absorption of amiloride from airway surfaces, or irreversible lung pathology. To distinguish between these possibilities, we used a novel, highly potent and long acting ENaC blocker, P643 (Parion Sciences), which is 35-60-fold more potent and ~5-fold longer acting than amiloride *in vitro*, and tested its therapeutic benefits in βENaC-Tg mice. Our studies show that, in contrast to amiloride, late intervention with P643 has significant therapeutic effects including reduction of airway mucus obstruction, neutrophilic inflammation and epithelial remodeling in established lung disease in adult βENaC-Tg mice. Taken together, our results demonstrate that early inhibition of Na^+ hyperabsorption with the classic ENaC blocker amiloride is an effective preventive therapy for CF-like lung disease *in vivo* and suggest that long acting ENaC blockers may have therapeutic effects in CF patients with chronic lung disease. Supported by EC (MEXT-2004-013666), CFF (MALL04G0) and Mukoviszidose e.V.

S7.2 – Airway Disease in the *CFTR*-targeted Pig

David A. Stoltz[1], David K. Meyerholz[2], Alejandro A. Pezzulo[1], Shyam Ramachandran[3], Paul B. McCray, Jr.[3], Joseph Zabner[1], Michael J. Welsh[1,4,5]

Departments of Internal Medicine[1], Pathology[2], Pediatrics[3], and Molecular Physiology and Biophysics[4]; and Howard Hughes Medical Institute[5], Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, IA

Lung disease causes most of the morbidity and mortality in cystic fibrosis (CF). However, understanding its pathogenesis has been hindered by lack of an animal model with characteristic features of CF. To overcome this problem, we recently generated pigs with targeted *CFTR* genes. We chose pigs for the development of a new CF model, because they are more similar to humans than are mice in terms of anatomy, physiology, immune system, biochemistry, life span, size, and genetics. Furthermore, their reproductive characteristics are favorable for experimental studies. Using recombinant adeno-associated viruses targeting *CFTR* in pig fetal fibroblasts and somatic cell nuclear transfer to porcine oocytes to generate *CFTR* +/- pigs we have now generated *CFTR* null pigs. All *CFTR* null pigs have meconium ileus and severe pancreatic involvement at birth. Nasal voltage measurements demonstrated lack of epithelial chloride conductance and differentiated cultures of tracheal epithelia also showed the lack of CFTR chloride current. At birth, *CFTR -/-* lungs show no evidence of inflammation in bronchoalveolar lavage fluid, histopathologic analysis, and microarray studies for inflammatory pathways. These findings suggest that *CFTR -/-* pigs may provide an opportunity to further investigate the mechanisms underlying the pathogenesis of CF lung disease and to develop new therapeutic tools for CF.

S7.3 – Characterization of a NEW CFTR Knockout Ferret Model

John F. Engelhardt

Department of Anatomy and Cell Biology, Department of Internal Medicine, and the Center for Gene Therapy, Carver College of Medicine,
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Programme note: Abstract details are not authorized for inclusion

S7.4 – Model Systems Based on Human Rectal Biopsies to Examine CFTR Modulators

JP Clancy, MD

University of Alabama at Birmingham

Thorough examination of CFTR modulators in pre-clinical model systems is a critical step in bringing new CFTR-restorative agents to CF patients. A number of model systems have been developed for this purpose and have provided necessary information to support early phase CF clinical trials in genotype-specific subpopulations. For example, studies in transgenic mice expressing *hG542X Cftr* in the gut have been used to demonstrate suppression of premature termination codons by aminoglycosides and PTC124, and these results provided important proof of principle in preparation for clinical trials of PTC124 in CF patients. As another example, primary Human Bronchial Epithelial cells (HBEs) derived from CF patients possessing the G551D CFTR mutation provided important preclinical results that helped guide the design and execution of a recently completed Phase II trial of VX-770. Unfortunately there are limitations to preclinical model systems currently in use. CF mice do not fully recapitulate the human CF phenotype and have differences in anatomy (eg: lack of airway submucosal glands) that can limit full translation of CFTR modulator effects from mice to men. Primary HBEs of varying genotypes are scarce, require meticulous growth conditions, demonstrate differences in behavior based on culture techniques, are limited to surface epithelium, and are often studied after months of ex vivo culturing.

Studies in rectal biopsies from CF patients are a potentially exciting and valuable preclinical model system that can be used to compliment these established assays and to assess modulator activity in human tissue. Advantages include genotype-specific (and patient-specific) assessment of single and combinations of reagents, the capability of examining a number of different readouts of CFTR modulation (ion transport, biochemical maturation of CFTR, transcript levels), and the ability to tailor modulator strategies to unique patient genotypes (eg: rare mutations that may have little preclinical data or established model systems to assess modulator effects).

In this session, we will summarize current research to standardize the use of rectal biopsies as a model system for CFTR modulator studies. We will discuss conditions to maintain tissue viability, reproducibility of measurements over time, activity of CFTR activators and blockers in biopsied rectal tissue, and the sensitivity of Intestinal Current Measurements (ICM), Western Blot, and RT-PCR to quantify CFTR activity and expression.

S7.5 - Generation of New Lung-Specific Tet-Dependent Activator Mice for Tight and Quantitative Control of Conditional Gene Expression in the Murine Lung

Julia Duerr[1], Maren Gruner[1], Susanne C Schubert[1], Uwe Haberkorn[2], Hermann Bujard[3], Marcus A Mall[1]

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The complex pathogenesis of cystic fibrosis (CF) lung disease remains incompletely understood. Conditional overexpression and deletion of genes in mice by combinatorial use of lung-specific promoter elements and the tetracycline-regulated system provides a powerful tool to elucidate the roles of candidate genes implicated in airway ion transport, inflammation, host defense and tissue remodeling in the in vivo pathogenesis of CF lung disease. However, the original version of the reverse tetracycline-dependent transactivator (rtTA) exhibited limited doxycycline sensitivity and residual affinity to its promoter (P_{tet}) producing leaky transgene expression in the absence of doxycycline impeding the use of this system for such mechanistic studies. We, therefore, used a new generation rtTA, designated rtTA2^S-M2, with no basal activity and increased doxycycline sensitivity, and the rat Clara cell secretory protein (CCSP) promoter to target its expression to pulmonary epithelia in mice. Novel CCSP-rtTA2^S-M2 founder lines were generated by pronuclear injection. For functional characterization, founder lines were crossed with bi-transgenic reporter mice expressing luciferase and Cre recombinase (LC-1). Background activity, doxycycline sensitivity, tissue and cell-type specificity, inducibility, and reversibility of doxycycline-dependent gene expression were determined by luciferase activity, immunohistochemistry, morphometry and bioluminescence measurements in neonatal and adult lungs. We generated two distinct novel CCSP-rtTA2^S-M2 activator mouse lines that confer tight and doxycycline dose-dependent regulation of transgene expression, with high inducibility, complete reversibility and no background activity, in airway and/or alveolar epithelia. We demonstrate that rtTA2^S-M2 enables quantitative control of conditional gene expression in respiratory epithelia of the murine lung. Our results predict that the new CCSP-rtTA2^S-M2 activator mouse lines will be useful to determine the specific roles of individual candidate genes in the complex pathogenesis of CF lung disease and might lead to the development of novel therapies.

Supported by: European Commission (MEXT-CT-2004-013666), Mukoviszidose e.V. (I06/04)

S7.6 - Plasma Lipidomics Reveals Phenotype-associated Differences within a Cohort of F508del Homozygous Patients

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Biomarkers obtained by rapid and non-invasive methods could be used in the precocious detection of exacerbations or as prognostic indicators in CF. A large body of evidence links CF with abnormal content and metabolism of certain lipids in plasma and tissues from patients and animal models. We have recently shown significant variations in the plasma levels of several phosphatidylcholine and lysophopshatidylcholine species related to the disease severity status in CF patients (Guerrera et al. PLoS One, 4(11):e7735, 2009). Our goal was to search for potential lipid signatures characteristic of CF patients presenting the same mutation (F508del) and different CF phenotypes, and to study their correlation with clinical parameters. We used a lipidomic approach based on multiple reaction monitoring (MRM) liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS/MS). Blood plasma samples from 44 F508del homozygous patients were collected. Organic extracts were subjected to targeted LC-ESI-MS/MS analysis and quantification of 50 lipid molecules or lipid classes, including individual fatty acids, phospholipids, and ceramides, cholesterol, total cholesterol-esters and total triglycerides. Five phosphatidylcholine species (PC32:2, PC34:2, PC34:1, PC36:5, PC36:3), two lysophosphatidylcholine (LPC18:0, LPC20:4n-6), one sphingomyelin (SM16:0) and six fatty acids (C22:6n-3, C16:0, C20:3n-9, C22:5n-6, C22:5n-3, C20:5n-3), as well as total LPC and the LPC/TG ratio, were significantly decreased in those patients presenting more severe phenotypes. In addition, ten fatty acids (C16:0, C16:1n-7, C18:0, C18:3n-3, C18:1n-9, C20:3n-6, C20:3n-9, C22:5n-6, C22:5n-3, C20:5n-3) were significantly and positively correlated with FEV1% at sampling. Concerning *Pseudomonas aeruginosa* infection, PC32:2, PC38:5 and C18:3n-3 were significantly decreased in patients presenting chronic infection, while cholesterol, cholesterol-esters and total triglycerides were significantly increased. FEV1% and chronic Pseudomonas infection were reevaluated two years after sampling and the potential predictive value of the analyzed lipids was assessed. Surprisingly, plasma cholesterol was negatively correlated with FEV1%, and total triglycerides were positively associated with chronic infection. These results confirm our previously reported findings and suggest a potential predictive value for neutral lipids (cholesterol and triglycerides) in plasma concerning CF severity. Further longitudinal prospective studies to confirm this potential, as well as mechanistic investigations aiming to explain these observations, are warranted.

Supported by: Vaincre la Mucoviscidose, EC LSHG-CT-2005-512044 (NEUPROCF), Agence Nationale de la Recherche (EICO-CF), and Legs Poix-University of Paris Descartes.

S7.7 - Evaluation of Safety and Gene Expression with a Single Dose of pGM169/Gl67A Administered to the Airways of Individuals with Cystic Fibrosis: The UK CF Gene Therapy Consortium 'Pilot Study'

Uta Griesenbach[1,2], Jane C Davies[1,2], Deborah R Gill[2,3], Nia Voase[1,2], Gwyneth Davies[1,2], Tracy Higgins[1,2], Alastair J Innes[2,4], Chris A Boyd[2,4], David Porteous[2,4], Stephen C Hyde[2,3], Eric WFW Alton[1,2]

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The UK CF Gene Therapy Consortium is working towards a multi-dose gene therapy study, using the best currently available non-viral gene transfer agent, and whose endpoint will be to detect clinical benefit rather than molecular or electrophysiological proof-of-principle. Based on extensive preclinical testing our selected product is pGM169, a CpG-free human *CFTR* plasmid with a CpG-free CMV enhancer and human elongation factor 1 alpha (hCEFI) promoter complexed with GL67A, a mixture of 3 lipids: GL67, DOPE and DMP-PEG5000. We are currently undertaking a single dose safety study (Pilot Study) because of a requirement to confirm safety of this 'first-in-man' product; however, the study design has been tailored also to assess gene expression *in vivo* in CF lungs.

A single nebulised dose of 20 ml (53 mg pGM169 and 286 mg GL67A) is delivered by an Aeroeclipse II breath-actuated device; a nasal dose of 10% of the nebulised volume is administered on the same occasion using a standard nasal spray device. The latter allows assessment of gene expression without the sampling issues inherent in lower airway assessment, as well as anchoring to our previous clinical trials. Safety measures include physical examination, lung physiology (spirometry, pulse oximetry), systemic and sputum inflammatory markers, renal and hepatic function and chest CT. Measurements are made at intervals prior to dosing and during a 28 day follow up period. Gene expression is assessed by a) quantitative Taqman RT-PCR for transgene mRNA on nasal and bronchial brushings, b) anti-CFTR immunohistochemistry, and c) nasal and lower airway potential difference measurements. Given inter-subject variability, paired measurements on individuals will be obtained; bronchoscopies are being performed prior to dosing and either 6 days or 14 days post-dosing. Nasal PD is measured on serial visits. To date, 13 patients have been treated with pGM169/GL67A and safety as well as efficacy data will be presented.

Funded by the UK Cystic Fibrosis Trust

Saturday 10 April – 14:30-16:15 **Room: Esmeralda**

Symposium 8 – CF genomic modifiers and worldwide genetics
Chairs: C. De Boeck (BE) & B. Tümmler (DE)

S8.1 – Modifier Genes in Cystic Fibrosis: Genome-Wide Association Study.

M. Knowles, M.D. *

The University of North Carolina at Chapel Hill, NC, USA

Cystic fibrosis (CF) is a recessive "monogenic" genetic disorder caused by mutations in CFTR. However, there is a wide range of organ involvement and disease severity in CF, even for patients who are homozygous ΔF508; therefore, there must be other non-CFTR genetic variants and/or environmental effects that account for this disease heterogeneity. Two studies of twins and sibs have assessed environmental versus genetic influences, and both concluded that genetic factors play a major role in lung disease severity (Mekus, F. et al; Hum Genet 2003; Vanscoy, LL, et al, Am J Respir Crit Care Med, 2007).

The search for gene modifiers in CF has been underway for more than a decade, using candidate gene approaches. Early studies were hampered by small sample size, and limitations of study design and phenotyping. These limitations have largely been overcome by 3 large studies in North America: 1) Johns Hopkins (twin/sib, family-based design), 2) UNC/CWRU (case-control, extremes-of-phenotype design in ΔF508 homozygotes); and 3) Canada (>70% all Canadian CF patients). These 3 groups now utilize standardized measures of lung function to more accurately define pulmonary phenotype. These groups have also reported heritability and/or modifiers of lung and other phenotypes using standardized methodologies. (Drumm, M, et al, 2005, N.Engl.J.Med; Bremer, LA, et al, 2008, Hum Mol Genet 2008; Collaco, JM, et al, 2008, JAMA; Dorfman, R, et al, 2008, JCI)

To overcome the limitations of the candidate gene approach, a North American Consortium to conduct genome-wide association studies (GWAS) was formed by investigators at UNC/CWRU, Hopkins, and Canada. A GWAS has been completed in >4,200 CF patients, plus ~1,000 parental samples, using the Illumina 610K Quad platform. Preliminary results suggest strong associations of lung disease severity to several genomic regions, and analyses of the GWAS data are ongoing. Because of the collaborative and complementary nature of studies being undertaken in CF, there is reason to be optimistic that more participants will be added, and emerging data will drive progress for better prognostic and therapeutic approaches.

This study is supported by U.S. CFF CUTTING06P0; CFF KNOWLE00A0; NIH R01HL068927; NIH R01HL68890, NIH RR00046, NIH R01DK66368; R37DK044003; Canadian CFF; Genome Canada.

*Reporting for the GMS investigators and the North American CF Gene Modifier Consortium.
(PIs: Garry Cutting MD; Mitch Drumm PhD; Peter Durie MD)

S8.2 - Mouse models as tools to evaluate CF modifiers

[1]Mitchell Drumm, [1]Craig Hodges, [2]Garry Cutting, [3]Michael Knowles

[1]Case Western Reserve University, Cleveland, Ohio [2]Johns Hopkins University, Baltimore Maryland, [3]University of North Carolina, Chapel Hill, North Carolina

The North American Gene Modifier Consortium has carried out whole genome typing of >500,000 single nucleotide polymorphisms on CF patients and families across the United States and Canada to identify genes that influence lung function, survival, and other clinical phenotypes associated with CF. The data have been assessed by case/control and linkage analysis study designs. The analyses have identified two regions clearly associating with lung function, one on chromosome 11 and another on chromosome 20, and many additional loci strongly suggestive of association. While these analyses have dramatically narrowed the regions of the genome to very small candidate intervals, they have not unambiguously defined single genes, but rather regions containing a few genes. Upon inspection of the literature, the genes in the associating intervals are of diverse function, including metabolism, immune function, neuronal synaptic plasticity, transcriptional regulation and sensitivity to hypoxia, most of which could readily be placed into a CF pathogenesis pathway. Thus, additional tools are needed to sort out which genes in these regions are responsible for the genotype/phenotype associations. To this end, we have begun characterizing these candidate pathways in CF mice and in mice with Cftr selectively inactivated in specific tissues. We find that growth is influenced most strongly by loss of Cftr from neuronal cell populations, consistent with neuroendocrine effects seen in “full CF” mice. The CF response to lung bacterial challenge is partly reproduced by absence of Cftr from myeloid cells, indicating a functional role for Cftr in immune cells.

The above strategy should help to define the pathways through which the modifying genes are acting, but they will not unambiguously determine how they exert their modifying effects. For this, we are crossing mutant alleles of the candidate modifiers with CF mice to determine if the modifying effect can be recapitulated in mice. However, the genetics do not indicate how the modifying effect is being imparted. That is, if variants are thought of as “high” and “low” function, one must consider two alternative mechanisms: “high” is protective and ”low” deleterious or “low” is protective and “high” is deleterious. As it is impractical to develop multiple alleles of the putative modifiers, we have taken an alternative approach to place null alleles of the modifiers on severe and mild Cftr backgrounds. In this way, loss of modifier function can be examined to determine if it makes mild CF mice more severely affected or severely affected mice milder. For these studies, we are crossing the modifier models with a Cftr null (*Cftr*^{tm1Unc}), ΔF508 and the mild mutation R117H.

S8.3 - Defining the clinical and functional consequences of CFTR mutations

Garry Cutting[1], Patrick Sosnay[1], Rachel Karchin[1], Mary Corey[2], Ruslan Dorfman[2] Chris Penland[3], Carlo Castellani[4]

[1]Johns Hopkins Medical Institutions, Baltimore; [2]Hospital for Sick Children, Toronto, [3]CF Foundation, Bethesda, [4]Cystic Fibrosis Center, Verona, Italy

CFTR, the gene responsible for CF, was identified almost 20 years ago and over 1600 variants have been found in this gene. While one mutation is common (delF508) and about 20 “less common” mutations reach a frequency of 1-2%, the remainder are rare, occurring sometimes in one or only a few families. Clinical studies and functional assays provide strong evidence that the common 20 mutations cause disease. However, the disease liability of most of the “rare” mutations is either unclear or unknown. The overall goal of the CFTR2 project is to assess the disease liability of the 1600 or so mutations in CFTR that have been reported in CF patients. Two major tasks have been completed; upgrading of the CF Mutation Database (CFMD) in Toronto to a new version called CFTR1 and the creation of a new phenotype-oriented database at Johns Hopkins that will be named CFTR2. CFTR1 will eventually house all known mutations (disease and non-disease causing variants) in the CFTR gene. The CFTR2 database currently has clinical data on 1099 mutations that occur in 39,614 patients CF patients from North America, Europe and Australasia. A committee of CF clinical experts approved the clinical data set obtained for every patient using uniform criteria. Sweat chloride level was selected as the primary metric. The first application of these criteria has been to mutations that occur in 9 or more patients worldwide which identified 160 mutations that account for 96% of the CF alleles in CFTR2. To complement clinical evidence of CF pathogenicity, the CFTR2 team is evaluating the functional consequences of the 160 mutations using 4 steps: 1) the nature of the mutation 2) studies of mutation effect on RNA splicing 3) studies of mutation effect upon CFTR processing and function and 4) absence in “non-CF” CFTR genes. Mutations that introduce a premature termination codon (point mutation or frameshift due to missing or additional nucleotides) are deemed to have sufficient evidence to be classified as functionally deleterious. Mutations that are predicted alter RNA editing (changes in splice site other than GT-AG) or to change one or more amino acids (substitutions) are being evaluated in steps 2 and 3. The final step will be a screen of healthy fertile male CF carriers (fathers of CF patients) to determine if any have a mutation of unknown disease association in their “healthy” CFTR gene. We are in the process of developing a high through put assay to genotype the 160 CFTR mutations in at least 2000 fathers. The results of the aforementioned methods are also being used to derive an algorithm that predicts the disease liability of the 160 mutations. The development of a predictive algorithm will provide an objective assessment of disease liability that will used to 1) corroborate clinical and functional data, 2) evaluate the disease potential of the remaining rare mutations and 3) enable prediction of disease causing potential of mutations discovered after the CFTR2 project has been completed.

Funded by the CF Foundation (Cutting09A0)

S8.4 - Histone Deacetylases (HDACs) and IFRD1 in CF Airway Epithelial Cell Models

Elise Blanchard[1], Laure Riffault[1], Annick Clement[1], Olivier Tabary[1], Jacky Jacquot[1]

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In cystic fibrosis (CF) patients, recent data suggest that Interferon related developmental regulator 1 (IFRD1) is a modifier gene for lung disease. IFRD1 acts in a histone-deacetylase (HDAC)-dependent manner to mediate transcriptional co-repression of NF-κB transactivation involved in inflammation. We therefore hypothesize that intrinsic alterations might occur in the balance of HDACs 1, 2 and 3 and IFRD1 expression in CFTR-deficient cells compared to normal and corrected CF airway cells.

We have examined the expression level of three HDACs(1-3) mRNA and IFRD1 mRNA (by qPCR) in two CF bronchial cell lines (IB3-1 and CFBE41o-) compared to CFTR-corrected and sufficient (S9 and 16HBE14o-) cell models. We also evaluated the level of HDACs(1-3) proteins by Western blotting and measured the total HDACs enzymatic activity under oxidative stress (IL-1β, 10 ng/ml and/or H_2O_2, 100 and 500 µM, 1h).

We show a lowest expression of HDACs(1-3) and IFRD1 mRNA in CFBE41o- cells compared to 16HBE14o- cells. We observe that the level of HDACs(1-3) proteins and particularly HDAC2 is reduced in CFTR-deficient cells compared to CFTR-sufficient cells in response to oxidative stress. We also demonstrate that total HDAC activity was lower in IB3-1 and CFBE41o- cells compared to S9 and 16HBE14o-cells at both basal and oxidative stress conditions.

Together, our data show significant difference in the HDACs(1-3)/IFRD1 signaling in CFTR deficient cells compared to normal and corrected CF airway cells. Understanding how loss of CFTR function leads to alterations in the regulation of IFRD1/HDACs complex and its role in exaggerated inflammatory response in CF airway cells require further investigation.

Supported by the French Cystic Fibrosis Association "Vaincre La Mucoviscidose".

S8.5 - Wobble Splicing at a NAGNAG Acceptor Site Induces a Mild Cystic Fibrosis Phenotype

Alexandre Hinzpeter[1], Abdel Aissat[1], Elvira Sondo[2], Catherine Costa[1], Nicole Arous[1], Christine Gameiro[1], Agathe Tarze[1], Laurence Weiss[3], Alix de Becdelièvre[1], Michel Goossens[1], Luis J. Galietta[2], Emmanuelle Girodon[1], Pascale Fanen[1]

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Around 30% of alleles causing genetic disorders induce premature termination codons (PTCs) (1) and are usually associated with severe phenotypes; however mild disease outcome can occur. We report mild to asymptomatic phenotypes in three cystic fibrosis homozygous patients for the E831X mutation (2623G>T). Analyses performed on nasal epithelial cell mRNA identified three distinct isoforms, a surprisingly more complex situation than expected for a single nucleotide substitution. Structure-function studies and in silico analyses provided the first experimental evidence of PTC removal by wobble splicing at a NAGNAG acceptor site. In addition to contributing to proteome plasticity (2), wobble splicing can therefore induce in vivo removal of a disease-causing UAG stop codon. This molecular study unravels a novel naturally occurring correction mechanism where the effect of either modifier genes or epigenetic factors could have been suspected. This finding is of importance for genetic counseling as well as for the choice of therapeutic strategies.

References:

1. Holbrook et al. 2004, Nat Genet 36:801-808.
2. Hiller et al. 2004, Nat Genet 36:1255-1257.

S8.6 - Abnormal CFTR mRNA Processing: Which Mutations Cause it, Which Are the Consequences and How Can it be Treated

Anabela S Ramalho[1,2], Margarida D Amaral[1,2]

[1]University of Lisboa, Faculty of Sciences, BioFIG (Center for Biodiversity, Functional and Integrative Genomics), Lisboa, Portugal, [2]National Institute of Health Dr. Ricardo Jorge, Department of Genetics, Lisboa, Portugal

Among the 1,600 variants described to occur in the CFTR gene [1], 13 % are considered to affect splicing, given their localization in consensus splicing sites. However, additional variants may impair or significantly decrease the efficiency of the splicing process, as usually mutations reported at the CFTR mutation database have not been functionally characterized. In fact, the impact of most CFTR variants at the expression and functional levels is still unknown. Yet, this missing information would be of utmost importance for disease diagnosis and prognosis. Moreover, with more recent "CFTR corrective" (or "CFTR-assist") therapies, this knowledge is also critical for appropriate selection of patients. To address this challenge, an international effort, called CFTR2 (Clinical and Functional TRanslation of CFTR) has been undertaken to characterize the pathophysiological consequences of the most common CFTR variants.

Our goal here, in coordination with the CFTR2 project, is to characterize the effects of mutations affecting splicing, not only at the mRNA level, but also in terms of CFTR protein processing and function. To this end, we are producing various CFTR intronic mini-genes (complete CFTR cDNA sequence with one or more introns) and expressing them in cell lines to be used as models for splicing.

Previously, we showed that the CFTR mutation (I1234V, 3832A>G) classified as "missense", actually affects splicing in native tissues from a CF patient with the I1234V/F508del genotype [2]. This mutation creates a "cryptic" donor splicing site in exon 19 (18 nts upstream the normal one) and our data showed that it completely abolishes usage of the normal donor. Here, we confirmed this result in BHK cells expressing a CFTR mini-gene containing intron 19 (IVS19) and the 3832A>G mutation. We also tested in this model how the "strength" of the two donors (normal and cryptic) influences splicing, by swopping the normal donor sequence into the location of the cryptic donor and vice versa. By RT-PCR we observed that when the sequences of both donors are the same (i.e., two equal donors at the cryptic and normal locations) only correctly spliced transcripts are generated. We conclude that the "strength" of the donor seems to play a major role in regulating alternative splicing at this CFTR locus.

Similarly, we produced BHK cell lines expressing CFTR mini-genes containing IVS5, IVS14b or IVS22 and results from qRT-PCR show that all these three introns are correct spliced. Western blot analysis is in progress to assess whether the resulting CFTR proteins are fully processed. Cell lines expressing the IVS5-CFTR construct with the 711+5G>A and 711+3A>G mutations were also generated and, in preliminary analyses, the normally spliced CFTR transcripts were detected.

These mini-gene systems can provide crucial information on the consequences of CFTR splicing mutations and will help to establish the most appropriate therapies to each mutation.

Work supported by PIC/IC/83103/2007 grant and pluriannual funding from BioFIG (FCT, Portugal).

References:

[1] The CFTR mutation database, 2009, http://www.genet.sickkids.on.ca/cftr

[2] Ramalho AS & Amaral MD (2007) Ped Pulmonol S30, p272

Saturday 10 April – 16:45-18:15 **Room: Esmeralda**

SYMPOSIUM 9 – Now and Future Modulators
Chairs: M.D. Amaral (PT) & F. Van Goor (US)

S9.1 - CFTR and CaCCs as pharmacological targets in cystic fibrosis

Nicoletta Pedemonte[1,2], Loretta Ferrera[1], Emanuela Caci[1], Antonella Caputo[1,2], Elvira Sondo[2], Valeria Tomati[2], Olga Zegarra-Moran[1], Luis J.V. Galietta[1,2]

[1]Laboratory of Molecular Genetics, Gaslini Institute, Genova, ITALY
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Recovery of mutant CFTR function is a major goal of pharmacological research in cystic fibrosis (CF). Correctors and potentiators are classes of chemical compounds able to overcome the trafficking and gating defects caused by F508del. Correctors may act by preventing the early degradation of F508del-CFTR in the endoplasmic reticulum and/or by slowing down its removal from the plasma membrane. Potentiators are instead compounds that enhance the activity of the mutant CFTR channel even under conditions of maximal cAMP elevation. Their mechanism of action is probably based on direct interaction with the CFTR protein. Potentiators are also useful for other CF mutations causing gating defect. The search for active compounds has been based on the screening of large chemical libraries using cell based functional assays. This strategy has been useful to identify a large number of very effective potentiators but less successful in the identification of good correctors. Our results indicate that the discovery of correctors by library screening is strongly influenced by the cell background. In most cases, correctors identified in one cell line do not show activity in other cell lines and in primary airway epithelial cells. These findings evidence the need for cells and/or assays that are more predictive of corrector activity in epithelial cells of CF patients. The correction of F508del mutation may also benefit from the identification of relevant molecular targets by proteomics or functional genomic strategies. A promising approach is the screening with siRNA or shRNA libraries looking for targets whose sliencing leads to enhanced F508del-CFTR rescue. Identification of these targets may help to develop more focused pharmacological tools.

An alternative target to restore chloride transport in CF is represented by calcium-activated chloride channels (CaCCs). Stimulation of CaCCs may be potentially beneficial for all CF patients because it is an approach independent of CFTR genotype. Recently, the protein TMEM16A (anoctamin-1) has been identified as an important component of CaCCs. TMEM16A is expressed in the airway epithelium and contributes to chloride secretion and mucociliary clearance. Its pharmacological stimulation may favour airway surface hydration. Activation of TMEM16A/CaCCs may be obtained by increasing intracellular calcium or, in theory, by direct channel activators. Identification of TMEM16A allows the search for specific activators using high-throughput screening approaches. In parallel, a better understanding of TMEM16A/CaCC function and regulation may help to design strategies to enhance chloride secretion by potentiating the activating stimulus or by preventing desensitization.

S9.2 - High-content Fluorescence Microscopy siRNA Screens to Track Function/Traffic of ENaC & CFTR

Margarida D. Amaral

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The information of complete genome sequences and the identification and systematic cloning of human cDNAs provide the challenging opportunity to analyse the complexity of biological processes on a large scale.

Here, the application of cutting-edge microscopy-based screening technology to study traffic and function of two CF-related membrane proteins, namely CFTR and ENaC will be described. For CFTR, novel lentiviral, inducible (Tet-ON) constructs (wt- and F508del) were generated, bearing both an mCherry-tag (fluorescent) fused at the N-terminus and a Flag-tag at CFTR 4th extracellular loop. These two tags allow determining on each individual cell the fraction of expressed CFTR which is residing in the cell membrane. For ENaC, a functional live-cell assay was selected, based on its activity of as Na^+ channel and using the FLIPR membrane potential (FMP) voltage-sensitive fluorescent dye in combination with the specific ENaC-blocker amiloride.

Data will be presented from ongoing studies regarding the application of these assays in the context of high-throughput "primary" screens to identify genes/proteins affecting the traffic of CFTR and function ENaC. In these screens, three high-content libraries of human siRNAs (Ambion) are used, namely: 1) the "kinome", targeting 710 different kinase genes; 2) the "secretome", targeting 1,552 genes involved in the trafficking of a temperature-sensitive VSVG variant (tsO45G); and 3) and the "druggable" targeting different 5,940 genes of relatively known function.

After "hit" validation by two additional siRNAs and "secondary" screens to classify them into ENaC/ CFTR pathways, some of the "hits" will be selected as possible drug targets for CF.

Work supported by TargetScreen2 (EU-FP6-LSH-2005-037365)grant.

S9.3 – Identification and Validation of Novel Drug Targets to treat Cystic Fibrosis: Modifiers of the Trafficking Defect of CFTR∆508

Fischer, David F.[1].; Tessari, Michela A[2]; Noel, Sabrina[3]; Roseboom, Marjet[1] ; Sammut, Sébastien[2]; Scaffidi, Amelia[1]; Frizzell, Raymond A.[3]; Janssen, Richard A.[2]; Ashlock, Melissa A.[4]

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Abnormal retention of the CFTR ∆F508 mutated protein in lung epithelial cells underlies the pathology in a large proportion of individuals with cystic fibrosis. A drug discovery alliance between CFFT and Galapagos was initiated with the aim to identify novel genes which upon shRNA-mediated knockdown were able to efficiently restore∆F508 CFTR activity. The final goal of this program is to prioritize these targets for entry into drug discovery.

BioFocus DPI's proprietary adenoviral shRNA libraries totaling 11,334 viruses against the human druggable genome were screened in a high-throughput functional assay in a human cystic fibrosis bronchial epithelial cell line (CFBE41o-). A total of 354 hits were identified and confirmed in the primary assay. Validation of the hits included a lack of cytotoxicity of the shRNA, expression of target in airway epithelial cells, identification of a second shRNA against the same target, and cell surface expression by biotinylation of CFTR∆F508 upon shRNA -mediated knock-down of the target. Most importantly, these shRNAs restored functional activity of the mutant channel in primary bronchial epithelial cells from ∆F508/∆F508 CF patients in transepithelial CFTR mediated current assays (Table 1). There was a very strong correlation between fully glycosylated band C expression in the CFBE41o- cells and functional activity in the primary cells of CF patients.

We will present an overview of the target discovery and validation program, resulting in a portfolio of 19 novel drug targets to treat CF. The identity of these novel targets also sheds light on the biology of trafficking of CFTR. For example, these data point to a role of TGF-beta signaling and inflammatory mediators in airways in the regulation of CFTR trafficking.

Acknowledgments
We thank Drs. Bill Guggino, Ineke Braakman, Kevin Foskett, John Hanrahan and Hugo De Jonge for helpful discussions. We acknowledge the support of Cystic Fibrosis Foundation Therapeutics.

S9.4 - SIV Vector Pseudotyped with Sev-F/HN Envelope Proteins Produces Long Lasting Expression in the Murine Lung, Is Readministrable and Transfects Human Airway Models

Uta Griesenbach[1,2], Makato Inoue[3], Cuixiang Meng[1,2], Andrea M Brum[1,2], Raymond Farley[1,2], Nikki Newman[1,2], Eiji Akiba[3], Mamoru Hasegawa[3], Eric WFW Alton[1,2]

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We have previously shown that simian immunodeficiency virus pseudotyped with F and HN protein from Sendai virus (F/HN-SIV) transduces murine nasal epithelium efficiently (3-5% of respiratory epithelial cells after perfusion with $5x10^8$ TU) and importantly can be repeatedly administered. We now show that expression in the nasal epithelium persists for the life-time of the animals (16-25 months, 9 of 9 C57Bl/6 mice). The vast majority of published studies have assessed lentiviral vectors in mouse nasal epithelium, and transduction of lung epithelium, particularly without pre-conditioning through polidocanol treatment or tissue damage, remains challenging. Here, we show that F/HN-SIV transduces lung epithelium efficiently and dose-dependently. In contrast to other studies, we show that lentivirus-mediated gene expression in the lung is stable for at least 13 months (latest time-point currently analyzed) (month 2: 362660±63922photons/sec, month 13: 431454±65647photons/sec, n=5-8 mice). We also show, for the first time, that lentivirus can be repeatedly administered to the lung (2 doses of F/HN-SIV-GFP followed by a 3rd dose of F/HN-SIV-Lux at monthly interval) without loss of activity compared to a single dose of F/HN-SIV-Lux (1 Dose: 40178±4843, 3 Doses: 39080±7490 RLU/mg protein, n=21/group). Importantly, we also show that gene expression increases with increasing number of doses (10 doses at daily interval) (Dose 1: 4693± 899, Dose 10: 239212±48362 RLU/mg protein, n=8/group, $p< 0.0005$). Importantly, we have not observed acute or chronic toxicity in 12 months follow-up studies in mice. We have also assessed the performance of F/HN-SIV in various airway ex vivo models. (a) The virus transduces human air liquid interface cultures efficiently with expression persisting for at least 4 months. (b) The virus transduces freshly obtained human nasal brushings dose-dependently. (c) The virus transduces human lung slices efficiently and expression persists for the life-span of the slices. These data suggest that, F/HN-SIV may be a suitable vector for cystic fibrosis gene therapy.

S9.5 - Effect of TS-04-95 on Primary Human Bronchial Epithelial Monolayers after a Repeated Dose Treatment

Marisa Sousa[1,2], Inna Uliyakina[1,2], Diana Faria[3], Yuemin Tian[3], Marta Palma[2], Alexis Traynor-Kaplan[4,5], Karl Kunzelmann[3], Margarida Amaral[1,2]

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A valid therapeutic strategy to circumvent the defective CFTR in CF airways involves activation of other, non-CFTR Cl^- channels, like the Ca^{2+}-activated Cl^- conductance (CaCC). Such "bypassing pharmacotherapy" [1] has the advantage of being equally applicable to CF patients with any CFTR genotype. Moreover, since hyperabsorption of Na^+ through ENaC has been described to be regulated by Cl^- transport [2], CaCC activators could serve to normalize both Cl^- and Na^+ transport in CF airways. Until recently, the most promising strategy to stimulate CaCC was through ATP- stimulation of purinergic receptors. However, other potential tools for a bypassing pharmacotherapy comprise manipulation of signaling pathways acting on ionic transport. Compounds in the inositol phosphate (InsP) pathway have been shown before to successfully inhibit Na^+ and fluid hyperabsorption across CF airway epithelial cells and also to enhance CaCC activity [2-4]. Here, we investigated the effects of an InsP-related compound (TS-04-95) to evaluate its therapeutic potential for CF.

We determined the efficacy of TS-04-95 in both stimulating Cl^- CaCC/TMEM16A-mediated currents (using the CaCC-01 inhibitor) and in inhibiting ENaC-mediated currents. Measurements were done in monolayers of polarized primary cultures of human bronchial epithelial (HBE) cells from lung explants [5] and human airway epithelial cell (H441) mounted in Ussing chambers. In addition double electrode voltage clamp (DEVC) experiments were performed in *Xenopus* oocytes and whole-cell patch-clamp experiments were performed in TMEM16A-expressing HEK293 cells. Results show that the repeated exposure of non-CF primary HBE monolayers (n=5) to TS-04-95 significantly ($p < 0.05$) enhanced the sustained ATP and CCH responses (in plateau phase 18.87±1.26 $\mu A/cm^2$ and 5.02±1.37 $\mu A/cm^2$, respectively). Experiments in HEK293 cells and *Xenopus* oocytes also indicated activation of TMEM16A by TS-04-95. No significant effects of TS-04-95 on ENaC-mediated Na^+ absorption or cAMP-mediated Cl^- secretion by CFTR were observed in non-CF primary HBE monolayers, while pronounced inhibition of ENaC by TS-04-95 was observed in H441 cells. These results suggest TS-04-95 as an efficient pharmacological tool for the treatment of the CF lung disease, whose efficacy in correcting epithelial salt transport will be further validated in primary cultures of HBE cells from human CF lungs as well as in native rectal biopsies from CF patients

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Work supported by TargetScreen2 (EU-FP6-LSH-2005-037365), PIC/IC/83103/2007 grants and BioFig (FCT, Portugal). MS is recipient of SFRH/BD/35936/2007 PhD fellowship (FCT, Portugal). Authors are grateful to A. Verkman for Ca01 compound.

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S9.6 - Effect of Small Molecule Correctors on Different Mutants of CFTR

Liudmila Cebotaru[1], William Guggino[2]

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Recent studies have identified small molecules which can rescue the processing of ΔF508. To gain insight into how these small molecules are working, we studied whether they can rescue two truncated forms of CFTR; TNR CFTR which is missing the second transmembrane domain and NBD2 and Δ264 CFTR which is missing the first 4 transmembrane segments of CFTR, and the missense mutation A455E which is associated with milder forms of CF.

Treating cells with cycloheximide demonstrated that the half life for both ΔF508 and Δ264 CFTR is short indicating that they are both rapidly degraded. The half life of A455E is much longer than that of ΔF508 CFTR but less than wild type CFTR consistent with A455E being a partial processing mutant. TNR CFTR, similar to wt CFTR is much more stable than either Δ264, ΔF508 CFTR or A455E over an 8 hour treatment period.

We observed that VRT 325 increased both B and C bands of ΔF508 CFTR in transiently transfected Cos 7 cells. The protein expression of Δ264 CFTR was significantly increased following treatment with 4A or VRT325. Both 4A and VRT325 increased the steady state levels of bands B and C of A445E. In contrast, protein expression of TNR CFTR was not affected either by 4A or VRT 325.

We conclude that mutant forms of CFTR such as ΔF508, Δ264 CFTR and A455E which are recognized as mutant proteins by the ER quality control mechanism can be corrected by 4A or VRT325. In contrast, TNR CFTR which resides stably in the ER cannot be corrected by these same small molecules. The data suggest that correctors 4A and VRT325 are likely to affect components of the quality control mechanism rather than correcting the folding of mutant CFTR.

Saturday 10 April – 18:45-19:45 **Room: Esmeralda**

Closing Lecture

CFTR modulators - Targets and Opportunities

JP Clancy / S. Rowe

The Gregory Fleming James Cystic Fibrosis Research Center
University of Alabama at Birmingham, and the Children's Hospital of Alabama
Birmingham, AL

Small molecules that have been developed to overcome genotype-specific defects in CFTR have advanced from high throughput screening to preclinical testing and now into clinical trials in CF patients. Currently there are three separate agents addressing three different classes of CF mutations that are under clinical investigation. Positive results reported over the past twelve months suggest that CFTR is a realistic target for drug development. In this symposium, Drs. Clancy and Rowe will review the recent and more distant history of preclinical and clinical trials with CFTR modulators over the past decade, including early studies of drugs to overcome F508del CFTR processing defects, suppression of premature termination codons in CFTR, and other studies that seek to use established therapeutics to overcome clinically relevant mutations in CFTR. In addition, the results of these studies and the recent availability of CFTR-active agents have lead researchers to examine how small molecules may overcome defects in mutant CFTRs, how to measure restored epithelial functions in preclinical model systems that predict clinical benefits, and how to bring novel strategies together to maximally enhance CFTR function in vitro and in vivo. This 'return to the bench' is sure to provide researchers with exciting clues to basic questions that underlie cystic fibrosis and CFTR defects, and provide logical strategies for future treatment endeavors.

Room: Safira
Posters – CFTR Structure and Function

P. 1

CFTR Controls the Chloride Ion Homeostasis in Rat Spinal Motoneurons during Postnatal Development

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Despite recent evidence that CFTR may be expressed in the central nervous system, its role in neuronal activity remains unclear. Our laboratory originally reported functional CFTR expression in motoneurons of neonatal rat lumbar spinal cord (Ostroumov et al., Brit J Pharmacol. 2007 Jan; 150(1): 47-57) where it can play an important role in regulation of neuronal chloride homeostasis. In the developing central nervous system neuronal chloride homeostasis is obtained via the opposite action of two principal cation-chloride cotransporters, namely NKCC1 and KCC2. The relative contribution to chloride transport (namely uptake and extrusion, respectively) by these transporters determines the direction and magnitude of the chloride current through GABA or glycine channels, which are important to control neuronal excitability via synaptic inhibition. Since during the first postnatal week GABA changes its action from depolarizing to hyperpolarizing, the expression changes and function of NKCC1, KCC2 and CFTR in the neonatal rat spinal cord were studied. Using real time RT PCR and immunohistochemistry, the present report shows that, in the lumbar region of the spinal cord, there was strong developmental control on the expression and location of CFTR and transporter proteins involved in chloride homeostasis (KCC2 and NKCC1). Interestingly, a positive correlation between age dependent rise in NKCC1 and CFTR gene expression was found. Electrophysiological techniques were used to investigate CFTR function in terms of its contribution to chloride regulation at different postnatal age. Sharp electrode experiments with the CFTR blocker glibenclamide (50 µM) showed this protein to be functional in motoneurons between P1 and P8 as this blocker increased motoneuron input resistance. Influence of this protein on the chloride reversal potential was examined with the whole cell patch-clamp technique applied to sagitally hemisected spinal cords. Lumbar motoneurons were approached blindly through the medial (cut) surface of the spinal cord and were identified by their antidromic response to ventral root stimulation. For estimation of chloride reversal potential, spontaneous inhibitory synaptic events were recorded in voltage clamp mode after blocking excitatory glutamatergic transmission. Under these conditions, block of CFTR by glibenclamide produced a negative shift in chloride reversal potential which was less intense at the end of the first postnatal week. Our data suggest that CFTR was functionally expressed by motoneurons of the rat lumbar spinal cord at the age between P1 and P8, when it was involved in chloride homeostasis in a developmentally-regulated fashion.

Supported by a grant from FVG regional government.

Understanding CFTR Biogenesis through the Deletion of the Regulatory Extension (RE) and Regulatory Insertion (RI)

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Structural knowledge of CFTR is crucial in order to gain a better understanding of how disease-associated mutations compromise the maturation and channel activity of the protein. Moreover, studies on NBD1 are of particular interest because this domain contains F508del the most prevalent CF-causing mutation, which causes defects in protein folding, plasma membrane trafficking and channel gating. Comparison of wt- and F508del-NBD1 crystal structures indicated that they do not substantially differ, except for their surface properties around the F508del residue [1,2]. Two segments may be responsible for such alteration, namely the regulatory extension (RE) and regulatory insertion (RI), both suggested to be conformationally dynamic, namely upon phosphorylation [1, 2]. The RI (Glu403-Leu435) is a ~30-residue insert between the first two ß-strands of the NBD1 ß-subdomain, and the RE (Ser654-Gly673) is at the C-terminus of NBD1, where it extends ~20 residues longer than canonical ABC domains forming a helix packing against NBD1 at the NBD1-NBD2 interface.

We hypothesized that the dynamic flexibility of these regions may also result in exposition of hydrophobic surfaces contributing to the low folding efficiency of both wt- and F508del-CFTR. Our first goal here was to study the structural/functional impact of removing the RE and the RI on full-length CFTR by assessing its *in vivo* processing. Secondly, we aimed to evaluate the effect on these variants of *in cis* mutations previously described to correct the defect of F508del-CFTR folding, namely: R1070W and G550E.

To this end, we produced ΔRE and ΔRI variants of wt- and F508del-CFTR, alone, together (ΔRE/ΔRI) and with those 2 point mutations by *in vitro* mutagenesis and used them to generate BHK stable cell lines. Results from Western blot (WB) show that wt-CFTR ΔRE, ΔRI and ΔRE/ΔRI are efficiently processed, although ΔRE/ΔRI exhibits lower levels of the mature form at steady-state. These data suggest that these variants acquire a native (folded) conformation *in vivo*, consistently with a previous study [3]. Their functional characterization by iodide efflux shows that ΔRE- and ΔRI-wt respond to agonists but not ΔRE- nor ΔRI-F508del. Moreover, removal of the RE causes a significant decrease in the function of wt-CFTR. By immunofluorescence, we show that ΔRE-wt is predominantly at the plasma membrane whereas ΔRE- and ΔRI-F508del are in the ER. Also, ΔRI-wt shows the same ER localization as F508del-CFTR with faint membrane staining. Preliminary results show that R1070W-ΔRI-F508del (but not R1070W-ΔRE-F508del) undergoes maturation. Additional studies on maturation efficiency and functional characterization of these (and of G550E) CFTR) variants are underway as well as studies with F508del-CFTR correctors.

Altogether, results from this study will help to elucidate the structural changes that account for the F508del defect and the mechanism of action of correctors.

Work supported by PIC/IC/83103/2007 grant and BioFIG - Centre for Biodiversity, Functional and Integrative Genomics (FCT, Portugal*). ACDP is a recipient of PhD fellowship SFRH/BD/17475/2004 (FCT,* Portugal*).*

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Trypsin-4 and Neutrophil Elastase Regulate CFTR Expression and Function in Cystic Fibrosis

Mathieu Le Gars[1,2,3], Delphyne Descamps[1], Naziha Bakouh[4], Gabrielle Planelles[4], SS Hong[5], Pierre Boulanger[5], Michel Huerre[2], Michel Chignard[1], Jean-Michel Sallenave[1,3]

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Introduction: Cystic fibrosis (CF) is a genetic disease caused by mutations in the CF transmembrane conductance regulator (CFTR). Defective membrane mutant CFTR (ΔF508) expression is instrumental in mediating lung inflammation. Lung stromal and inflammatory cells secrete serine-proteases which have well described deleterious effects on the integrity of lung tissue. However, the effect of proteases on CFTR has not yet been addressed. The aim of the present study was to characterize the effect of tissue (trypsin-4) and neutrophil-derived (Elastase or NE) serine-proteases on CFTR both at structural and functional levels.

Methods:

1) Epithelial cells (human bronchial NCI-H292 and alveolar A549 cells) were infected with adenovirus over-expressing wild-type (WT) and mutant ΔF508 CFTR for 48 h and subsequently incubated with increasing levels of either NE (NCI-H292) or trypsin-4 (A549). After 7 h, the effect of protease incubation was assessed by measuring CFTR expression (Western Blot analysis) and IL-8 secretion (ELISA).

2) *Xenopus laevis* oocytes were injected with RNA encoding for WT CFTR protein (CFTR-oocytes) or with water (control oocytes). Current voltage (I/V) was measured 3 days later in presence of Ringer with or without forskolin+IBMX supplemented or not with NE for 15 min.

Results: We show here in bronchial and alveolar epithelial cells that NE and trypsin-4 up-regulate IL-8 production 1.3 to 1.5 fold in CF cells (over-expressing CFTR ΔF508), respectively, when compared to WT cells. This induced IL-8 up-regulation was correlated with the NE (but not trypsin-4) generation of a cleaved 95 kDa CFTR molecular species, suggesting that the IL-8 pro-inflammatory phenotype is exacerbated by CFTR degradation. In WT CFTR-transfected oocytes, Forskolin+IBMX enhanced the slope of linear shaped I/V curves, consistent with the functional expression of CFTR. In WT CFTR-oocytes also incubated with NE, increase in conductance was 2 to 2.5 fold lower.

Conclusions: We demonstrate here that while both serine-proteases NE and trypsin-4 up-regulate IL-8 in bronchial and alveolar epithelial cells, only NE is able to significantly degrade the WT CFTR protein. This degradation also occurred with ΔF508 CFTR, suggesting that NE may exacerbate the CFTR phenotype in CF. We are currently dissecting the mechanisms involved.

Keyword: CFTR, Elastase

Role of the C-terminal Extremity of CFTR NBD1 in the Control of CFTR Activity

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Cystic fibrosis (CF) is caused by mutations of the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). Mutations of the *CFTR* gene affect the trafficking of the protein and/or its function. It has already been shown that the fragment of NBD1 comprising the amino acids 622 to 634 is crucial for the maturation process of CFTR proteins (Chan et al., 2000). In this study we have investigated the role of the last β hairpin of NBD1 and its direct environment in the activation of CFTR channel. We performed a molecular dissection of CFTR by examining several CFTR mutants constructed by site directed mutagenesis and expressed as GFP-tagged proteins in HEK293 and BHK cells. These mutants, located in the last β hairpin of NBD1 or in contact with it, were studied downstream the β_c5 (H620Q), inside the hairpin (E621G), and within the β_c6 (S623A & S624A) or in contact with H620 (S459A in the Walker A of NBD1and F640A). Patch clamp analysis using whole cell configuration revealed a time and voltage independent Cl^- current stimulated by forskolin (Fsk) for all CFTR mutants. All the currents were blocked by 10 µM of $CFTR_{inh}$-172. The Cl^- current (I) elicited by E621G and S624A mutants was similar to wt current. We determined I at +40 mV for wt, 83.3±9.7 pA/pF; E621G, 84.2±10.1 pA/pF; S624A, 81.8±7.7 pA/pF. On the contrary the mutations introduced on both sides of the hairpin (H620Q & S623A) or in conjunction with the amino acid H620 (S459A & F640A) results in a CFTR channel with an strong increased Cl^- channel activity (I at +40 mV: H620Q, 124.6±19.4 pA/pF; S623A, 142.8±16.7 pA/pF; S459A, 192.6±15.1 pA/pF; F640A, 178.3±5.8 pA/pF). Moreover, all the mutated channels with an increase Cl^- current present a faster time course of activation. These results indicated that the orientation of the two latest β strands and the link between this hairpin and other domains, like the walker A of NBD1, could play a role in the control of CFTR channel activation. All these results suggest an important influence of the C-terminal extremity of NBD1 into the control of CFTR channel gating.

Supported by Vaincre La Mucoviscidose, Ministère de l'enseignement supérieur et de la recherche and CNRS

Room: Safira
Posters – CFTR Biogenesis, Quality Control and Trafficking

P. 5

Spleen Tyrosine Kinase (SYK) Phosphorylates CFTR NBD1 and Interacts in vivo with CFTR

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Regulation of CFTR intracellular trafficking as well as channel activation is the result of a complex network of CFTR interacting proteins, that include molecular chaperones, glycosidases, the basal trafficking machinery and other factors, among which protein kinases and phosphatases are particularly relevant. Regulation of CFTR biogenesis/traffic by phosphorylation and dephosphorylation is still poorly understood. Spleen Tyrosine Kinase (SYK) is a non-receptor tyrosine kinase, described to have a role in signal transduction, and its consensus phosphorylation site consists of a tyrosine followed two negatively charged residues. In CFTR sequence, this consensus appears once at Tyr512 (i.e., very close to residue F508, deleted in most CF patients).
Our aim here was to identify the putative role of SYK on CFTR biogenesis and trafficking.
Firstly, by qRT-PCR analysis, we demonstrate that SYK is endogenously expressed in epithelial respiratory cells (Calu-3 and virally transduced wt-or F508del-CFBE), thus arguing for the physiological significance of this kinase in human airway epithelial cells. Next, data from CFTR co-immunoprecipitation followed by western blot (WB) for SYK (and vice-versa) showed that SYK interacts *in vivo* with CFTR. Then, *in vitro* phosphorylation, using immunoprecipitated SYK and purified CFTR-NBD1 or recombinant NBD1-Y512F, revealed that SYK undergoes auto-phosphorylation but that it also phosphorylates CFTR-NBD1 but not NBD1-Y512F. Furthermore, data also show that phosphorylation is abolished when dead-kinase SYK is used in this assay.
To further elucidate the biochemical and functional consequences of mutating Tyr512, we generated cell lines stably expressing CFTR mutants, where Y512 was substituted by either a neutral residue (Y512A and Y512F, mimicking the bulky side group of tyrosine) or by an acidic residue (Y512D, Y512E) both in the wt- and F508del-CFTR backgrounds. Pulse-chase experiments followed by CFTR immunoprecipitation and WB in these cells lines show that Y512A/D-CFTR have both decreased steady-state levels and efficiency of processing vs those of wt-CFTR, whereas Y512F-CFTR does not evidence significant differences neither in immature form turnover nor in its maturation efficiency. CFTR function of these mutants was also determined using the iodide efflux assay. Results show that cells expressing Y512A-CFTR and Y512D-CFTR have, respectively ~15% and ~60% reduction in the IBMX/Fsk-stimulated iodide efflux vs those expressing wt-CFTR.
Altogether, our data suggest phosphorylation of wt-CFTR by SYK plays a positive role in its stability and maturation and that this effect seems to be mediated by the consensus site for SYK at Y512-CFTR.

Work supported by TargetScreen2 (EU- FP6-LSH-2005-037365) grant and pluriannual funding of BioFig (FCT, Portugal). SL is recipient of PhD fellowship SFRH/BD/47445/2008 (FCT, Portugal).

Mechanisms of F508del-CFTR Rescuing by Genetic Revertants, Low Temperature and Small Molecules

Carlos M Farinha[1,2], Marisa Sousa[1,2], Inna Uliyakina[1,2], Ana Carina Da Paula[1,2], Margarida D Amaral[1,2]

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CFTR bearing F508del, the most common CF-causing mutation, is retained intracellularly at the endoplasmic reticulum (ER) due to ineffective folding and sent to premature degradation through the ubiquitin proteasome pathway (UPP). Accordingly, most mutant protein fails to reach its proper location at the plasma membrane. Several approaches have been used in the recent years to rescue the misfolding and trafficking defects of the F508del-CFTR mutant and to understand the underlying mechanisms. These strategies either improve folding or circumvent the ER quality control (ERQC) that targets F508del-CFTR to UPP, or both. They include: 1) low temperature incubation (26ºC); 2) presence of genetic revertants (second-site mutations that either remove ERQC retention signals or stabilize its folded conformation); and 3) small molecules (interacting directly with F508del-CFTR to favour correct folding and/or indirectly to promote traffic). Understanding how F508del-CFTR is rescued to the cell surface by distinct agents enables us to mimic such effects by small molecules. Moreover, identifying distinct rescuing mechanisms, will possibly allow usage of different therapeutic molecules for enhanced results.
Our aim is to assess the synergistic/additive effects of F508del-CFTR correctors with those of several genetic revertants of F508del-CFTR and of low temperature incubation to learn more about their mechanism of action (MoA). As an example, we tested here the VRT-325 small molecule.
To this end, the following single F508del-CFTR revertants (stably expressed in BHK cells) were tested: I539T; G550E; R553M; R553Q; R555K; R1070W. Also the following multiple revertants: 4RK; G550E/R553Q; R553M/R555K; R553Q/R555M; 4RK/G550E; F494N/Q637R; and F429S/F494N/Q637R. Western Blot was performed after incubating these cell lines at 26ºC for 48h and/or treatment with VRT-325 (6.7µM; 24h). Densitometry was used to calculate for each variant the percentage of band C vs. the total of CFTR expressed. Data were normalized to band C percentage in BHK cells expressing wt-CFTR. Results so far regarding efficacy of F508del-CFTR rescuing show that: i) R553M is the most effective single genetic revertant; ii) R553Q/R555K is the most effective double revertant combination; iii) VRT-325 is less efficient than low temperature. Regarding additivity of different rescuing agents; iv) low temperature additivity is evident for all the genetic revertants tested except R553M and is most evident for G550E; v) VRT-325 exhibits highest additivity with the G550E revertant; vi) the only genetic revertants exhibiting additivity with both low temperature and VRT-325 are R1070W, R553M/R555K and G550E/R553Q; vii) 4RK/ G550E restores F508del-CFTR processing to wt-CFTR levels. Results iv) and v) suggest that rescuing by these agents takes place by distinct MoA.
Altogether, these data contribute to further elucidate the MoA of F508del-CFTR correctors, as exemplified here for VRT-325, and its relationship with specific residues critical for CFTR folding.

Work supported by PIC/IC/83103/2007 grant and BioFig pluriannual funding (FCT, Portugal). MS is recipient of SFRH/BD/35936/2007 fellowship (FCT, Portugal). Authors thank CFF-USA for correctors (B.Bridges, Chicago, USA) and antibody (J.Riordan, UNC, USA) as well as P.Haggie (UCSF, CA USA) for the YFP plasmid.

Effect of Small Molecule Correctors on Different Mutants of CFTR

Liudmila Cebotaru[1], William Guggino[2]

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Recent studies have identified small molecules which can rescue the processing of ΔF508. To gain insight into how these small molecules are working, we studied whether they can rescue two truncated forms of CFTR; TNR CFTR which is missing the second transmembrane domain and NBD2 and Δ264 CFTR which is missing the first 4 transmembrane segments of CFTR, and the missense mutation A455E which is associated with milder forms of CF.

Treating cells with cycloheximide demonstrated that the half life for both ΔF508 and Δ264 CFTR is short indicating that they are both rapidly degraded. The half life of A455E is much longer than that of ΔF508 CFTR but less than wild type CFTR consistent with A455E being a partial processing mutant. TNR CFTR, similar to wt CFTR is much more stable than either Δ264, ΔF508 CFTR or A455E over an 8 hour treatment period.

We observed that VRT 325 increased both B and C bands of ΔF508 CFTR in transiently transfected Cos 7 cells. The protein expression of Δ264 CFTR was significantly increased following treatment with 4A or VRT325. Both 4A and VRT325 increased the steady state levels of bands B and C of A445E. In contrast, protein expression of TNR CFTR was not affected either by 4A or VRT 325.

We conclude that mutant forms of CFTR such as ΔF508, Δ264 CFTR and A455E which are recognized as mutant proteins by the ER quality control mechanism can be corrected by 4A or VRT325. In contrast, TNR CFTR which resides stably in the ER cannot be corrected by these same small molecules. The data suggest that correctors 4A and VRT325 are likely to affect components of the quality control mechanism rather than correcting the folding of mutant CFTR.

Preferential Sumoylation of ΔF508 CFTR and NBD1 Leads to Protein Degradation

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The cystic fibrosis transmembrane conductance regulator (CFTR) was the first mammalian membrane protein implicated as a substrate for endoplasmic reticulum associated degradation (ERAD), and it has served as a model for the folding or disposal of polytopic membrane proteins. Steps in ERAD usually include the recognition and removal of misfolded proteins from the ER, followed by their ubiquitylation and proteasome-dependent degradation. Due to its complex folding and domain assembly requirements, much of WT CFTR and ~100% of the common folding mutant, F508del CFTR, are degraded in most systems.

Small heat shock proteins (sHsps) bind destabilized proteins during cell stress and disease, but their physiological functions are less clear. Hsp27 is expressed in airway epithelial cells, and it selectively interacted with the common CFTR mutant, F508del, and targeted it for proteasomal degradation. This action of Hsp27 appears to result from its ability to interact physically with the SUMO E2 conjugating enzyme, Ubc9, and like Hsp27, Ubc9 selectively promoted F508del CFTR proteolysis. Similarly, the knockdown of Hsp27 or the SUMO E1 enzyme, SAE1/2, increased CFTR expression 2-3 fold. Hsp27 expression promoted the sumoylation of F508del CFTR in vivo, and disabling the SUMO pathway via SUMO E1 knockdown reduced the ability of Hsp27 to degrade mutant CFTR.

To begin to evaluate the properties of CFTR that lead to its sumoylation/degradation, we determined the modification of CFTR NBD1 by SUMO-1 in vitro. The reaction mixture included the purified components: E1, E2, SUMO-1, WT or F508del NBD1 and ATP. NBD1 sumoylation was detected either by the molecular mass shift observed upon blotting with an NBD1 antibody or from blots performed using anti-SUMO-1. SUMO modification of NBD1 increased as a function of time over a 0-120 min assay period, with significantly greater modification observed for F508del vs. WT NBD1. Reactions run at different ATP concentrations demonstrated the expected ATP dependence of NBD1 modification, and showed also that WT NBD1 sumoylation increased at low ATP whereas the F508del NBD was less sensitive to ATP in the range 0.1-2 mM. Thus, the native NBD1 conformation, as exemplified by the WT domain at high ATP, was a poor substrate for SUMO modification relative to F508del NBD1. These observations, together with the eight degree lower melting temperature of the F508del NBD1, suggest that the native NBD1 conformation is not modified by SUMO, whereas non-native conformational intermediates are preferentially sumoylated.

These findings link sHsp-mediated mutant CFTR sumoylation to protein degradation, and they raise the possibility that folding intermediates formed during CFTR biogenesis are stabilized by SUMO addition until the native conformation is obtained.

Room: Safira
Posters – Epithelial Ion and Fluid Transport

P. 9

Expression and Functional Roles of TRP Channels in Normal and CF Airway Epithelial Cells

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The Transient Receptor Potential (TRP) calcium-permeable cation channels are a novel superfamily of cation channels many of which enable cells to sense changes in their local environment, such as pH, temperature and mechanical forces. There is growing evidence for the involvement of TRPs in various disease states, including respiratory diseases. However, the expression patterns and functional roles of TRPs in the respiratory epithelium remain poorly characterised. The aim of this project was to investigate the expression and functional roles of several TRPs, while primarily focusing on TRPA1, in airway epithelial cells derived from healthy subjects and patients with cystic fibrosis (CF).

TRP expression was examined in primary nasal epithelial cells obtained by nasal brushings from healthy volunteers (HNEC) and patients with CF who are homozygous for F508del (CFNEC), in primary bronchial epithelial cells obtained by bronchial brushing from healthy volunteers (PBEC) and in human bronchial epithelial (16HBE14o-, HBE) and cystic fibrosis bronchial epithelial (CFBE41o-, CFBE) cell lines (gift from Dieter C. Gruenert, University of San Francisco).

Preliminary data show that gene transcripts for TRPA1, TRPM8 and TRPV1 are detected in all cell types using RT-PCR (n=3, except CFNEC, n=1). Protein expression was confirmed in all cell types using western blotting (n=3, except CFNEC, n=1). Preliminary immunocytochemical studies revealed the presence of TRPM8 (n=2) and TRPV1 (n=1) in HNEC and CFNEC and the presence of TRPA1 in PBEC (n=1).

For functional studies on TRPA1, PBECs were loaded with fluo-4AM ester (0.025% (w/v) pluronic) for 30 min prior to imaging, imaged using laser confocal microscope (BioRad) and stimulated with the TRPA1 specific agonist, cinnamaldehyde (10 - 300µM). There was a dose dependent increase in $[Ca^{2+}]_i$. The responses at maximally effective agonist concentration (~ 100 µM) were ~50% of the responses to the calcium ionophore, 1 µM A23187. The best-fit EC_{50} value for mean concentration-response data (n= 10 cells per condition) was 50 µM which was similar to that in HEK293 cells expressing TRPA1 (40 µM, n= 10 cells per condition).

These preliminary data suggest that TRP channels (TRPA1, TRPM8 and TRPV1) are similarly expressed in epithelial cells (both primary cells and cell lines) from healthy subjects and patients with CF. Further work is required to determine whether channel function in maintained in CF cells. This is potentially important as several of these channels respond to noxious stimuli causing airways inflammation.

F508del-CFTR Increases Intracellular Ca^{2+} Signaling that Causes Enhanced Calcium-dependent Cl^- Conductance in Cystic Fibrosis

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Increase in intracellular calcium $[Ca^{2+}]_i$ activates a chloride conductance (CaCC) that has been shown to be enhanced in cystic fibrosis (CF). An ongoing controversy exists as to the reason of enhanced CaCC in CF. As shown earlier it may be due to infection of CF airways as well as accumulation of mutant cystic fibrosis transmembrane conductance regulator (F508del-CFTR) in the endoplasmic reticulum (ER), which may cause a ER stress response. In nasal epithelial cells of patients homozygous for F508del-CFTR we did not detect an increase in expression of the Ca^{2+} activated Cl^- channels TMEM16A and bestrophin 1. Also expression of plasma membrane localized TMEM16A or ER-localized bestrophin 1 was not different between CF bronchial epithelial (CFBE) cells stably expressing wtCFTR or F508del-CFTR. However, purinergic calcium signaling was enhanced in CFBE/F508del-CFTR cells. Exposure of CFBE cells to bacterial lipopolysaccharides (LPS) had only marginal effects on intracellular calcium signaling.
However, the $[Ca^{2+}]_i$ augmenting effect of F508del-CFTR was confirmed in the human airway epithelial cell line A549 after induction of expression of F508del-CFTR. We found that UTP-induced increase in $[Ca^{2+}]_i$ was significantly reduced in chloride-depleted cells. Cl^- dependence of the intracellular Ca^{2+} signaling is reminiscent of the role of bestrophin1 for intracellular Ca^{2+} signaling. As shown earlier (Barro-Soria et al, Pflügers Archive 1999), bestrophin1 is an ER-localized Cl^- channel that facilitates Ca^{2+} release from the ER by acting as a counter ion channel. Our data suggest that ER-trapped F508del-CFTR may similarly function as an ER-localized Cl^- channel, which may be the mechanism how F508del-CFTR enhances intracellular Ca^{2+} signaling. An additional reason for the increase in $[Ca^{2+}]_I$ in F508del-CFTR expressing cells may be binding of F508del-CFTR to the inositol-1,4,5-trisphosphate [IP3] receptor binding protein released with IP_3 (IRBIT). As shown previously IRBIT binds to and regulates CFTR (Yang et al, JCI 2009). Overexpression of IRBIT attenuated UTP, i.e. Ca^{2+} activated chloride conductance in *Xenopus* oocytes; and this effect that was compensated by coexpression of F508del-CFTR. Taken together these data suggest that enhanced calcium-dependent chloride secretion in CF airways is due to an increase in intracellular Ca^{2+} signaling, which may be due to an ER-chloride conductance by ER-trapped F508del-CFTR and/or binding of IRBIT to F508del-CFTR and enhanced IP_3-signaling.

This study was supported by DFG SFB699-A7 and TargetScreen2 (EU-FP6-2005-LH-037365). JRM is the recipient of a fellowship from FCT, Portugal.

CFTR Activates Calcium Activated Chloride Channels in *Xenopus* Oocytes through Extracellular Acidosis

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Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) is a cAMP/PKA regulated chloride channel in charge of epithelial Cl^- secretion. It is known for long that CFTR also controls the activity of several other transmembrane proteins such as Na^+/H^+ exchangers, epithelial sodium channels (ENaC), anion exchangers such as SLC26A9, aquaporin water channels, and calcium activated chloride channels (CaCC). CaCC is a chloride channel that is activated by an increase in intracellular Ca^{2+}. CFTR inhibits endogenous Ca^{2+} activated Cl^- currents (CaCC) in *Xenopus* oocytes, bovine pulmonary artery endothelium and isolated parotid acinar cells by an unknown mechanism. Additional studies demonstrated that upregulation or downregulation of CFTR resulted in a parallel up- and downregulation of cAMP-, Ca^{2+}-, and volume regulated Cl^- conductance. Although these data suggested a clear functional and even molecular correlation of different anion conductances, they were regarded as separate molecular entities by others. In the present study we found that CFTR is also able to activate CaCC through extracellular acidification.

In *Xenopus* oocyte, extracellular acidification (pH 5.5) activated a Cl^- current that shared the properties of the endogenous Ca^{2+} activated Cl^- current in oocytes that is now known as TMEM16A (Anoctamin, ANO). This current, however, could only be activated in oocytes expressing wtCFTR or expressing CFTR with residual function such as ΔF508-CFTR. Notably, in *Xenopus* oocytes a fraction of ΔF508-CFTR is trafficked to the cell membrane where it operates as a cAMP/PKA-regulated Cl^- channel. In contrast in oocytes expressing G551D-CFTR, ΔR-CFTR lacking the R-domain of CFTR or in water injected controls oocytes no CaCC was activated by extracellular acidosis. The acid-induced current had an ion permeability of $I^->NO_3^->Cl^-$ and was completely inhibited by 4,*4*-diisothiocyanostilbene-2,2′-disulfonic acid, 2Na (DIDS) and niflumic acid (NFA) but not $CFTR_{inh}172$. Removal of extracellular Ca^{2+} did not reduce the current induced by extracellular acidic pH. However depletion of intracellular endoplasmic reticulum Ca^{2+} stores with BAPTA, completely abolished extracellular acidosis-inducing currents. In conclusion, we present the novel finding that extracellular acidosis is activating CaCC with the help of CFTR. The mechanism by which low pH activates CaCC remains currently unclear and needs further examination.

Acknowledgement: We would like to thank Michael J. Welsh (M.D.) Department of Internal Medicine, University of Iowa College of Medicine for ΔRCFTR construction. This work is supported by research grant DFG SFB699 A6, DFG KU756/8-1

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TMEM16A is Involved in Intestinal Tumor Development in APC $^{min/+}$ Mice

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Membrane ion channels have been demonstrated to control cell proliferation and to participate in the development of cancer. Cellular expression and function of these ion channels undergo changes during cancer development. The Ca^{2+} activated Cl^- channel TMEM16A (Anoctamin 1 , ANO1) has turned out to be of exceptional importance for epithelial Cl^- secretion and has been recognized as a potential drug target for cystic fibrosis (CF) therapy, since it may serve as an alternative secretory pathway. However, previous studies on TMEM16A (also known as DOG1) have shown that its expression is tightly correlated with cancer development, since it is upregulated in gastrointestinal stromal tumors as well as in head and neck cancers. Previous studies from our group have shown that TMEM16A is essential for cellular volume regulation by facilitating regulatory volume decrease (RVD). RVD-related conductances may be upregulated during both cell proliferation and apoptosis.

We used a murine model expressing a nonsense mutation in the adenomatous polyposis coli (APC) tumor suppressor gene, predisposing mice to multiple adenoma formation, as a model for studying polyp growth and TMEM16A-expression in the intestine. We hypothesized that TMEM16A levels would be increased in polyps resulting in cell proliferation. Surprisingly, expression of TMEM16A was shown to be downregulated in polyps of the distal colon of $APC^{min/+}$ mice. Furthermore, treatment of $APC^{min/+}$ mice with the mTOR-inhibitor rapamycin, which has been shown earlier to reduce formation of polyps, resulted in an upregulation of TMEM16A-expression in both distal and proximal colon. These findings suggest that TMEM16A may be involved in apoptosis and counteracts growth of polyps. Further investigations should demonstrate whether activation of TMEM16A induces apoptosis and whether this prevents TMEM16A from being a drug target in cystic fibrosis.

Gap Junctions Contribute to Airway Surface Liquid Homeostasis in Human Airway Epithelial Cells

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The airway surface liquid (ASL) plays a critical role in lung defense and is required to ensure hydrated and clearable mucus. It is determined by active transepithelial salt transport, mainly involving cystic fibrosis transmembrane conductance regulator (CFTR) activation. CFTR activity is regulated by protease-activated receptors (PAR) at the basolateral membranes and adenosine receptors (ADO-Rs) at the apical membrane; both pathways involved the release of prostaglandin E2 (PGE2) and stimulation of their basolateral receptors (EP-Rs). Thus coordination of the signaling generated in airway cells by these G-protein coupled receptors (GPCRs) is critical for appropriate regulation of ASL height and efficient mucociliary clearance. In this context, gap junction (GJ) channels may contribute to the coordination of intercellular signaling since they provide direct aqueous pores between neighboring cells. Therefore GJs, which are formed by connexin proteins, allow intercellular spread of ions, nucleotides and second messengers for coordinating the tissue activity. In the submucosal gland Calu-3 cell line, we found a parallel regulation of CFTR and GJs in response to GPCRs stimulation. Indeed, activation and inhibition of ADO and PGE2 pathways enhanced and decreased, respectively, the extent of GJ intercellular communication. Moreover, inhibition of GJs prevented CFTR currents induced by PAR in Ussing chambers. Then, to investigate the consequences of GJ-CFTR interaction on fluid secretion in Calu-3 cells, we monitored ASL volume by confocal microscopy and images 3D reconstruction. Stimulation of Calu-3 cells with PGE2 increased ASL volume. This effect was dependent on CFTR activity, because PGE2 failed to enhance ASL volume in genetically modified Calu-3 cell line lacking CFTR. Interestingly, the PGE2-induced ASL volume increase was abolished in the presence of the GJ blocker, indicating that GJs mediate fluid secretion in Calu-3 cells via CFTR. Finally, to confirm the importance of GJs on ASL volume regulation in a cell system closer to the human airway physiology, we repeated the experiment on primary cultures of well polarized human airway epithelial cells (HAECs). HAEC cultures showed active Na^+-dependent absorption of ASL, which was blocked in the presence of amiloride. In agreement with the results obtained on Calu-3 cells, PGE2 induced an increase of ASL volume, which was prevented with the GJ blocker. Thus, our results show that GJs coordinate a signaling network, comprising CFTR, ADO-Rs, PARs and EP-Rs, which is required for ASL volume homeostasis in airway epithelial cells.

Supported by VLM, FNRS, FFC, Schmidheiny and Novartis Consumer Health.

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The Switch of Intestinal Slc26 Exchangers from Anion Absorptive to HCO_3^- Secretory Mode is Dependent on CFTR Anion Channel Function

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CFTR has been recognized to function both as an anion channel and a key regulator of Slc26 anion transporters in heterologous expression systems. Whether this regulatory relationship between CFTR and Slc26 transporters is seen in native intestine, and whether this effect is coupled to CFTR transport function or other features of this protein has not been studied. The duodenum of anesthetized CFTR-, NHE3-, Slc26a6-, and Scl26a3-deficient mice and WT littermates were perfused and duodenal HCO_3^- secretion (DBS) and fluid absorptive or secretory rates were measured. The selective NHE3 inhibitor S1611 or genetic ablation of NHE3 significantly reduced fluid absorptive rates and increased DBS. Slc26a6 (PAT1) or Slc26a3 (DRA) ablation reduced the S1611-induced DBS increase and reduced fluid absorptive rates, suggesting that the effect of S1611 or NHE3 ablation on HCO_3^- secretion may be an unmasking of Slc26a6 and Slc26a3-mediated Cl^-/HCO_3^- exchange activity. In the absence of CFTR expression or after application of the CFTR(inh)-172, fluid absorptive rates were similar to WT, but S1611 induced virtually no increase in DBS, demonstrating that CFTR transport activity, and not just its presence, is required for Slc26-mediated duodenal HCO_3^- secretion. A functionally active CFTR is an absolute requirement for Slc26-mediated duodenal HCO_3^- secretion, but not for Slc26-mediated fluid absorption, in which these transporters operate in conjunction with the Na^+/H^+ exchanger NHE3. This suggests that Slc26a6 and Slc26a3 need proton recycling via NHE3 to operate in the Cl^- absorptive mode, and Cl^- exit via CFTR to operate in the HCO_3^- secretory mode.

Regulation of ENaC by the Stress Response Protein SERP1

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Cystic Fibrosis (CF) is characterized by enhanced Na^+ absorption in the airways, which contributes to reduced airway surface liquid (ASL) and compromised mucociliary clearance. Therapeutic strategies have been developed to overcome enhanced Na+ absorption by directly inhibiting the epithelial Na+ channel (ENaC). Current results from those strategies demonstrate only limited success, which prompted us to search for alternative strategies. In the present study we identified SERP1 (stress-associated ER protein 1, also known as RAMP4, ribosome-associated membrane protein 4) as a novel binding partner of ENaC in a split-ubiquitin screening using ß-ENaC as bait. Expression of SERP1 was previously shown to be enhanced during cellular stress, causing accumulation of unfolded proteins in the endoplasmic reticulum (ER). SERP1 is homologous to yeast suppressor of SecY 6 protein (YSY6), a suppressor of a secretory pathway mutant, which suggests a role in pathways controlling membrane protein biogenesis at the ER level. SERP1/RAMP4 also controls biogenesis by interacting with the molecular chaperone calnexin.
Here, we examined the role of SERP1 in regulating ENaC-expression. Using amiloride-induced change in FMP fluorescence as a read-out for the amount of amiloride sensitive epithelial Na+ conductance (FMPAmil), we found that treatment of both H441 and A549 cells with 3 different SERP1 siRNAs significantly enhanced FMPAmil. This enhancement, however, seems to be attenuated (A549 cells) in the presence of a cocktail of protease inhibitors, but not by the endocytosis inhibitor dynasore. Curiously, steady-state expression of ßEnaC is reduced. In contrast, overexpression of SERP1 largely reduced FMPAmil. Amiloride-sensitive transport was also measured in Ussing chamber experiments on H441 cells grown on permeable supports. Treatment of the cells by SERP1-siRNA increased the amiloride short-circuit current. SERP1 was found to co-localize in the ER with all 3 ENaC subunits as well as with calnexin. Taken together SERP1 appears to be a novel regulator of ENaC expression and a potential new target for drug therapy of CF.

Work supported by TargetScreen2 (EU-FP6-LSH-2005-037365) grant; SFB699 A6. DF, JA and SL are recipients of PhD fellowships SFRH/BD/43313/2008, SFRH/BD/29134/2006 and SFRH/BD/47445/2008, respectively (FCT, Portugal)

Development of a Western-Blot Assay for siRNA Screens to Identify Proteins Affecting ENaC Traffic / Function

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Sodium (Na^+) absorption in epithelia is mainly mediated by the epithelial Na^+ channel (ENaC) expressed at the apical plasma membrane in many epithelial tissues throughout the body. The channel is constituted by 3-mer combinations of four distinct subunits (a, b, g and d). Defects in ENaC function play a major role in diseases such as Liddle's syndrome, type I pseudohypoaldosteronism, high-altitude pulmonary edema, and also cystic fibrosis (CF). Understanding the regulation of this channel is critical to understand its contribution in various clinical phenomena [1].

Our goal was to establish a robust, simple and sensitive medium-throughput Western blot (WB) assay to analyse ENaC expression levels at steady-state in epithelial cell lines, to be used as a secondary test after high-content siRNA screen for proteins involved in ENaC traffic / function.

Three different cell lines were tested: H441, A549 and A549 stably expressing the mCherry-Flag-ßENaC under an inducible (Tet-ON) promoter. WB was performed essentially as previously [2] applying in each lane of a 7% (w/v) polyacrylamide SDS-PAGE minigel (BioRad Mini-PROTEAN® Tetra), the contents of 1-8 wells from a 96-well plate (approximately 12-100 mg of total protein). After electrophoresis and electroblotting, the nitrocellulose membrane was blocked with 5% skimmed milk and incubated at 4ºC o.n. with the following commercially available antibodies: H-95 (Santa Cruz Biotechnology), Rb AB3530P (Chemicon) and 324870 (Calbiochem) for αENaC; H-190 (Santa Cruz Biotechnology) for ßENaC; H-110 (Santa Cruz Biotechnology) for γENaC; anti-Flag M2 (Sigma-Aldrich) and Anti-RFP (MBL) for Flag and mCherry tags, respectively. Following the incubation with Anti-Mouse IgG or Anti-Rabbit IgG (BioRad) secondary antibodies, signal detection was carried out using a West Pico® Detection System (Pierce). As a control for the correct ENaC band identification, we used lysates from cells treated with siRNAs for α- and ßENaC, (Silencer Select, Ambion, refs: s12543, s12545, s12546, s12547).

Our results indicate that cell lysates from two wells of a 96-well plate (approximately $8x10^4$-10^5 cells) are enough to detect each one of the three ENaC subunits by WB in the above cell lines. We found that the best Abs to detect ßENaC subunit by WB were H-190 and anti-RFP to specifically identify endogenous ßENaC protein and mCherry-Flag-ßENaC construct, respectively. We found that bands detected by these Abs were significantly decreased 72h post-transfection with 2.4pmol of ßENaC siRNA.

In conclusion, this medium-throughput WB assay seems to be robust enough as a secondary screen to assess the effect upon ENaC subunit expression of knocking-down (by siRNAs) genes that were identified in primary microscopy-based screen as regulating ENaC function and/or traffic.

Work supported by EU Grant FP6-LSH-2005-037365 (TargetScreen2)

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A High-Content Microscopy-based siRNA Screen to Identify Novel Proteins Affecting ENaC Function/Traffic

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Ion channels play a critical role in a variety of physiological processes. The cystic fibrosis transmembrane conductance regulator (CFTR) is a protein kinase A (PKA) and ATP-regulated Cl^- channel that is mutated in cystic fibrosis (CF). CFTR also controls other membrane conductances, such as the activity of the epithelial Na^+ channel (ENaC) and an enhanced Na^+ absorption is observed in CF airways.
The aim of this work was to identify novel therapeutic targets for CF, in particular proteins whose inhibition would lead in turn to reduced ENaC activity.
We developed a high-throughput microscopy-based screening assay, using human alveolar type 2 epithelial A549 cells and a FLIPR membrane potential sensitive (FMP) dye, whose fluorescence can be quenched due to hyperpolarization of the cell membrane by inhibiting Na^+ absorption with amiloride. A ratio of FMP fluorescence before and after amiloride was used as a measure of the amount of active ENaC. We used three different human siRNA libraries, the *kinome library* that consisted of siRNAs targeting 710 different kinase genes, the *druggable* library that targeted 5940 different human genes and the *secretome library* identified as targeting 1552 genes involved in the trafficking of a temperature sensitive membrane transport marker, tsO45G.
From the *kinome* and *secretome* screens, we identified 183 siRNA-targeted genes that inhibit ENaC, being 43% of them validated with two additional siRNAs. From the *druggable* screen, we identified 228 different genes whose inhibiton by siRNA transfection leads to a reduction of ENaC activity. Further experiments are being performed at the moment to characterize the relevance of the interaction detected between ENaC and some of the hits, namely whether they can be used as drug targets for CF.

Work supported by TargetScreen2 (EU-FP6-LSH-2005-037365) grant; SFB699 A6. *JA is recipient of PhD fellowship SFRH/BD/29134/2006 (FCT, Portugal)*

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Connexin 26 is Involved in the Regulation of the Initial Steps of Airway Epithelium Repair

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Functional integrity of the airway epithelium is altered in cystic fibrosis (CF). Epithelial integrity depends on the expression and assembly of specific proteins into specialized junctional structures. Gap junctions, made of connexin (Cx) hexamers, play crucial roles in these interactions by contributing to the ability of cells to share signaling factors directly between adjacent cells. The pattern of Cx expression in Human Airway Epithelial Cell (HAEC) cultures is associated with the differentiation state. Thus, Cx26 decreases to undetectable level with HAEC differentiation to a polarized airway epithelium. Airway epithelium repair is characterized by successive steps of adhesion and migration early after injury. This is followed by a proliferation phase and a pseudostratification leading to a renewed epithelium. We questioned whether a specific pattern of Cx26 expression was associated with the different phases of epithelium repair. A model of HAEC repair was established by wounding mechanically well polarized cultures. Wound healing was monitored for several days by time-lapse imaging. In parallel, the cultures were fixed at different times allowing for immunostaining and confocal analysis. The number of proliferating cells markedly increased following wound injury, as revealed by Ki-67 detection. Concomitant with the cell ability to proliferate, Cx26 was transiently expressed at the wound area and in basal cells behind the wound. Decreased cell proliferation with mitomycine C or methotrexate led to a down-regulation of Cx26 expression. Reciprocally, gap junction inhibition delayed the epithelial repair and reduced HAEC proliferation, suggesting interdependence between Cx26 and cell proliferation during airway epithelium repair. The inhibition of Cx26 expression was also related to morphological changes that might link Cx26 to adhesion and migration. However, the actin polymerization inhibitor Y27632 did not affect the pattern of Cx26 expression following wound injury. These results suggest that Cx26 plays a key role in the initial steps of airway epithelium repair but its exact contribution remains to be determined.

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Transforming Growth Factor Beta 1 (TGF ß1) in Induced Sputum and Serum in Cystic Fibrosis

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Background: Previous studies have provided evidence that high-producer TGFß1 genotypes are associated with severe lung disease in cystic fibrosis (CF), but data on TGFß1 levels and its impact on CF lung disease is scarce.
Aim: Assessing the relationship between TGFß1 and lung disease in CF.
Methods: 24 patients delta F 508 homozygous (median age 20.5 y, m/f 14/10, BMI 20,35 kg/m^2, Shwachman score 75, FEV_1(%) 83) and 8 controls (median age 27,5 y, m/f 5/3, BMI 21.6 kg/m^2) were examined. TGFß1 was assessed in serum and induced sputum (IS) by an ELISA kit (R&D Systems, Wiesbaden, Germany).
Results: TGFß1 in IS (CF/controls median 76,5/ 59,1 /µl, p< 0.074) were higher in CF. In CF TGFß1 in IS was significantly higher than in serum (median IS/serum 76.5/35.5 /µl, p=0.0001). There was a negative correlation between TGFß1 in serum and lung function (FEV1) (r=-0,488, p=0,025), MEF 25 (r=-0.425, p=0.055), VC (r=-0.572, p=0.007)), but there was no correlation TGFß1 in IS and lung function in CF.
Conclusion: TGFß1 in serum but not in IS correlates with lung function. Our data might support the idea that hematopoetically derived TGFß1 contributes critically to lung function in CF.

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Effect of EGFR Inhibition on Basal and CSE Induced IL-8 Release from CF and Non-CF Airway Epithelial Cells

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Cigarette smoke extract (CSE) can reduce CFTR gene and protein expression. In addition, CSE has been shown to induce IL-8 release from mucoepidermoid cells via the EGFR pathway [1]. However, the mechanism(s) by which spontaneous and CSE elicited IL-8 release is regulated in CF airway epithelial cells has not yet been fully elucidated. Consequently, this study aimed to compare the effects of the EGF receptor specific kinase inhibitor, AG-1478, on basal and CSE stimulated IL-8 release from 2 bronchial epithelial cell lines (CFBE41o- and 16HBE14o-) and from primary nasal epithelial cells isolated from F508del CF patients and healthy volunteers.

Nasal epithelial cells were obtained via nasal brushings of the inferior turbinate of CF patients; F508del homozygote (n=3), and healthy volunteers (n=3). The cells were expanded in culture and used at passage 3. Cell lines non-CF (n=4) and CF (n=4) (gift from Dieter C. Gruenert, USF) and primary nasal cells were treated for 4 h with or without 5% CSE in the presence or absence of increasing concentrations of AG-1478 (50 nM - 1 μM). Culture supernatants were harvested and IL-8 analysed by ELISA (R & D Systems).16HBE14o- cells were highly susceptible to the effects of EGFR inhibition, as exposure to increasing concentrations of AG-1478, significantly reduced basal IL-8 release from 132.8±8.667 pg/ml (Vehicle control -DMSO 0.01%) to 71.60±6.77 pg/ml (250 nM), 69.92±4.94 pg/ml (500 nM) and 70.99±4.53 pg/ml (1 μM) from these cells (all $p< 0.05$). Furthermore, AG-1478 significantly reduced 5% CSE induced IL-8 release from these cells from 125.0±2.001 pg/ml (Vehicle control -DMSO 0.01% + 5% CSE) to 68.92±1.95 pg/ml (1 μM + 5% CSE) ($p< 0.05$). In contrast, AG-1478 only significantly reduced 5% CSE induced IL-8 release from the CFBE41o- cells (from 360±59.93 pg/ml to 174±24.99 pg/ml) at the highest concentration of AG-1478 utilised (1 μM) in the presence of 5% CSE ($p< 0.05$). Although a decrease in IL-8 release was observed in the nasal epithelial cells after exposure to AG-1478 this did not reach statistical significance.

These data suggest that both spontaneous and CSE induced IL-8 release from non-CF and CF immortalized cell lines and primary airway epithelial cells derived from healthy and F508del CF patients, may in part be regulated by the activation of the EGFR signaling pathway. These data are consistent with the findings of Sabauste *et al.,* which demonstrated that activation of the EGF receptor plays a role in IL-8 release from airway epithelial cells [2].

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NF-κB Inflammatory Signalling is Suppressed by CFTR in a Number of Cell Models

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Introduction: Little is understood on how defects in the CFTR gene can cause persistent chronic pulmonary infection and inflammation. Numerous studies have shown that inflammatory signalling through the NF-κB pathway is increased in CF lungs and that this is linked to the production of pro-inflammatory cytokines such as interleukin 8 (IL-8)**.** However, there is little consensus on the mechanism(s) which link CFTR and its inheritable mutant forms to chronic lung inflammation. Recent work both in our lab and by Vij et al suggests that wild type CFTR can suppress inflammation (Vij, Mazur et al. 2009). Here we demonstrate that this idea holds true in a variety of cell models.

Methods: We used 2 different cell lines (NCI-H441 cells, a human lung adenocarcinoma cell line of bronchiolar (Clara) cell lineage and H57 cells, a HeLa cell derived cell line which stably over expresses the NF-κB luciferase construct) to examine the role of wild-type CFTR on inflammation. For luciferase assay cells were seeded in 12-well plates. The 4-κB-luciferase reporter plasmid contains four NF-κB binding elements 3' (TGGGGACTTTCCGC) 5' upstream of the thymidine kinase minimal promoter region that drive expression of the firefly luciferase reporter gene. H441cells were transfected with 400 ng of 4-κB-luciferase reporter plasmid using lipofectamine 2000 reagent. H57 cells stably express the 3Enhancer-κB-conA-Luc plasmid. Both cell lines were transfected with increasing amounts of CFTR up to 400 ng. To rule out a DNA dose effect, transfections were supplemented with empty vector plasmid to keep total DNA constant (for example, the 100 ng transfection of CFTR contained 300 ng of empty vector). 24h post transfection cells were incubated for 4 hours with fresh media or stimulated with 10 ng/ml TNFα. Supernatants were retained for IL-8 Elisa assays and cells lysed for luciferase assays.

Results: Our results show that in H441 cell, the level of CFTR expression correlates inversely with endogenous and stimulated NF-κB activity and IL-8 release. We also find the basal NF-κB activity in the HeLa derived H57 cell line is suppressed by increasing amounts of CFTR. We find that transfection of $2x10^5$ H441 cell or $1x10^5$ H57 cells with 400ng of CFTR in a well of a 12 well plate results in approximately 40% reduction in NF-κB activity.

Conclusions: These data together with that of Vij et al in HEK293 cells suggest that this reduction in inflammation following wild type CFTR transfection suggests that wild-type CFTR induced inflammation suppression is a broad phenomenon. These data indicate that CFTR has anti inflammatory properties and that the hyper-inflammation found in CF may be due to a disruption of the suppressive signalling link between CFTR and NF-κB and that one cause of the inflammation observed in CF is due lack of functioning CFTR at the cell membrane. We are focussing on the signalling link in current work.

This work was funded by the CF Trust PJ549

Reference: Vij, N., S. Mazur, et al. (2009). "CFTR is a negative regulator of NFkappaB mediated innate immune response." PLoS ONE **4**(2): e4664.

IL-8 Production by Calu-3 Cells Depressed for CFTR and in CF Human Airway Epithelial Cells

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A pro-inflammatory state of the human airway epithelium has been proposed to explain the exaggerated recruitment of neutrophils to the airways of patients with cystic fibrosis (CF). From early clinical observations to the latest research, a polemic issue has been whether mutant CFTR, or presence of a dysfunctional CFTR, triggers an enhanced production of the chemokine IL-8 by airway epithelial cells. As an attempt to address this question, we have compared IL-8 release in Calu-3 cells where CFTR was knocked-down by siRNA (kdCFTR cells) or knocked-out by shRNA (SH3 cells). The production of the cytokine was evaluated in parallel experiments in parental Calu-3 cells and in Calu-3 cells expressing an altered shRNA CFTR sequence (Alter cells). We also measured IL-8 release by well polarized cultures of human airway epithelial cell (HAECs) from non-CF and CF origin. We have first verified for CFTR expression and function in the Calu-3 cell lines. CFTR was virtually undetectable by Western blot in SH3 cells and short-circuit currents (Isc) were absent in Ussing chambers (1.7 ± 0.5 mA/cm^2, n=7, as compared to 18.7 ± 6.3 mA/cm^2, n=7 in Alter cells). CFTR was still expressed in kdCFTR cells but Isc currents were strongly reduced (3.7 ± 1 mA/cm^2, n=9, as compared to 16 ± 4 mA/cm^2, n=18 in Calu-3 cells). IL-8 was measured in the Calu-3 cell lines grown on plastic dishes in the absence or presence of TNF-a. However, no difference in basal or stimulated IL-8 release was observed between the different cell lines, whatever expression of CFTR was reduced or abolished. These experiments were repeated on well polarized Calu-3 cells grown on Transwell filters, a culture condition which again did not affect the production of the chemokine between the cell lines. We next evaluated IL-8 release by HAEC cultures on Transwell filters. HAECs could be amplified up to 3 passages, and for each passage differentiated at the air-liquid interface into a mucociliated pseudostratified epithelium within 30 days. The production of IL-8, which is highly elevated in CF HAEC cultures, decreased with time of differentiation to the level of non-CF cultures. At this time of the culture, we then measured the release of IL-8 induced by TNF-a as a function of cell passage. Although CF HAECs showed enhanced basal and stimulated release of IL-8 at early passage, this difference vanished for higher passages. Finally, secretions of 7- and 30-days old cultures generated from passage 2 HAECs were subjected to a cytokine array. Seven days CF HAEC cultures showed increased production of GM-CSF, GRO-a, IL-5, IL-6, IL-7 and MCP-1 but decreased production of TIMP-1 as compared to non-CF cultures. This difference, with the exception of IL-6, disappeared in 30 days HAEC cultures. Thus, our results revealed major differences in cytokine production in non-differentiated CF HAEC as compared to non-CF cells; this difference was abolished in well polarized HAECs. They also indicate that there is no intrinsic CFTR-dependent anomaly of cytokine secretion by CF HAECs or CFTR-depressed Calu-3 cell lines.

Supported by "VLM" and FNRS

Mitochondrial Dysfunction in CF Models: A Possible Role in the Abnormal Inflammatory Response?

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A hallmark of cystic fibrosis (CF) is excessive inflammation and oxidative stress is known as a mechanism possibly involved in overproduction of inflammatory cytokines. Reduced glutathione (GSH) is a significant component of the antioxidant defence mechanism and has been shown to be decreased not only in the lung epithelial lining fluid but also in mitochondria of epithelial cells with impaired CFTR function. In addition, deficits in mitochondrial GSH were reported elsewhere to lead to respiratory chain dysfunction.
The purpose of this study was to evaluate the possible relationship between loss of CFTR function, mitochondrial redox state and mitochondrial respiratory chain complexes activity in several CF models and to evaluate if mitochondrial dysfunction could be involved in the altered inflammatory response in CF models. Aiming to correct possible mitochondrial and cellular defects, we studied the efficacy of the antioxidant GSH monoethyl ester, a drug able to selectively restore mitochondrial GSH.
Mitochondria from murine colonic epithelia *(Cftr$^{-/-}$ vs. Cftr $^{+/+}$* mice) and from two CF human bronchial epithelial cell lines (IB3-1 and CFBE 41o-) compared to their CFTR corrected counterparts (C38 and CFBE 41o- WTCFTR) were isolated using differential centrifugation. The redox state was assessed by i) mitochondrial and cytosolic GSH concentrations measured by HPLC coupled with electrochemical detection; ii) ROS levels as indicated by CM-H_2DCFDA fluorescence and iii) mitochondrial aconitase activity measured by spectrophotometry. Complex I enzymatic activity was monitored by using a spectrophotometry assay and its expression was analysed by blue native PAGE. IL-8 production was quantified by ELISA.
We observed a 30 % decrease in mitochondrial GSH concentrations in CF *vs.* WT cells whereas cytosolic GSH was unchanged. This was associated with an increase in cellular ROS production and a decrease in mitochondrial aconitase activity. Complex I activity was decreased by 50 % in all three CF models and this decrease was not due to a decrease in complex I expression. Basal IL-8 production showed higher levels in CF *vs.* WT. Treatment of CF cells with GSH monoethyl ester restored mitochondrial GSH concentrations, mitochondrial aconitase activity, cellular ROS production and complex I activity. Basal and TNFα induced IL-8 levels were also reduced by GSH monoethyl ester.
We suggest that reversible redox alterations in the mitochondrial compartment and complex I inhibition could be linked. A decrease in mitochondrial GSH could be implicated in the exaggerated basal or stimulated cytokine production in CF models. Furthermore, antioxidants targeting mitochondria could be useful in restoring complex I activity and controlling the redox balance in CF cells.

Modulation of Sphingolipids Metabolism Reduces the Neutrophil Chemotaxis in Human and Murine Respiratory Models *in vitro* and *in vivo*

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Investigation on novel targets for the treatment of CF lung inflammation is a major priority, considering that at present no fully satisfactory anti-inflammatory treatment is available. Growing evidence suggests sphingolipids (SLs) as novel targets for the treatment of pulmonary disorders, including CF (Uhlig, 2008). The accumulation of the SL ceramide has been identified as one of the key regulators of inflammation and infection in CF airways in different CFTR-/- mouse models (Teichgraber, 2008). Miglustat, an inhibitor of the synthesis of glycoshingolipids (GSLs), used for treating type I Gaucher disease and others GSLs storage disease, produces an anti-inflammatory effect in bronchial epithelial cells (Dechecchi, 2008). In this study we tested the effect of miglustat in murine models of lung infection and inflammation. C57BL/6 mice were challenged *with* aqueous solution of miglustat (2 mg/dose) by intraesophageal gavage at 72, 48 and 24 hours before intranasal instillation of LPS or a treatment schedule of 400mg/kg one hour before the i.t. inoculum with *P. aeruginosa* strain PAO1. Bronchoalveolar lavage fluid (BALF) was collected 4 h post LPS challenge or PAO1 infection and the inflammatory response in terms of leukocyte recruitment and MPO activity in the airways was analyzed. Miglustat significantly reduces the amount of neutrophils recruited in BALF and MPO activity both in LPS stimulated and PAO1 infected mice. To support the hypothesis that the anti-inflammatory effect of miglustat is related to changes in SLs metabolism, CF bronchial cells were treated with amitriptyline, an inhibitor of two key enzymes of the SLs metabolism: acid sphingomyelinase and acid ceramidase. Amitriptyline (10 microM), added to IB3-1 and Cufi-1 cells 4 hours before infection with *P.aeruginosa*, significantly inhibits the expression of IL-8 mRNA by about 50 %, in agreement with the anti-inflammatory effect obtained *in vivo* in CF mice (Teichgraber, 2008; Becker, 2009). To demonstrate that miglustat and amitriptyline affect the immune response through changes in cellular ceramide levels, the analysis of ceramide, expressed on the cellular membrane of cells infected by *P. aeruginosa,* was measured by FACS analysis with anti-ceramide antibody MAS 0020 (Glycobiotech). Ceramide staining in IB3-1 and CuFi-1 cells increases upon infection with *P.aeruginosa.* Both miglustat and amitriptyline significantly reduce the increase of ceramide expression induced by *P.aeruginosa.* Measurement of apoptotic cells demonstrate that miglustat does not produce any significant effect on apoptosis. Collectively these results strongly suggest that miglustat, tested at concentrations much lower than those necessary to correct lysosomal storage diseases, could be a promising molecule to correct the excessive neutrophil chemotaxis observed in the lung of patients affected by CF, without producing significant pro-apoptotic effects and strengthen the hypothesis that SLs metabolism represents a target to restore a normal inflammatory response in CF epithelium.

***Supported by**: Italian Cystic Fibrosis Research Foundation (#12/2008 and CFaCore) with the contribution of "Delegazione FFC di Vicenza con le scuole vicentine".*

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Hepatic Corticosteroid-binding Globulin Expression in CF Patients

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Bronchial inflammation is a characteristic hallmark of Cystic Fibrosis lung disease. Glucocorticoids (GC) are powerful anti-inflammatory molecules with widespread spectrum of action. They are commonly used to fight lung inflammation despite side effects and controversial efficiency among patients. To better understand possible actions of these molecules, our study focuses on plasma corticosteroid-binding globulin (CBG). CBG is a plasma transport glycoprotein produced by the liver that binds GC with high affinity; about 90% of circulating GC is bound to CBG. According to the free hormone hypothesis, CBG is thought to primarily regulate the bioavailability and metabolic clearance of GC. CBG could also address GC directly to sites of inflammation. To investigate how CBG is regulated in inflammation and CF context, its expression was assayed by qPCR in the liver of CF patients (n=9) *vs* healthy donors (n=6). Transcript measurements show a 14 times increase in CF patients ($p < 0.001$) compared to donors. We are currently studying in hepatic cell lines the regulation of CBG by inflammatory and/or anti-inflammatory molecules to better understand possible consequences of such up-regulation on GC efficiency. Interestingly, preliminary results suggest that inflammation up-regulate CBG expression in Hep3B cell line while dexamethasone treatment doesn't cause any change in CBG transcripts level.

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

Role of A20 Signalling in Inflammation in Cystic Fibrosis Epithelium

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Introduction: A20 is regulated by and responsible for the termination of NF-κB signalling through immune receptors including TLRs. Mice deficient in A20 display persistent activation of NF-κB by TLRs and TNF-R, causing multiorgan inflammation in adults.[1] A20 is a negative regulator of TLR induced inflammatory responses in the airway in response to bacterial and viral stimuli.[2] In order to effectively inhibit NF-kB signalling, A20 forms a complex with Ring Finger protein (RNF)11, the E3 ligase Itch and a binding protein for TAX called TAX1BP1. A20 is reliant on these complex members for binding and degradation of target proteins.[3] We propose that in the chronically inflamed CF epithelium, A20 and the A20 signalling complex is compromised, thereby contributing to pro-inflammatory signalling through the NF-κB pathway.
Methods: Non-CF (HBE) and CF (CFBE) bronchial epithelial cell lines grown to 80% confluency on collagen-coated plates and were stimulated with 50µg/ml LPS from *Pseudomonas aeruginosa* (Sigma) for 0-24h. A20 and NF-κB (p50 and p65) levels were measured by FACS. Nuclear extracts for NF-κB staining were obtained using a DNA CycleTest kit (BD Biosciences). Cells were treated with a commercially available p65 inhibitor (JSH-23, CalioBiochem) to determine which NF-kB subunit regulates IL-8 processing. RNF11, Itch and TAX1BP1 expression was assessed by qPCR (Roche LightCycler). IL-8 release was measured by ELISA (Peprotech). Statistics were calculated by ANOVA, with n=4-6.
Results: CFBE cells had significantly greater ($P< 0.05$) release of IL-8 than HBE cells. A20 expression peaked 1h post-stimulation in HBE cells and after 4h in CFBE cells. Expression of A20 in CFBE cells fell below basal levels by 12h exposure, while HBE cells showed sustained A20 expression. Basal NF-κB (both p50 and p65) was significantly greater in CFBE than in HBE cells ($P< 0.0001$). p50 levels remained unchanged in HBE cells following LPS stimulation, while CFBE cells showed significant increases (2 and 4h post-stimulation, $P< 0.001$) which returned to basal levels 8 and 12h post-stimulation. p65 expression increased significantly in both HBE and CFBE cells following LPS exposure ($P< 0.05$-0.001). However, these increases were more pronounced in CFBE cells. Notably, p65 expression remained significantly up-regulated at 12h (when A20 was inhibited) in CFBE cells and not in HBE cells. Inhibition of p65 found that IL-8 release in CFBE cells is p65 dependent. qPCR revealed that mRNA expression of RNF11, Itch and TAX1BP1 differed between HBE and CFBE cells. Expression of all three genes was enhanced in HBE cells following LPS exposure. However, in CFBE cells, expression of RNF11, Itch and TAX1BP1 remained either unchanged or was reduced following stimulation with LPS.
Conclusion: In CF epithelium, the A20 signalling complex is compromised permitting increased NF-κB (p65)-driven inflammatory signalling.

This work was supported by a grant from the CF Trust UK (PJ541).
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Is CFTR Involved in Toll-like-Receptor Regulation?

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Chronic bacterial infection and inflammation are hallmarks of CF lung disease. The exaggerated inflammatory response is triggered by cell wall components such as lipopolysaccharide (LPS) and peptidoglycan (PGN) that bind to Toll-Like Receptors(TLR)-4 and 2 respectively, causing increased release of inflammatory mediators including interleukin (IL)-8. Using tracheobronical epthielial cell lines we previously showed that CFTE cells (homozygous F508delta) produced significantly more IL-8 than non-CF (HTE) cells following stimulation with TLR-4 ligand *Pseudomonas aeruginosa* LPS. Together with reduced CFTR mRNA ($p < 0.01$) this may suggest a link between CFTR and TLR expression/signalling. The aim of this study was to explore the link between diminished CFTR expression and the regulation of TLR expression.

Protein expression (FACS) of TLR-4 and 2 on the cell surface was followed over 24hrs after stimulation with *P.aeruginosa* LPS (SE22, clinical isolate, 100 ng/ml). At basal level, TLR-4 and 2 were significantly increased in CFTE compared to HTE cells (TLR-4 $p < 0.05$, TLR-2 $p < 0.01$). In CFTE cells TLR-2 surface expression increased over time peaking at 8h ($p < 0.01$) compared to HTE cells. While TLR-4 surface expression decreased in both cell types (8h), in HTE cells it remained significantly below basal levels at 24h ($p < 0.05$). Conversely, TLR-4 expression in CFTE cells returned to basal level within 24h and was associated with significantly increased IL-8 release in these cells. To create a model of repeated stimulation, cells were first stimulated with LPS as before, after 24h, the supernatant was removed, cells washed and again stimulated with LPS. Following this repeated stimulation, HTE cells showed a significant decrease in IL-8 release at 48h compared to 24h ($p < 0.05$), while CFTE cells produced similar amounts of IL-8 at both time points. This suggests that in HTE cells the reduced TLR-4 expression may act as a protective mechanism, whereas CFTE cells can be repeatedly stimulated.

To investigate if the loss of CFTR in CFTE cells is involved in the dysregulation of TLR-4 surface expression, HTE cells were treated with CFTR inhibitor (172Inhib, 10 mM, 16 h) or CFTR was knocked down by siRNA. After CFTR inhibition, TLR-4 expression did not change at basal levels, but was significantly higher 24h after stimulation ($p < 0.05$ vs. untreated HTE). While these cells showed increased IL-8 release after 24h, this did not reach significance. Basal TLR-4 expression was increased in CFTR negative HTE cells, which following LPS stimulation was again increased significantly (2-fold) over LPS stimulated controls ($p < 0.001$ vs. scambled). In this instance TLR-4 expression in CFTR negative HTE cells was similar to expression in CFTE cells.

These results suggest that loss of CFTR is associated with the basal expression of TLR-2 and TLR-4. The inability of CFTE cells to down-regulate TLR-4 surface expression after stimulation renders the cells prone to repeated LPS stimulation. Therapeutic intervention that reduces basal TLR-4 expression and normalises its kinetic profile may help to control inflammation in CF.

Future work will investigate the TLR-4 signalling pathway leading to IL-8 release in these cells.

Trappin-2, an Antimicrobial Peptide, Induces a Flagellin/TLR5-Dependent Modulation of Alveolar Macrophage & Epithelial Cell Activity

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Introduction: Epithelial-derived endogenous antimicrobial molecules such as defensins, SLPI and trappin-2 have the potential to positively affect the phenotype of phagocytic myeloid cells (macrophages, neutrophils, dendritic cells), by modifying their differentiation, chemotactic properties and activation in different lung pathologies. Recently, it has been shown that trappin-2 (T2, R&D System) can improve the clearance of *Pseudomonas aeruginosa* (*P.a*) by alveolar macrophages (AM) in vitro and in vivo (Wilkinson *et al*. Am. J. Pathol, 2009, 174:1338-46). The aim of the present study is to study T2 opsonizing effect on the clearance of *P.a* by AM and a potential modulation on cytokine productions by AM and lung epithelial cells stimulated with *P.a*.

Methods:

Measurement of the early clearance of *P.a* by AM

PAK (a wild-type P.a. strain), PAK∆*fliC* (a flagellum-deficient PAK) or PAKL88 (a PAK strain with a TLR5 binding site deficient flagellum) were pre-incubated or not with T2 (100 nM, a sub-lytic concentration) for 30 min at 37°C. These suspensions were then added to murine AM (MH-S cell line) at a multiplicity of infection of 0.1. Co-cultures were then incubated for 2 h before lysis with 0.1% Triton X-100. Serial dilutions were subsequently plated on LB agar to determine remaining bacterial CFU.

Modulation of AM & epithelial cell activation by *P.a* or purified *P.a* flagellins

AM, Beas2B or A549 cells were stimulated with *P.a* or flagellins pre-incubated with PBS or T2. TNF-α or IL-8 secretions were then measured by ELISA.

Results: We confirm that T2 enhances the early clearance of wild-type *P.a* by AM but not that of PAK∆*fliC* or PAKL88. In addition, T2-pre-incubated PAK but not PAK∆*fliC* increases AM or lung epithelial cells TNF-α and IL-8 secretions. Moreover, Beas2B epithelial cells stimulated with PAK-flagellin pre-incubated with T2 secreted higher levels of IL-8, when compared with cells incubated with flagellin alone.

Conclusion: Our data suggest that T2, by 'opsonising' *P.a* and by increasing cytokine production of AM or lung epithelial cells may constitute an important modulator of innate immune responses. Moreover, T2 activity requires *P.a* flagellin-TLR5 interaction. We are currently assessing whether T2 could improve the potential defective bacterial clearance of *P.a* observed in cystic fibrosis.

Pseudomonas aeruginosa Infection Drives Functionally Unopposed Matrix Metalloproteinase Activity In Lung Parenchymal And Inflammatory Cells

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Introduction: Cystic fibrosis (CF) lung disease is characterised by bronchiectasis; progressive destruction and dilatation of the airways. This abnormal remodelling of the airways occurs in the setting of chronic infection, particularly with Pseudomonas aeruginosa (PA), and is associated with loss of elastin and cartilage, with progressive submucosal fibrosis. Matrix Metalloproteinases (MMPs) are a group of enzymes which have the ability to degrade all known components of the extracellular matrix, and may therefore contribute to the abnormal matrix turnover that occurs in CF. Net MMP activity is dependent on the molar ratio of MMPs:Tissue Inhibitor of Metalloproteinases (TIMPs).
Hypothesis: Infection with PA drives MMP secretion by both inflammatory cells and parenchymal cells in CF, resulting in subsequent airway remodelling.
Methods: Human bronchial epithelial cells (HBE), cystic fibrosis bronchial epithelial (CFBE) cells and peripheral blood monocytes were stimulated for 4 hours with increasing doses of PAO-1-the well-characterised laboratory strain of PA, or conditioned media from PA infected monocytes (coMPA) for 72 hours and supernatants collected. Recombinant human TIMP-1 (the major inhibitor of MMP-9) was incubated directly with PA to investigate whether PA could directly destroy this protein. MMP-9 was measured by gelatin zymography and ELISA, and TIMP-1 was measured by ELISA. Intracellular signalling was investigated with Western blotting.
Results: PA infection caused a dose dependant reduction in TIMP-1 by HBE cells (1.6+/-0.19ng/ml for MOI (Multiplicity of Infection)-100 vs 2.5+/- 0.19ng/ml in controls $p< 0.033$), and monocytes (124.3 +/- 10.95pg/ml for MOI 100 vs 384.1+/- 39.90 pg/ml in controls $p< 0.001$). Given the rapid reduction in TIMP-1 we investigated whether PA could directly destroy TIMP-1. After 4 hours of incubation with PA, TIMP-1 was no longer detectable by ELISA ($p< 0.001$). CoMPA significantly increased MMP-9 induction from both HBE cells (10.29+/-0.6ng/ml coMPA vs 5.2+/-0.3ng/ml for controls $p< 0.001$) and CFBE cells (16.6+/-2ng/ml coMPA vs 6.0+/-0.85ng/ml for controls $p< 0.001$). TIMP-1 was significantly reduced in CFBE following coMPA stimulation ($p=0.001$) and reduced in the supernatants of HBE cells, however this failed to reach significance. Our data also suggests that the secretion of MMP-9 in response to PA infection is regulated by p38, JNK and ERK mitogen activated protein kinases.
Conclusion: Direct PA infection and coMPA stimulation both induced significant production of MMP-9 by lung epithelial and monocytic cells,and is accompanied by a rapid loss of TIMP-1; its major inhibitor. These data suggest that PA infection can induce functionally unopposed MMP-9 activity both in the lung parenchyma, and from newly recruited inflammatory monocytes,and may represent a mechanism for the excessive tissue damage observed in CF.

The Effect of Budesonide in a Refined Rat Model of Respiratory Infection with *Pseudomonas Aeruginosa*

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Striking a balance between effective suppression of excessive inflammation whilst maintaining host defense remains a key challenge in the treatment of cystic fibrosis. We evaluated whether therapeutic budesonide treatment could attenuate inflammation without modulating bacterial load in a refined rat model of respiratory infection with Pseudomonas aeruginosa (P.a.). *P.a.* PAO1V strain (University of Colorado) was embedded in agar beads in the presence of 0.01% v/v of the surfactant SPAN® 80. Under isofluorane anaesthesia, male Sprague Dawley rats (250-300g; n=16/group) were inoculated (i.t.) with agar beads containing 10^5 colony forming units (cfu) of P.a. Sham groups were given sterile beads. Rats were dosed with 3 mg/kg (p.o.) budesonide or vehicle 16 hr post inoculation, subsequently dosed twice a day and culled on days 2 or 5. Cfu counts were performed on lung tissues; cell counts were performed on bronchoalveolar lavage fluid (BALF). Data are expressed as mean ± S.E.M. and analysed using a Kruskal-Wallis test with a Dunn's post test and a Mann Whitney test where appropriate. P.a. infection significantly elevated neutrophils 2 and 5 days post infection (**Table 1**, P< 0.05). Budesonide augmented the P.a.-induced BALF neutrophils 2 (P< 0.05) and 5 days (P< 0.01, **Table 1**) post infection. Budesonide significantly increased bacterial load at 2 days (**Table 1**, P< 0.05, n=8) but not at 5 days post inoculation. In conclusion budesonide enhanced inflammatory cell infiltrate and increased bacterial load. As patients may be intermittently treated with antibiotics these findings may warrant further evaluation. Future studies will focus on evaluating the effect of a combination of budesonide and antibiotics.

	Day 2				Day 5			
	Sham Vehicle	Sham Budesonide	P.a. Vehicle	P.a. Budesonide	Sham Vehicle	Sham Budesonide	P.a. Vehicle	P.a. Budesonide
Log cfu	1.5±0.0	1.5±0.0	4.4±0.3	5.4±0.2$	1.5±0.0	1.5±0.0	5.2±0.2	5.6±0.1
Total cells	4.0±0.6	4.0±0.6	17.0±7.0	41.0±7.0$	6.0±1.0	7.0±1.0	20.0±3.0*	28.0±3.0$
Neutrophils	0.3±0.1	0.3±0.1	12.0±4.0*	29.0±6.0$	0.1±0.03	0.3±0.1	3.0±1.0*	13.0±3.0$$

[Table 1: Lung Tissue cfu and BALF cells (x105).]

* P< 0.05 versus sham; $ P< 0.05; $$ P< 0.01 versus P.a. vehicle

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To Ivestigate the Virulence Factors that Are Associated with the Pathogenesis of *Pandoraea spp* in Cystic Fibrosis (CF) Patients

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Pandoraea species have emerged as one of the organisms that have been recently been isolated from both the lungs and from blood cultures of CF patients. The specific bacterial factors that might contribute to virulence in CF have not been identified even though many have been proposed. These include lipopolysaccharide (LPS), biofilm formation, adherence to epithelial cells, stimulation of pro-inflammatory response, ability to invade lung epithelial cells and exported products such as siderophores and proteases.

Previously, our group have shown that certain *Pandoraea* strains were able to invade A549 lung epithelial cells and promote a potent pro-inflammatory response. (Caraher et al, 2008) The ability of *Pandoraea* to disrupt tight junctions in polarized monolayers and translocate through the monolayer was also examined. (G. Herbert et al, unpublished data). A qualitative method was used to investigate whether the disruption to tight junctions was as a result of proteolytic cleavage of tight junction proteins; the level of expressed extracellular protease was examined in a range *Pandoraea* isolates which all exhibited a high protease activity as indicated by large one of clearance on skim milk agar. The protease activity was further characterized using a range of protease inhibitors, PMSF (serine protease inhibitor) and EDTA (Metallo-protease inhibitor) and E-64 (cysteine protease inhibitor). Results indicated that all isolates exhibited serine protease activity. The mechanism by which these proteases may contribute to virulence and also their potential to interfere with host defence mechanism is currently being examined. One of the effects of *Pandoraea* isolates on lung epithelial cells studied was that of the host immune response. All the *Pandoraea* strains examined stimulated the production of pro-inflammatory cytokines, IL-6 and IL-8 and TNF-alfa in the two human bronchial epithelial cell lines examined; HBE and CFBE cells, with high secretion of IL-8 by all examined isolates in mostly HBE cells with secretion of IL-8 exceeding 1.5ng ml-1 in HBE cells and 1.1ng ml-1 in CFBE cells. The effect of bacteria cell associated components or toxins released from the bacterial cells on cytokine induction were also examined using bacterial culture supernatants (with and without heat treatment). Results demonstrated that heat treated cells induced the lowest level of cytokines in HBE and CFBE cells suggesting that a heat labile component is potentially triggering the production of pro-inflammatory cytokines. The overall expression of these cytokines by all the *Pandoraea* strains examined indicates that this is an important mechanism of virulence and is likely to contribute to its persistency in CF patients. Studies investigating the mechanism (s) that are responsible for this immune response are ongoing.

This project is funded under TSR PRDSP 2008.

Investigation of the Potential Mechanisms of Antibiotic Resistance in the Cystic Fibrosis Pathogen, *burkholderia dolosa*

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Burkholderia cepacia complex (Bcc), are a group of opportunistic Gram-negative bacteria that cause chronic pulmonary infections in patients with Cystic Fibrosis (CF). They are known to demonstrate resistance to the most clinically useful antibiotics. *B. cenocepacia* and *B. multivorans* are the most clinically relevant species as they are the most predominant isolates recovered from CF patients. However, *B. dolosa* has emerged as a virulent and transmissible species, even though it represents a low percentage of Bcc isolates recovered from CF patients.

A common mechanism of antibiotic resistance in Gram-negative bacteria is antibiotic efflux. An antibiotic efflux gene cluster known as the "*ceo*" operon, which confers resistance to chloramphenicol, trimethoprim and ciprofloxacin has been previously identified in *Burkholderia cenocepacia* (Nair *et al*, 2004). The cluster contains five open reading frames consisting of a single transcriptional unit, *llpE* encoding a lipase-like protein, *ceoA* encoding a putative periplasmic linker protein, *ceoB* encoding a putative cytoplasmic membrane protein and *opcM* encoding a previously described outer membrane protein. There is also a putative LysR-type transcriptional regulatory gene, *ceoR*, which is transcribed upstream of the structural gene cluster. PCR amplification of the genes from the *ceo* gene cluster have been shown to be present in five genomovars; *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis* and *B. vietmaniensis*.

Initially, we examined the antibiotic susceptibility of different *B. dolosa* isolates to tobramycin, ceftazadime and ciprofloxacin on 4, 8 and 12 day old biofilms and found they were less susceptible to treatment with increasing maturation and development of the biofilms. Efflux pumps can be highly active in bacterial biofilms, thus we investigated whether the *ceo* gene cluster was present in *B. dolosa* strains. *B. cenocepacia* strains LMG 18826 (BC7) and LMG 18863 (K56-2) were used as positive controls. Our results demonstrated that all five genes were not present in *B. dolosa*, unlike that for *B. cenocepacia*. This may be due to *B. dolosa* having increased susceptibility to some of the antibiotics that this particular gene cluster encodes, thus explaining the presence of some of the *ceo* genes in *B. dolosa* isolates. It is therefore, possible that there is a different efflux pump present in *B. dolosa* that could confer resistance to other antibiotics.

In conclusion, all five ceo genes are not present in the *B. dolosa* isolates examined in this study. Further studies are required to determine whether efflux pumps present in *B. dolosa* contribute to the antibiotic tolerance of *B. dolosa* biofilms. Greater understanding of the mechanisms involved in Bcc antibiotic resistance should lead to improved antibiotic therapy, especially for patients with Cystic Fibrosis.

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Development of a Multi-Cellular Co-Culture Model of Normal and Cystic Fibrosis Human Airways *in vitro*

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Introduction: Understanding the disease pathogenesis of CF currently relies on animal and/or single cell culture models. Whilst these approaches partially enable the study of CF, the models have inherent limitations.
Objectives: We intend to overcome some of these limitations by developing a multi-cellular co-culture model of human airways *in vitro*. Our co-culture model for non-CF airways uses human pulmonary fibroblasts (HPF) and human bronchial epithelial cells (Calu-3) grown at air liquid interface (ALI). This model is adapted to mimic CF by replacement of Calu-3 with IB3-1 (the CF cell line bearing delta F508/W1282X).
Methods:
Cell Viability: Optimal conditions for co-culture growth were defined by light microscopy, flow cytometry and a fluorescence-based assay after exposure to fresh and conditioned medium.
Cell Morphology: Cytokeratin (CK) antibodies were used to detect the specific epithelial cell markers, CK5 and CK8 in single and co-cultures. The presence and location of fibroblasts were confirmed with 1B10, an antibody specific for fibroblast surface antigens. Zonulae occludens-1 (ZO-1) staining was used as evidence of tight junction formation.
Cell Physiology: Differentiation to a "tight" epithelial cell barrier was monitored by measuring transepithelial electrical resistance (TER) over time at ALI. Apical fluid secreted by co-cultures was examined for the presence of mucin using a MUC5AC antibody.
Results and Discussion:
Cell Viability: Suitable culture medium has been defined for both co-culture models, demonstrated by maintenance of typical morphology, viability and growth characteristics.
Cell Morphology: CK staining indicates basal and differentiated epithelial cell populations after ALI has been established. ZO-1 staining indicates that tight junctions are present at the apical side of co-cultures, confirming that a polarised cell layer is formed.
Cell Physiology: Increasing TER indicates an increase in the barrier function of epithelial cell cultures and co-cultures but not for HPF in mono-culture. Mucin is secreted apically and preliminary results indicate mucus hypersecretion in CF models compared to non-CF.
These results are an invaluable starting point for developing an *in vitro* model employing multiple, human cell types for further investigation of CF. We envisage that our models will be useful for comparing the response of CF and non-CF airways to infection and inflammation.

We would like to thank the Humane Research Trust for financial support.

Lung Function Measurement in a CF Mouse Model

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The usefulness of cystic fibrosis (CF) knock out mice to study lung disease has been a topic of controversy. In order to use these KO mice to study the mechanisms of airway inflammation in CF, we assessed lung function measurements to test whether these mice develop lung disease. Lung function measurement in mice is a well established method to study lung pathophysiology in models such as asthma and emphysema.

In 20 weeks old males and females *Cftr*^{tm1Unc}-Tg$^{(FABPCFTR)}$ Jaw mice (Cftr^{KO}) (n=7) (Jackson laboratories) and wild type (WT) littermates (n=5) we measured baseline lung function via the invasive forced oscillation technique of the flexiVent system (SCIREQ, Montreal, PQ, Canada). The 'single compartment' snapshot perturbation was imposed to measure resistance (R), elastance (E) and compliance (C=1/E) of the whole respiratory system (lung and thorax). Consequently, the more specific 'constant phase' forced oscillation perturbation (primewave-8) was applied, resulting in airway resistance (Rn), tissue damping (tissue resistance, G) and tissue elasticity (H).

With the 'snapshot' perturbation, we observed that there was a significant increased resistance (R) and elastance (E) of the respiratory system in Cftr^{KO} mice compared to WT ($p=0.0025$ and $p=0.0303$, respectively). Applying the primewave-8 perturbation, showed that both airway resistance (Rn) as well as tissue resistance (G) and tissue elasticity (H) are significantly increased ($p=0.048$, $p=0.0051$ and $p=0.048$, respectively).

Lung function measurement parameters indicate CF like changes in the lung of KO mice, proving that these KO mice could serve as a good model to study inflammation and structural changes.

Generation of New Lung-Specific Tet-Dependent Activator Mice for Tight and Quantitative Control of Conditional Gene Expression in the Murine Lung

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The complex pathogenesis of cystic fibrosis (CF) lung disease remains incompletely understood. Conditional overexpression and deletion of genes in mice by combinatorial use of lung-specific promoter elements and the tetracycline-regulated system provides a powerful tool to elucidate the roles of candidate genes implicated in airway ion transport, inflammation, host defense and tissue remodeling in the in vivo pathogenesis of CF lung disease. However, the original version of the reverse tetracycline-dependent transactivator (rtTA) exhibited limited doxycycline sensitivity and residual affinity to its promoter (P_{tet}) producing leaky transgene expression in the absence of doxycycline impeding the use of this system for such mechanistic studies. We, therefore, used a new generation rtTA, designated rtTA2^{S}-M2, with no basal activity and increased doxycycline sensitivity, and the rat Clara cell secretory protein (CCSP) promoter to target its expression to pulmonary epithelia in mice. Novel CCSP-rtTA2^{S}-M2 founder lines were generated by pronuclear injection. For functional characterization, founder lines were crossed with bi-transgenic reporter mice expressing luciferase and Cre recombinase (LC-1). Background activity, doxycycline sensitivity, tissue and cell-type specificity, inducibility, and reversibility of doxycycline-dependent gene expression were determined by luciferase activity, immunohistochemistry, morphometry and bioluminescence measurements in neonatal and adult lungs. We generated two distinct novel CCSP-rtTA2^{S}-M2 activator mouse lines that confer tight and doxycycline dose-dependent regulation of transgene expression, with high inducibility, complete reversibility and no background activity, in airway and/or alveolar epithelia. We demonstrate that rtTA2^{S}-M2 enables quantitative control of conditional gene expression in respiratory epithelia of the murine lung. Our results predict that the new CCSP-rtTA2^{S}-M2 activator mouse lines will be useful to determine the specific roles of individual candidate genes in the complex pathogenesis of CF lung disease and might lead to the development of novel therapies.

Supported by: European Commission (MEXT-CT-2004-013666), Mukoviszidose e.V. (I06/04)

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Wobble Splicing at a NAGNAG Acceptor Site Induces a Mild Cystic Fibrosis Phenotype

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Around 30% of alleles causing genetic disorders induce premature termination codons (PTCs) (1) and are usually associated with severe phenotypes; however mild disease outcome can occur. We report mild to asymptomatic phenotypes in three cystic fibrosis homozygous patients for the E831X mutation (2623G>T). Analyses performed on nasal epithelial cell mRNA identified three distinct isoforms, a surprisingly more complex situation than expected for a single nucleotide substitution. Structure-function studies and in silico analyses provided the first experimental evidence of PTC removal by wobble splicing at a NAGNAG acceptor site. In addition to contributing to proteome plasticity (2), wobble splicing can therefore induce in vivo removal of a disease-causing UAG stop codon. This molecular study unravels a novel naturally occurring correction mechanism where the effect of either modifier genes or epigenetic factors could have been suspected. This finding is of importance for genetic counseling as well as for the choice of therapeutic strategies.

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Abnormal CFTR mRNA Processing: Which Mutations Cause it, Which Are the Consequences and How Can it be Treated

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Among the 1,600 variants described to occur in the CFTR gene [1], 13 % are considered to affect splicing, given their localization in consensus splicing sites. However, additional variants may impair or significantly decrease the efficiency of the splicing process, as usually mutations reported at the CFTR mutation database have not been functionally characterized. In fact, the impact of most CFTR variants at the expression and functional levels is still unknown. Yet, this missing information would be of utmost importance for disease diagnosis and prognosis. Moreover, with more recent "CFTR corrective" (or "CFTR-assist") therapies, this knowledge is also critical for appropriate selection of patients. To address this challenge, an international effort, called CFTR2 (Clinical and Functional TRanslation of CFTR) has been undertaken to characterize the pathophysiological consequences of the most common CFTR variants.

Our goal here, in coordination with the CFTR2 project, is to characterize the effects of mutations affecting splicing, not only at the mRNA level, but also in terms of CFTR protein processing and function. To this end, we are producing various CFTR intronic mini-genes (complete CFTR cDNA sequence with one or more introns) and expressing them in cell lines to be used as models for splicing.

Previously, we showed that the CFTR mutation (I1234V, 3832A>G) classified as "missense", actually affects splicing in native tissues from a CF patient with the I1234V/F508del genotype [2]. This mutation creates a "cryptic" donor splicing site in exon 19 (18 nts upstream the normal one) and our data showed that it completely abolishes usage of the normal donor. Here, we confirmed this result in BHK cells expressing a CFTR mini-gene containing intron 19 (IVS19) and the 3832A>G mutation. We also tested in this model how the "strength" of the two donors (normal and cryptic) influences splicing, by swopping the normal donor sequence into the location of the cryptic donor and vice versa. By RT-PCR we observed that when the sequences of both donors are the same (i.e., two equal donors at the cryptic and normal locations) only correctly spliced transcripts are generated. We conclude that the "strength" of the donor seems to play a major role in regulating alternative splicing at this CFTR locus.

Similarly, we produced BHK cell lines expressing CFTR mini-genes containing IVS5, IVS14b or IVS22 and results from qRT-PCR show that all these three introns are correct spliced. Western blot analysis is in progress to assess whether the resulting CFTR proteins are fully processed. Cell lines expressing the IVS5-CFTR construct with the 711+5G>A and 711+3A>G mutations were also generated and, in preliminary analyses, the normally spliced CFTR transcripts were detected.

These mini-gene systems can provide crucial information on the consequences of CFTR splicing mutations and will help to establish the most appropriate therapies to each mutation.

Work supported by PIC/IC/83103/2007 grant and pluriannual funding from BioFIG (FCT, Portugal).

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Histone Deacetylases (HDACs) and IFRD1 in CF Airway Epithelial Cell Models

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In cystic fibrosis (CF) patients, recent data suggest that Interferon related developmental regulator 1 (IFRD1) is a modifier gene for lung disease. IFRD1 acts in a histone-deacetylase (HDAC)-dependent manner to mediate transcriptional co-repression of NF-κB transactivation involved in inflammation. We therefore hypothesize that intrinsic alterations might occur in the balance of HDACs 1, 2 and 3 and IFRD1 expression in CFTR-deficient cells compared to normal and corrected CF airway cells.

We have examined the expression level of three HDACs(1-3) mRNA and IFRD1 mRNA (by qPCR) in two CF bronchial cell lines (IB3-1 and CFBE41o-) compared to CFTR-corrected and sufficient (S9 and 16HBE14o-) cell models. We also evaluated the level of HDACs(1-3) proteins by Western blotting and measured the total HDACs enzymatic activity under oxidative stress (IL-1β, 10 ng/ml and/or H_2O_2, 100 and 500 µM, 1h).

We show a lowest expression of HDACs(1-3) and IFRD1 mRNA in CFBE41o- cells compared to 16HBE14o- cells. We observe that the level of HDACs(1-3) proteins and particularly HDAC2 is reduced in CFTR-deficient cells compared to CFTR-sufficient cells in response to oxidative stress. We also demonstrate that total HDAC activity was lower in IB3-1 and CFBE41o- cells compared to S9 and 16HBE14o-cells at both basal and oxidative stress conditions.

Together, our data show significant difference in the HDACs(1-3)/IFRD1 signaling in CFTR deficient cells compared to normal and corrected CF airway cells. Understanding how loss of CFTR function leads to alterations in the regulation of IFRD1/HDACs complex and its role in exaggerated inflammatory response in CF airway cells require further investigation.

Supported by the French Cystic Fibrosis Association "Vaincre La Mucoviscidose".

Plasma Lipidomics Reveals Phenotype-associated Differences within a Cohort of F508del Homozygous Patients

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Biomarkers obtained by rapid and non-invasive methods could be used in the precocious detection of exacerbations or as prognostic indicators in CF. A large body of evidence links CF with abnormal content and metabolism of certain lipids in plasma and tissues from patients and animal models. We have recently shown significant variations in the plasma levels of several phosphatidylcholine and lysophopshatidylcholine species related to the disease severity status in CF patients (Guerrera et al. PLoS One, 4(11):e7735, 2009). Our goal was to search for potential lipid signatures characteristic of CF patients presenting the same mutation (F508del) and different CF phenotypes, and to study their correlation with clinical parameters. We used a lipidomic approach based on multiple reaction monitoring (MRM) liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS/MS). Blood plasma samples from 44 F508del homozygous patients were collected. Organic extracts were subjected to targeted LC-ESI-MS/MS analysis and quantification of 50 lipid molecules or lipid classes, including individual fatty acids, phospholipids, and ceramides, cholesterol, total cholesterol-esters and total triglycerides. Five phosphatidylcholine species (PC32:2, PC34:2, PC34:1, PC36:5, PC36:3), two lysophosphatidylcholine (LPC18:0, LPC20:4n-6), one sphingomyelin (SM16:0) and six fatty acids (C22:6n-3, C16:0, C20:3n-9, C22:5n-6, C22:5n-3, C20:5n-3), as well as total LPC and the LPC/TG ratio, were significantly decreased in those patients presenting more severe phenotypes. In addition, ten fatty acids (C16:0, C16:1n-7, C18:0, C18:3n-3, C18:1n-9, C20:3n-6, C20:3n-9, C22:5n-6, C22:5n-3, C20:5n-3) were significantly and positively correlated with FEV1% at sampling. Concerning *Pseudomonas aeruginosa* infection, PC32:2, PC38:5 and C18:3n-3 were significantly decreased in patients presenting chronic infection, while cholesterol, cholesterol-esters and total triglycerides were significantly increased. FEV1% and chronic Pseudomonas infection were reevaluated two years after sampling and the potential predictive value of the analyzed lipids was assessed. Surprisingly, plasma cholesterol was negatively correlated with FEV1%, and total triglycerides were positively associated with chronic infection. These results confirm our previously reported findings and suggest a potential predictive value for neutral lipids (cholesterol and triglycerides) in plasma concerning CF severity. Further longitudinal prospective studies to confirm this potential, as well as mechanistic investigations aiming to explain these observations, are warranted.

Supported by: Vaincre la Mucoviscidose, EC LSHG-CT-2005-512044 (NEUPROCF), Agence Nationale de la Recherche (EICO-CF), and Legs Poix-University of Paris Descartes.

TMEM45a: a Hypoxia-Regulated Centriole/Basal Body Protein Implicated in Development of Cilia and CF Pathogenesis

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In a previous microarray study of differential gene expression in native nasal epithelial cells we found TMEM45a to be 3-fold upregulated in CF ($p < 0.00001$) compared to healthy subjects. This 32 kDa transmembrane protein of still unknown function is upregulated following hypoxia [1] and ER stress [2], and downregulated by asthma and smoking [3], being therefore implicated in pathways relevant to CF pathophysiology.

Our goal here was to define the functional implications of the upregulation of TMEM45a in CF.

Firstly, we performed qRT-PCR in human bronchial epithelial cell lines to confirm the microarray data. Surprisingly, we found that levels of TMEM45a transcripts, although sensitive to CFTR genotype, were down-regulated in CFBE vs HBE cells: 2-fold down in presence of F508del-CFTR ($p < 0.01$). We also confirmed that in these cells TMEM45a is up-regulated by hypoxia (4-fold; $p < 0.0001$). Next, using a polyclonal antibody against TMEM45a (C-13, Santa Cruz) we determined the intracellular localization of this protein. Results from immunofluorescence demonstrated a punctate expression pattern for TMEM45a in HBE, CFBE and 293-HEK cells corresponding to that expected for a protein found at the centriole. Partial co-localization with γ-tubulin further suggests that TMEM45a is present in the pericentriolar matrix (PCM), a mass of proteinaceous material that organizes microtubules in the cytoplasm and mitotic spindle [4]. Our data also show that the TMEM45a stained structure is dynamic, sometimes ring-shaped, sometimes taking the form of a pair of centrioles, and sometimes dispersing in the cytoplasm, e.g., during spindle formation and mitosis. The condensed structure is generally localized within the Golgi complex, as shown by co-localization with Golgi-P58 protein.

Since centrioles are associated with the formation of basal bodies which nucleate cilia, we also investigated the intracellular localization of TMEM45a in human native nasal epithelial ciliated cells. Data show that the pattern of TMEM45a is profoundly different in these native cells: it strongly associates with individual basal bodies underlying the apical membrane. It also co-localizes to the cilia themselves and with a large cytoplasmic fibrous structure lying between the Golgi and the apical membrane. We have documented the co-localization of TMEM45a in both native nasal epithelial cells and cell lines with a variety of markers suggesting its function as a regulator of or component in the development of cilia. This would be consistent with a mechanistic or sensory function which may be altered in CF, e.g., in response to mechanical signals transmitted by defective ASL clearance.

In conclusion our data suggest that TMEM45a plays a role as a centriole/PCM and Golgi-related protein and place it at the intersection of a series of systems perturbed in CF pathophysiology, and we suggest it as a novel potential modulator of CF.

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Posters – Therapeutic Strategies to Correct Ion Transport Defects

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Effect of TS-04-95 on Primary Human Bronchial Epithelial Monolayers after a Repeated Dose Treatment

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A valid therapeutic strategy to circumvent the defective CFTR in CF airways involves activation of other, non-CFTR Cl^- channels, like the Ca^{2+}-activated Cl^- conductance (CaCC). Such "bypassing pharmacotherapy" [1] has the advantage of being equally applicable to CF patients with any CFTR genotype. Moreover, since hyperabsorption of Na^+ through ENaC has been described to be regulated by Cl^- transport [2], CaCC activators could serve to normalize both Cl^- and Na^+ transport in CF airways. Until recently, the most promising strategy to stimulate CaCC was through ATP- stimulation of purinergic receptors. However, other potential tools for a bypassing pharmacotherapy comprise manipulation of signaling pathways acting on ionic transport. Compounds in the inositol phosphate (InsP) pathway have been shown before to successfully inhibit Na^+ and fluid hyperabsorption across CF airway epithelial cells and also to enhance CaCC activity [2-4]. Here, we investigated the effects of an InsP-related compound (TS-04-95) to evaluate its therapeutic potential for CF.

We determined the efficacy of TS-04-95 in both stimulating Cl^- CaCC/TMEM16A-mediated currents (using the CaCC-01 inhibitor) and in inhibiting ENaC-mediated currents. Measurements were done in monolayers of polarized primary cultures of human bronchial epithelial (HBE) cells from lung explants [5] and human airway epithelial cell (H441) mounted in Ussing chambers. In addition double electrode voltage clamp (DEVC) experiments were performed in *Xenopus* oocytes and whole-cell patch-clamp experiments were performed in TMEM16A-expressing HEK293 cells. Results show that the repeated exposure of non-CF primary HBE monolayers (n=5) to TS-04-95 significantly ($p < 0.05$) enhanced the sustained ATP and CCH responses (in plateau phase 18.87±1.26 $\mu A/cm^2$ and 5.02±1.37 $\mu A/cm^2$, respectively). Experiments in HEK293 cells and *Xenopus* oocytes also indicated activation of TMEM16A by TS-04-95. No significant effects of TS-04-95 on ENaC-mediated Na^+ absorption or cAMP-mediated Cl^- secretion by CFTR were observed in non-CF primary HBE monolayers, while pronounced inhibition of ENaC by TS-04-95 was observed in H441 cells. These results suggest TS-04-95 as an efficient pharmacological tool for the treatment of the CF lung disease, whose efficacy in correcting epithelial salt transport will be further validated in primary cultures of HBE cells from human CF lungs as well as in native rectal biopsies from CF patients.

Work supported by TargetScreen2 (EU-FP6-LSH-2005-037365), PIC/IC/83103/2007 grants and BioFig (FCT, Portugal). MS is recipient of SFRH/BD/35936/2007 PhD fellowship (FCT, Portugal). Authors are grateful to A. Verkman for Ca01 compound.

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Screening for F508del-CFTR Correctors Identified by *Pharmacophore Modelling* through Western blot and NBD1 Folding Assays

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Pharmacophore modelling based on virtual screening of compounds is a ligand-based approach particularly useful when the target protein 3D structure is unavailable. Using a shape fingerprint of the low-energy conformation of known CFTR correctors we screened ~6 million commercial compounds. The resulting hits were clustered by pharmacophoric fingerprint to yield a diverse set of 250 compounds which were subjected to biological evaluation. In parallel, we built a predictive protein docking model based on recently published X-ray structures to identify putative binding sites for F508del-CFTR correctors and compared the diverse set with the sites identified by the model.

The 250 small-molecule library was screened by two parallel medium-throughput methodologies: 1) quantitative Western blot (WB) to assess rescuing of F508del-CFTR processing in CFBE cells [1] (by appearance of its mature form); and 2) wt- and F508del-NBD1 folding and stability assays, using differential scanning fluorimetry. The latter determines the compound impact in NDB1 thermodynamic and kinetic stabilities, by measuring respectively, the shift in the melting temperature (DT_m), and aggregation rate at 37°C. A T_m increase reflects higher thermodynamic stability and a decrease in aggregation rate suggests higher the kinetic stability. "Hit" compounds should increase stability, reduce aggregation propensity and promote maturation.

Sensitivity of the WB method was given by incubating cells with known F508del-CFTR correctors: VRT-325 (6.7mM;24h); C4a (10mM;6h), showing that 5-6 wells of a 96-well plate (~2-3x10^5 cells) suffice to detect F508del-CFTR maturation at 37ºC (9%;12%, respectively). In the screen, cells from 5 wells were pooled after compound incubation (10mM;24h, 37ºC and 26ºC), extracts prepared and WB performed with the 596 anti-CFTR antibody. The screen identified 4 primary "hit" compounds causing extensive rescue and/or an impressive stabilization of NBD1, respectively (% of band C rescue vs DMSO control; DT_m) TS-01-01-C9 (70%; 3.4ºC); TS-01-01-B9 (160%; 1.6ºC); TS-01-01-C5 (90%; 1.5ºC), TS-01-01-E12 (114%; 1.5ºC).

The effect of these compounds on F508del-CFTR trafficking was also assessed by a microscopy-based assay performed at 26°C in A549 cells expressing a double tagged flag/mCherry CFTR construct under an inducible (Tet-ON) promoter. The ratio of total CFTR at the membrane (assessed by an anti-Flag antibody without cell permeabilization) over the total CFTR expressed (given by the mCherry fluorescence) was determined per cell. Values were (±SEM): DMSO - 0.415 ± (0.008); Corr-4a - 0.589 (± 0.009); C9 - 0.445 (± 0.004); C5 - 0.373 (± 0.004); E12 - 0.497 (± 0.005); B9 - 0.387 (± 0.004). We could confirm by this strategy rescue of F508del-CFTR by TS-01-01-E12, which showed additive rescuing to low temperature. A secondary library of 46 compounds derived from these 4 lead primary "hits" is currently under assessment.

Pharmacophore modeling appears as a powerful method to rapidly identify new compounds correcting F508del-CFTR maturation/folding defects and substantially reduces the number of compounds screened.

Work supported by TargetScreen2 (EU-FP6-LSH-2005-037365) grant. Authors thank CFF-USA for correctors (B.Bridges, Chicago, USA) and antibody (J.Riordan, UNC, USA) and the CFTR Folding Consortium (P.Thomas, Texas, USA) for NBD1.

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SIV Vector Pseudotyped with Sev-F/HN Envelope Proteins Produces Long Lasting Expression in the Murine Lung, Is Readministrable and Transfects Human Airway Models

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We have previously shown that simian immunodeficiency virus pseudotyped with F and HN protein from Sendai virus (F/HN-SIV) transduces murine nasal epithelium efficiently (3-5% of respiratory epithelial cells after perfusion with $5x10^8$ TU) and importantly can be repeatedly administered. We now show that expression in the nasal epithelium persists for the life-time of the animals (16-25 months, 9 of 9 C57Bl/6 mice). The vast majority of published studies have assessed lentiviral vectors in mouse nasal epithelium, and transduction of lung epithelium, particularly without pre-conditioning through polidocanol treatment or tissue damage, remains challenging. Here, we show that F/HN-SIV transduces lung epithelium efficiently and dose-dependently. In contrast to other studies, we show that lentivirus-mediated gene expression in the lung is stable for at least 13 months (latest time-point currently analyzed) (month 2: 362660±63922photons/sec, month 13: 431454±65647photons/sec, n=5-8 mice). We also show, for the first time, that lentivirus can be repeatedly administered to the lung (2 doses of F/HN-SIV-GFP followed by a 3rd dose of F/HN-SIV-Lux at monthly interval) without loss of activity compared to a single dose of F/HN-SIV-Lux (1 Dose: 40178±4843, 3 Doses: 39080±7490 RLU/mg protein, n=21/group). Importantly, we also show that gene expression increases with increasing number of doses (10 doses at daily interval) (Dose 1: 4693± 899, Dose 10: 239212±48362 RLU/mg protein, n=8/group, $p< 0.0005$). Importantly, we have not observed acute or chronic toxicity in 12 months follow-up studies in mice. We have also assessed the performance of F/HN-SIV in various airway ex vivo models. (a) The virus transduces human air liquid interface cultures efficiently with expression persisting for at least 4 months. (b) The virus transduces freshly obtained human nasal brushings dose-dependently. (c) The virus transduces human lung slices efficiently and expression persists for the life-span of the slices. These data suggest that, F/HN-SIV may be a suitable vector for cystic fibrosis gene therapy.

Evaluation of Safety and Gene Expression with a Single Dose of pGM169/Gl67A Administered to the Airways of Individuals with Cystic Fibrosis: The UK CF Gene Therapy Consortium 'Pilot Study'

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The UK CF Gene Therapy Consortium is working towards a multi-dose gene therapy study, using the best currently available non-viral gene transfer agent, and whose endpoint will be to detect clinical benefit rather than molecular or electrophysiological proof-of-principle. Based on extensive preclinical testing our selected product is pGM169, a CpG-free human *CFTR* plasmid with a CpG-free CMV enhancer and human elongation factor 1 alpha (hCEFI) promoter complexed with GL67A, a mixture of 3 lipids: GL67, DOPE and DMP-PEG5000. We are currently undertaking a single dose safety study (Pilot Study) because of a requirement to confirm safety of this 'first-in-man' product; however, the study design has been tailored also to assess gene expression *in vivo* in CF lungs.

A single nebulised dose of 20 ml (53 mg pGM169 and 286 mg GL67A) is delivered by an Aeroeclipse II breath-actuated device; a nasal dose of 10% of the nebulised volume is administered on the same occasion using a standard nasal spray device. The latter allows assessment of gene expression without the sampling issues inherent in lower airway assessment, as well as anchoring to our previous clinical trials. Safety measures include physical examination, lung physiology (spirometry, pulse oximetry), systemic and sputum inflammatory markers, renal and hepatic function and chest CT. Measurements are made at intervals prior to dosing and during a 28 day follow up period. Gene expression is assessed by a) quantitative Taqman RT-PCR for transgene mRNA on nasal and bronchial brushings, b) anti-CFTR immunohistochemistry, and c) nasal and lower airway potential difference measurements. Given inter-subject variability, paired measurements on individuals will be obtained; bronchoscopies are being performed prior to dosing and either 6 days or 14 days post-dosing. Nasal PD is measured on serial visits. To date, 13 patients have been treated with pGM169/GL67A and safety as well as efficacy data will be presented.

Funded by the UK Cystic Fibrosis Trust

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Proteomics Characterization of Nasal Epithelial Cells: Their Use for Biomarkers Discovery in CF

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Background: CF is a monogenic multiorgan disorder but progressive lung disease accounts for 95% of morbidity and mortality among CF patients. Nasal respiratory epithelium is representative of airway system and epithelial cells account for approximately 80-95% of nasal brushing (NB) cellular content. Since nasal epithelium reflects characteristic ionic transport abnormalities of lower airways in CF dysfunction, characterization and comparison of NB cells proteome obtained from CF patients with mild or severe lung disease and healthy volunteers by a high-throughput proteomics technology is of great importance.
Experimental: Epithelial cells were collected by a brushing procedure[1] from nasal mucosa of non-CF healthy individuals (n=27), carriers of a single mutated *CFTR* allele (n=18) and CF patients with 2 identified mutations presenting Mild (n=10) or Severe (n=17) lung disease. Cytological characterization was achieved by Leishman staining and light microscopy and samples were pooled according to above criteria. Pooled NB samples were fractionated into soluble- or membrane-enriched fractions by differential centrifugation and global proteome profiling was achieved by using complementary gel-based and gel-free mass spectrometry (MS) proteomics: two-Dimensional Difference In Gel Electrophoresis (2D-DIGE) coupled to Matrix Assisted Laser Desorption/Ionization-Time Of Flight MS (MALDI-TOF/TOF) and 2D-Liquid Chromatography coupled to tandem MS (2D-LC-MS/MS) using a label-free strategy. Differentially expressed proteins were analyzed concerning their function, biological processes and cellular localization using dedicated software packages.
Results: Overall, 719 and 2525 proteins were identified by at least 2 peptides in soluble and membrane enriched fractions by LC-MS/MS, respectively. Of the identified proteins, 306 were present in both fractions. Proteins with positive identification in all groups under study were used to characterize this biological sample using Ingenuity Pathway Analysis (IPA) retrieving processes such as oxidative phosphorylation, cellular organization and locomotion or molecular transport. Epithelium-specific proteins such as KRT5, 8, 19, MUC-5AC, SCCA1 were identified as previously studied by our group[2]. Biological processes in which the differentially expressed proteins participate in are related to glycolysis/gluconeogenesis, glutathione metabolism, protein folding or pentose phosphate pathway for the soluble fraction and oxidative phosphorylation, metabolism of xenobiotics by Cyt. P450, retinol metabolism and cell junction signaling for the membrane fraction. Differentially expressed proteins are involved in cytoskeleton organization (b-actin), chronic inflammation (PLUNC, cathepsins or lysozyme C) or acting like molecular chaperones (GRP75 and HSP90AB1).
Proteins such as CBR1 and ALDH1A1 previously found by our group as associated with abnormal responses of CF mutant mice to injury [3] were also identified as desregulated in CF NB. The protein profiling of NB soluble fraction by 2D-DIGE was analyzed by dedicated software Progenesis SameSpots™ and the differentially expressed spots are currently under identification by MALDI-TOF/TOF MS.
Conclusions: Cell fractionation together with gel-based and gel-free proteomics approaches provided in-depth characterization of NB proteome that could be potentially involved in CF pathophysiology, allowing identification of candidate biomarkers for clinically manifested CF and distinguish it from other respiratory diseases.

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Detection and Discrimination of CFTR Mutants by Flow Cytometry

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Background: Cystic fibrosis is a heterogeneous disease and caused by dysfunction of CFTR, an epithelial-expressed regulator of transmembrane conductance. Over 1000 CFTR mutations are subcategorized into five classes which discriminate between severe and mild mutations. Patients carrying similar mutations show distinct disease phenotypes which may be attributed to differences in residual CFTR expression and function. We have currently designed an assay that measures CFTR protein levels by flow cytometry in nasal epithelial cells. To validate this assay and to find mutation-specific mAb staining patterns, we have generated multiple HEK293 cells stably expressing CFTR or various mutants. We analyzed the capacity of mAb L12B4 (AA 386-412) and 24.1 (1377-1480) to detect CFTR by Western blot, flow cytometry and confocal microscopy.
Methods: Mutant CFTR constructs were generated using Quick-change PCR on a wtCFTR-pcDNA template kindly provided by Dr. I. Braakman (University of Utrecht, The Netherlands). These constructs were sequence verified, transfected in HEK293, and stable lines were generated using neomycin selection. For immunofluorescent analysis, cells were fixed and permeabilized, incubated with anti-CFTR antibodies, and subsequently with fluorescently-labeled secondary antibody.
Results: We observed expression of wild type and mutant CFTR in our stable HEK293 cells. Using mAbs that recognize different epitopes within CFTR, we were able to improve CFTR staining intensity. When staining intensity of mAb L12B4 and 24.1 were compared, we could discriminate between CFTR stop mutants and other mutations such as D508F.
Conclusion: CFTR expression levels can be assessed by immunofluorescence and flow cytometry. Our data indicate that CFTR mutational status can be predicted by staining patterns of mAbs that recognize distinct epitopes of CFTR. The analyses of CFTR expression level by flow cytometry may be significant for CF diagnosis, prediction of disease progression and may predict the magnitude of pharmacotherapy that directly targets the expression of CFTR such as PTC124.

Analysis of CFTR Expression in Nasal Epithelial Cells by Flow Cytometry

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Background: The diagnosis Cystic fibrosis (CF) is based on clinical symptoms, genotype and functional tests including sweat chloride levels and nasal potential difference (NPD). CFTR mutations lead to loss-of-function gene products and have been classified into five classes. This classification discriminates between severe and mild mutations and is associated with clinical disease severity. However, CF patients with a similar CFTR mutation can display completely different phenotypes. We hypothesize that CFTR expression levels in individual patients play a role in the variation of clinical outcome. Nasal epithelial cells express CFTR protein as has been shown by semi-quantitative techniques such as confocal microscopy and western blot. These cells can simply be derived by nasal brushings, however, yields are insufficient for reliable western blot analysis and cellular purity is affected by contaminating leukocytes. Therefore, we developed a flow cytometric-based assay that allows for the quantification of CFTR in single cells using various monoclonal antibodies.
Objective: To quantify CFTR expression levels in CF patient nasal epithelial cells using flow cytometry.
Methods: With a cytological brush, nasal cells were taken from healthy volunteers and CF patients. After fixation and permeabilisation, the cells were incubated with anti- CFTR antibodies and anti-pancytokeratin antibodies to discriminate epithelial cells. Multiple CFTR antibodies were tested including mouse monoclonal L12B4 and 24.1 antibodies.
Results: Preliminary data indicate higher CFTR expression levels in healthy volunteers compared to patients. Within the patient population a diversity of expression between the different types of mutations is observed.
Conclusion: CFTR expression levels can be determined using minimal invasive methods. Technique variability should still be addressed using an internal standard. In the future, CFTR expression levels in patient derived cells might be correlated to other functional assays such as NPD, sweat chloride and disease severity. In addition, CFTR expression analysis in patient nasal cells may predict the effect of novel pharmacotherapy that directly affect CFTR expression.

Abbreviations: CF; cystic Fibrosis, CFTR; cystic fibrosis transmembrane conductance regulator.
Keywords: cystic fibrosis, CFTR expression, nasal epithelial cells