2012
European Cystic Fibrosis Society

New Frontiers in Basic Science of Cystic Fibrosis

Conference Programme & Abstract Book

Sainte Maxime, France

Chairpersons

Luis Galietta, Aleksander Edelman and John Hanrahan

28 March - 01 April 2012

CONTENTS

CONFERENCE SPONSORSHIP & SUPPORT

The ECFS thanks the following for their support

Supported by Gilead Sciences Europe Ltd who provided funding. Gilead Sciences Europe Ltd has had no input into the content of the materials and presentations used at this event.

Unconditional Grant

Support for Young Investigators from France

Support for Young Investigators from the UK

University of Nice – Logistical Support

Welcome from the ECFS President

Dear Friends and Colleagues,

It is a great pleasure to welcome you to the 9th European Cystic Fibrosis Conference entirely dedicated to Basic Science which, this year, takes place in Sainte Maxime, France. This year we are delighted to welcome Prof. Luis Galietta as the conference Chairperson and who is supported by Prof. Aleksander Edelman and Prof. John Hanrahan as co-chairpersons.

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

I extend a very warm welcome to an exciting conference.

Stuart Elborn
President
European Cystic Fibrosis Society

Conference Chairpersons' Welcome

We are very happy to welcome you to the 9th Basic Science of CF conference 2012, which will take place in the town of Sainte Maxime, France. On the French Riviera, overlooking the Gulf of St Tropez, lies the picturesque town of Sainte Maxime. With the clear waters of the Mediterranean to the south, and the shelter of the Maures Mountains to the north, this town boasts breathtaking views. The weather too is exceptional, with over 300 days of sun annually, and it is this combination that has drawn artists, writers and poets to the town for generations.

With this conference, you can expect to hear about recent unpublished results and engage in discussions of data and ideas in an informal and interactive environment. A key characteristic of the conference is the high attendance of young participants, both PhD students and post-docs, who contribute enormously to the success of this annual event with their enthusiasm and vivid discussions. Here you will have excellent opportunities to discuss your data and interact in a great atmosphere with the best of European and International experts in this field.

This year's programme includes a number of symposia with international experts covering topical aspects of basic research in cystic fibrosis together with invited talks from submitted abstracts; there are also two 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 all scientists, not just from the field of CF research, but also from a diverse range of related topics, to an exciting conference of high scientific quality.

Luis Galietta
University of Genoa
Italy

Aleksander Edelman
University of Paris Descartes
France

John Hanrahan
McGill University
Canada

2012 ECFS Conference
New Frontiers in Basic Science of Cystic Fibrosis

France, 28 March-31 March 2012

Programme

Chairpersons: Luis Galietta (Genoa, Italy), Aleksander Edelman (Paris, France), John Hanrahan (Montreal, Canada),

Wednesday, 28 March 2012 (Day 1)	
14:00-17:30	Registration, Light Meal Set-up of Posters in room Mimosa
17:30-18:00	**Official opening of the meeting by the Conference Chairpersons** – Room Provence
18:00-19:00	**Opening Keynote Lecture –** Seeking CFTR Stability - John Riordan (US)
19:00-20:00	**Welcome Cocktail**
20:00-21:30	*Dinner*

Thursday, 29 March 2012 (Day 2)	
07:30-08:45	*Breakfast*
08:45-10:30	**Symposium 1 – Exploring CFTR Structure and Function** **Chairs: J. Riordan (US) / D. Sheppard (UK)**
08:45-09:10	Full-length CFTR structure at 9 Angstrom resolution: New insights into function – Robert Ford (UK)
09:10-09:35	Coupling between catalytic cycle and pore gating in the CFTR channel – László Csanády (HU)
09:35-10:00	On the structure of the regulatory domain of the CFTR – Oscar Moran (IT)
10:00-10:15	Abstract 1: Structure-Function Analysis of Interhelical Interfaces Comprised of the Intracellular Loops of CFTR - Steven Molinski (CA)
10:15-10:30	Abstract 2: Conserved NBD-TMD interactions regulate the biosynthesis of ABC transporters - Patrick Thibodeau (US)
10:30-11:00	*Coffee break* & **Poster viewing** – La Villa Rose
11:00-12:45	**Symposium 2 – The Wild and Mutant CFTR Network** **Chairs: M. Amaral (PT) / P. Barbry (FR)**
11:00-11:25	Correction of CFTR Misfolding Via Modulation of Protein Quality Control Machines – Doug Cyr (US)
11:25-11:50	Intermediate filaments and CFTR/F508delCFTR: K8/K18/CFTR functional interaction – Aleksander Edelman (FR)
11:50-12:15	ER Stress, GRASP, and Unconventional Trafficking of CFTR - Min Goo Lee (KR)
12:15-12:30	Abstract 48: Pharmacological Rescue of Mutant CFTR Detected Using a Novel Fluorescence Platform - Raymond A Frizzell (US)
12:30-12:45	Decoding Proteostasis in Cystic Fibrosis - W. Balch (US)
12:45-14:30	*Lunch*
14:30-16:15	**Symposium 3 – Potentiators and Correctors; Drugs for a Sick Channel** **Chairs: C. Bear (CA) / W. Skach (US)**
14:30-14:55	Drug discovery : integrating epigenomics, pharmacogenomics and diagnostics for future personalized medicines for cystic fibrosis patients – Frédéric Becq (FR)
14:55-15:20	Differential effects of CFTR correctors and potentiators on the CF mutants F508del and A561E – David Sheppard (UK)
15:20-15:45	Probing the interaction of small molecule modulatory compounds with full length reconstituted CFTR protein - Christine Bear (CA)
15:45-15:55	Abstract 3: Keratin 8 - NBD1 of CFTR complex: structural studies - Anna Kupniewska (FR)
15:55-16:05	Abstract 4: CFTR corrector C18 targets the first membrane-spanning domain during synthesis - Bertrand Kleizen (NL)
16:05-16:15	Abstract 45: Proteostasis modulation as a novel therapeutic approach in cystic fibrosis -

	Nicoletta Pedemonte (IT)
16:15-16:45	*Coffee Break* & **Poster Viewing** – La Villa Rose
16:45-18:15	Room: Provence **Special Group Discussion-I – CFTR Biogenesis, Quality Control, and Drug Targets** **Moderators: A. Harris (US) / W. Skach (US)** Room: Mimosa **Special Group Discussion-II – Understanding the CF Airway Milieu and Host Defence** **Moderators: J. Stutts (US) / G. Pier (US)**
20:30-21:30	*Dinner*
21:30-23:00	Flash Poster Session Even Numbers Evening Poster Session – Posters with Even Numbers – Room: La Villa Rose

Friday, 30 March 2012 (Day 3)

07:30-08:45	*Breakfast*
08:45-10:30	**Symposium 4** – **Epithelial Ion Transport Mechanisms** **Chairs: R. Frizzell (US) / J. Stutts (US)**
08:45-09:10	Analysing the contribution of ENaC and CFTR to airway surface fluid homeostasis – Olga Zegarra-Moran (IT)
09:10-09:35	Bicarbonate-Dependent Chloride Transport Drives Fluid Secretion by the Human Airway Epithelial Cell Line Calu-3 – John Hanrahan (CA)
09:35-10:00	Why mouse submucosal gland serous acinar cells secrete so poorly in response to cAMP – Kevin Foskett (US)
10:00-10:10	Abstract 9: HCO3- permeability of ANO1/TMEM16A - Jinsei Jung (KR)
10:10-10:20	Abstract 13: 17β-Estradiol Acts Upon the C-terminal Domain of STIM1 to Inhibit Calcium Influx - John T Sheridan (US)
10:20-10:30	Abstract 19: Causative role of SLC26A9 in pathogenesis of bronchiectasis? - Isabelle Sermet-Gaudelus (FR)
10:30-11:00	*Coffee break* & **Poster viewing** – La Villa Rose
11:00-12:45	**Symposium 5 – The Airway Surface Liquid Battleground** **Chairs: K. Foskett (US) / L. Galietta (IT)**
11:00-11:25	The ASL Battleground – Robert Tarran (US)
11:25-11:50	Host Defense in the CF airways – how disease impairs innate immunity – Paul McCray (US)
11:50-12:15	Role of the HVCN1 proton channel in acid secretion by the airway epithelium – Horst Fischer (US)
12:15-12:25	Abstract 11: Rapid Effect of 17β-Estradiol on Airway Surface Liquid Hydration in Normal and Cystic Fibrosis Bronchial Epithelia.- Vinciane Saint-Criq (IE)

12:25-12:35	Abstract 12: The ENaC Inhibitory Domain of SPLUNC1 Restores ASL Height in CF Airway Epithelia - Carey A Hobbs (US)
12:35-12:45	Abstract 17: Role of CFTR and bicarbonate in CF mucus secretion and microrheology - Pierre Lesimple (CA)
12:45-14:00	Lunch
14:00-18:00	**Free Afternoon**
18:00-19:45	**Symposium 6 – Inflammation in CF: the Chicken or the Egg?** **Chairs: G. Döring (DE) / A. Edelman (FR)**
18:00-18:25	Inflammation in CF: a walk on the lipid side – Mario Ollero (FR)
18:25-18:50	TMEM16A or CFTR channel activity suppresses proinflammatory cytokines secretion of human cystic fibrosis respiratory epithelia – Gergely Lukacs (CA)
18:50-19:15	Evidence that Absence of Functioning CFTR Does Not Intrinsically Dysregulate Autonomous Epithelial Cell Inflammatory Responses – Scott Randell (US)
19:15-19:25	Abstract 23: A role of TLR9 in Pseudomonas aeruginosa-induced lung inflammation - Fatima Ben Mohamed (FR)
19:25-19:35	Abstract 28: Targeting enzymes involved in the metabolism of glucosylceramide to modulate transcription of IL-8 gene in CF bronchial epithelial cells - Maela Tebon (IT)
19:35-19:45	Abstract 29: Neutrophil elastase degrades CFTR in vitro and in vivo and disables CFTR channel function in a P.aeruginosa lung infection model - Jean-Michel Sallenave (FR)
20:00-21:30	*Dinner*
21:30-23:00	Flash Poster Session Odd Numbers Evening Poster Session – Posters with Odd Numbers – Room: La Villa Rose

Saturday, 31 March 2012 (Day 4)

07:30-08:45	Breakfast
08:45-10:30	**Symposium 7 – Models of CF disease** **Chairs: J. Hanrahan (CA) / H. De Jonge (NL)**
08:45-09:10	Use of A porcine Model of CF to Investigate Bacterial Erradication Defect – Joseph Zabner (US)
09:10-09:35	Early CFTR-Dependent Innate Immunity in the Ferret Lung – John Engelhardt (US)
09:35-10:00	CF mouse models: similarities and differences with human CF - Hugo De Jonge (NL)
10:00-10:10	A functional CFTR assay using primary cystic fibrosis intestinal organoids - Jeffrey Beekman (NL)
10:10-10:20	Abstract 36: A Novel Approach to the Development of a Murine Model of CF Associated Chronic Pulmonary Bacterial Infection - Avril Monahan (UK)
10:20-10:30	Abstract 37: KCNN4 potassium channel inactivation decrease lethality in a cystic fibrosis animal model.- Carlos A Flores (CL)

10:30-11:00	*Coffee break* & **Poster viewing** – La Villa Rose
11:00-12:00	Keynote Lecture – Novel Immunological Aspects of Cystic Fibrosis - Gerd Döring (DE)
12:00-13:30	*Lunch*
13:30-15:15	**Symposium 8 – Fighting Bacteria in CF** **Chairs: P. McCray (US) / H. Fischer (US)**
13:30-13:55	Vaccination strategies against CF pathogens – Gerald Pier (US)
13:55-14:20	Current developments in anti-biofilm strategies and assessing their efficacy with an ex vivo sputum biofilm model – Mario Vaneechoutte (BE)
14:20-14:45	Exposure of CFTRΔF508 airway epithelial cells to Pseudomonas aeruginosa biofilms, but not their non-CF counterparts, leads to neutrophil recruitment due to increased p38α MAPK activation – Simon Rousseau (CA)
14:45-15:00	Abstract 26: Lipoxin A4 Delays the Invasion of Human Bronchial Epithelial and Human Cystic Fibrosis Bronchial Epithelial Cells by the pathogen Pseudomonas aeruginosa - Gerard Higgins (IE)
15:00: 15:15	Abstract 21: Anti-Aspergillus Activity of Human Respiratory Epithelial Cells - Michel Chignard (FR)
15:15-15:30	*Break*
15:30-17:00	Room: Provence **Special group discussion-III – New Approaches for Exploring the CFTR Interactome** **Moderators: M. Amaral (PT) / P. Fanen (FR)** Room: Mimosa **Special group discussion-IV – Anti-Inflammatory and Antimicrobial Strategries** **Moderators: A. Mehta (UK) / S. Rousseau (CA)**
17:00-17:30	*Coffee Break &* Poster Viewing – *Villa Rose*
17:30-19:15	**Symposium 9 – Cellular Functions: CFTR and Beyond** **Chairs: P. Fanen (FR) / G. Lukacs (CA)**
17:30-17:55	Differential fragmentation of wild type, F508del and CK2-related CFTR mutants – Anil Mehta (UK)
17:55-18:20	microRNA-based silencing of Delta/Notch signaling promotes multiple cilia formation – Pascal Barbry (FR)
18:20-18:45	Stress Activated MAP KINASE Pathway(s) Regulates ER Quality Control Threshold – Alberto Luini (IT)
18:45-19:00	Abstract 50: F508del-CFTR ER retention, hypoxia and saturated fatty acid accumulation: a cross road to cystic fibrosis pathogenesis - Laurie-Anne Payet (FR)
19:00-19:15	Abstract 49: The F508del mutation in CFTR gene impacts bone formation – Jacky Jacquot (FR)
20:30	*Dinner / Social Event*

POSTER TITLES & AUTHORS

P.1 **Structure-Function Analysis of Interhelical Interfaces Comprised of the Intracellular Loops of CFTR**
Steven Molinski, Ling Jun Huan, Christine Bear

P.2 **Conserved NBD-TMD interactions regulate the biosynthesis of ABC transporters**
Chelsea Crum, Kara Stonebraker, Patrick H. Thibodeau

P.3 **Keratin 8 - NBD1 of CFTR complex: structural studies**
Anna Kupniewska, Sara Bitam, Grazyna Faure, Oscar Moran, Michal Dadlez, Aleksander Edelman

P.4 **CFTR corrector C18 targets the first membrane-spanning domain during synthesis**
Bertrand Kleizen, Floor Peters, Adrien Melquiond, Alexandre Bonvin, Ineke Braakman

P.5 **Hsp27 Selectively Targets F508del CFTR for Degradation via the SUMO Pathway**
Raymond A Frizzell, Xiaoyan Gong, Annette Ahner, Wael Rabeh, Gergely L Lukacs

P.6 **Study of long-range regulatory mechanisms of the CFTR gene**
Stéphanie Moisan, Claude Férec

P.7 **A Role for in Cystic Fibrosis (CF) by Direct Interaction of Nucleoside Diphosphate Kinase (NDPK) with the First Nucleotide Binding Domain (NBD1) of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Protein**
Mathieu Kerbiriou, Pascal Trouvé, Claude Férec, Anil Mehta, Louise Robson, Richmond Muimo

P.8 **Study of the chloride channel ANO1 in cystic fibrosis context**
Manon Ruffin, Solenne Marie, Céline Charlier, Harriet Corvol, Annick Clement, Olivier Tabary

P.9 **HCO_3^- permeability of ANO1/TMEM16A**
Jinsei Jung, Joo Hyun Nam, Hyun Woo Park, Min Goo Lee

P.10 **Lipoxin A_4 stimulated potassium currents increase airway epithalial repair in cystic fibrosis**
Paul J Buchanan, Valerie Urbach, Brian J Harvey

P.11 **Rapid Effect of 17β-Estradiol on Airway Surface Liquid Hydration in Normal and Cystic Fibrosis Bronchial Epithelia.**
Vinciane Saint-Criq, Sung Hoon Kim, John A Katzenellenbogen, Brian J Harvey

P.12 **The ENaC Inhibitory Domain of SPLUNC1 Restores ASL Height in CF Airway Epithelia**
Carey A Hobbs, M Jackson Stutts, Robert Tarran

P.13 **17β-Estradiol Acts Upon the C-terminal Domain of STIM1 to Inhibit Calcium Influx**
John T Sheridan, Michael J Watson, Robert Tarran

P.14 **Regulation by CFTR activity of OAG activated Ca^{2+} influx in cystic fibrosis cells**
Laura Vachel, Caroline Norez, Frédéric Becq, Clarisse Vandebrouck

P.15 **Lipoxin A_4 increases the Airway Surface Liquid layer height in Cystic Fibrosis Bronchial Epithelial cells via an apical ATP release activating a P2Y receptor pathway**
Gerard Higgins, Valia Verriere, Brian J Harvey, Paul McNally, Valerie Urbach

P.16 **Fusion Dependent Activation of Vesicular P2X4 in Alveolar Type II Cells Leads to a Cell Volume Increase-Coupling Secretion and Fluid Homeostasis in the Lung?**
Kristin Thompson, Elena Hecht, Oliver Wittekindt, Pika Miklavc, Christine Kranz, Paul Dietl, Manfred Frick

P.17 **Role of CFTR and bicarbonate in CF mucus secretion and microrheology**
Pierre Lesimple, Maria Kilfoil, John W Hanrahan

P.18 **MiR-449 MicroRNAs Control Biogenesis Of Motile Cilia In Human Airway Epithelium**
Benoit Chevalier, Laure-Emmanuelle Zaragosi, Imène Henaoui, Karine Robbe-Sermesant, Lisa Chami-Giovannini, Rainer Waldmann, Laurent Kodjabachian, Christelle Coraux, Pascal Barbry, and Brice Marcet

P.19 **Causative role of SLC26A9 in pathogenesis of bronchiectasis?**
Naziha Bakouh, Thierry Bienvenue, Jordi Ehrenfeld, Delphine Roussel, Philippe Duquesnoy, Huguette Liote, Nicolette Farman, Annick Thomas, Aleksander Edelman, Gabrielle Planelles, Isabelle Sermet-Gaudelus

P.21 **Anti-aspergillus activity of human respiratory epithelial cells**
Viviane Balloy, Jean-Paul Latgé, Michel Chignard

P.22 ***P. aeruginosa* signaling molecules abolished hypoxia-inducible factor-1 (HIF-1) signaling pathway in human cystic fibrosis airway epithelial cells.**
Claire Legendre, Jerry Reen, Marlies Mooij, Claire Adams, Fergal O'Gara

P.23 **A role of TLR9 in *Pseudomonas aeruginosa*-induced lung inflammation**
Fatima Ben Mohamed, Mathieu Medina, Yong Wu, Dominique Leduc, Sophia Maschalidi, Michel Chignard, Bénédicte Manoury, Lhousseine Touqui

P.24 **Investigating the effects of neutrophils on biofilm formation and development of Burkholderia cepacia complex (Bcc)**
Mark Murphy, Máire Callaghan, Emma Caraher

P.25 **Evaluation of an azithromycin analog (CYS0073) in lung inflammatory response**
Ambre Deveaux, Viviane Balloy, Olivier Tabary, Philippe Le Rouzic, Michel Chignard, Annick Clement, Harriet Corvol, Michael Burnet, Loïc Guillot

P.26 **Lipoxin A_4 Delays the Invasion of Human Bronchial Epithelial and Human Cystic Fibrosis Bronchial Epithelial Cells by the pathogen *Pseudomonas aeruginosa***
Gerard Higgins, Brian J Harvey, Paul McNally, Valerie Urbach

P.27 ***P.aeruginosa*-dependent kinase signaling network in bronchial epithelial cells**
Valentino Bezzerri, Anna Tamanini, Maria Cristina Dechecchi, Maela Tebon, Valentina Lovato, Giulio Cabrini

P.28 **Targeting enzymes involved in the metabolism of glucosylceramide to modulate transcription of IL-8 gene in CF bronchial epithelial cells**
Maela Tebon, Valentino Bezzerri, Valentina Lovato, Anna Tamanini, Seng Cheng, Maria Cristina Dechecchi, Giulio Cabrini

P.29 **Neutrophil elastase degrades CFTR in vitro and in vivo and disables CFTR channel function in a *P.aeruginosa* lung infection model**
Mathieu Le Gars, Delphyne Descamps, Delphine Roussel, Emilie Saussereau, Saw-See Hong, Pierre Boulanger, Marc Paulais, Laurette Malleret, Azzaq Belaaouaj, Alexander Edelman, Michel Huerre, Michel Chignard, Jean-Michel Sallenave

P.30 **CF epithelial cells lack the anti-inflammatory zinc finger domain of A20 and the adaptor protein TAX1BP1**
Catriona Kelly, Mark TS Williams, Kathryn Mitchell, Stuart J Elborn, Madeleine Ennis, Bettina C. Schock

P.32 **Synthesis and screening for anti-biofilm activity of new homoserine lactones (HSL) analogs of Pseudomonas aeruginosa.**
Aurelie Furiga Chusseau, Barbora Lajoie, Saloma El Hage, Geneviève Baziard, Christine Roques

P.33 **Altered virulence features of sequential clonal variants of *B. cenocepacia* isolated from a CF patient**
Jean S Tyrrell, Andreia Madeira, Isabel Sá Correia, Siobhán McClean, Máire Callaghan

P.34 **The *Pseudomonas aeruginosa* Density in Airways of Cystic Fibrosis Patients Treated for an Exacerbation is not Linked to Clinical Improvement**
Pieter Deschaght, Petra Schelstraete, Leen Van Simaey, Sabine Van daele, Frans De Baets, Mario Vaneechoutte

P.36 **A Novel Approach to the Development of a Murine Model of CF Associated Chronic Pulmonary Bacterial Infection**
Avril Monahan1, Paul Mc Gurnaghan2, Ronan Mc Mullan2, Stuart Elborn1, Rebecca Ingram1

P.37 **KCNN4 potassium channel inactivation decrease lethality in a cystic fibrosis animal model.**
Carlos A Flores, Texia T Riquelme, Viviana Bustos, Pablo Cid, Francisco V Sepúlveda

P.39 ***AGER* -429T/C is associated with an increased lung cystic fibrosis disease severity**
Julie Beucher, Pierre-Yves Boelle, Pierre-François Buisson, Annick Clement, Harriet Corvol, French CF Modifier Gene Study Investigators

P.40 **Genetic investigation of Cystic Fibrosis Transmembrane Regulator (CFTR) Mutations in a Cohort of Consecutive Patients Candidate for Assisted Reproductive Techniques**
Mauro Rongioletti, Fabrizio Papa, Maria Bernardetta Majolini, Cinzia Vaccarella, Ilaria Simonelli, Valentina Mazzucchi, Patrizio Pasqualetti, Giancarlo Liumbruno

P.41 **Mechanism Of The CFTR-VX-770 Interaction: Direct Binding And ATP-Independent Modulation Of Channel Activity**
Paul D.W. Eckford, Canhui Li, Elyse K. Watkins, Christine E. Bear

P.42 **The iminosugar IsoLAB corrects the defective trafficking of F508del-CFTR in cystic fibrosis cells**
Johanna Bertrand, Clément Boinot, Geoge W.J Fleet, Frédéric Becq, Caroline Norez

P.43 **Modulation of annexin A5 expression by Gonadotropin-Releasing Hormone (GnRH) in human bronchial epithelial cell lines: consequences on CFTR protein**
Nathalie Benz, Pascal Trouvé, Claude Férec

P.44 **Evaluation of F508del-CFTR correctors efficacy in CFBE41o- cells**
Valeria Tomati, Elvira Sondo, Emanuela Caci, Nicoletta Pedemonte, Luis JV Galietta

P.45 **Proteostasis modulation as a novel therapeutic approach in cystic fibrosis**
Nicoletta Pedemonte, Valeria Tomati, Elvira Sondo, Emanuela Pesce, Emanuela Caci, Luis J. V. Galietta

P.46 **Rescuing both trafficking and chloride channel function of ΔF508 CFTR**
Liudmila Cebotaru, Owen Woodward, William Guggino

P.47 **ΔF508 Correction in CF Cells using ZFN Homology-Directed Repair**
Hollywood JA, Lee CM, Flynn R, Kaschig K, Scallan MF, Harrison PT

P.48 **Pharmacological Rescue of Mutant CFTR Detected Using a Novel Fluorescence Platform**
John P. Holleran, Matthew L. Glover, Kathryn W. Peters, Carol A. Bertrand, Simon C. Watkins, John W. Jarvik, Raymond A. Frizzell

P.49 **The F508del mutation in CFTR gene impacts bone formation**
Carole Le Henaff, Annelise Gimenez, Eric Hay, Caroline Marty, Caroline Norez, Frederic Becq, Pierre Marie, Jacky Jacquot

P.50 **F508del-CFTR ER retention, hypoxia and saturated fatty acid accumulation: a cross road to cystic fibrosis pathogenesis**
Laurie-Anne Payet, Linette Agbomenou, Sébastien Giraud, Jean Marc Berjeaud, Sandra Mirval, Frédéric Becq, Clarisse Vandebrouck, Thierry Ferreira

P.51 **Establishment of an F508del pseudoislet model for the study of CF-related Diabetes**
Neville H NcClenaghan, Peter Flatt, Alan G.S. Harper, Catriona Kelly

P.52 **Cystic fibrosis airway epithelial regeneration is abnormal in absence of endogenous infection and inflammation**
Damien Adam, Jacqueline Roux, Sarah Lingée, Béatrice Nawrocki-Raby, Philippe Birembaut, Christelle Coraux

AWARD WINNERS

ECFS Young Fellows Travel Award

Pieter Deschaght, BE
Gerard Higgins, IE
Carey Hobbs, US
Jinsei Jung, KR
Pierre Lesimple, CA
Stéphanie Moisan, FR
Jean Tyrrell, IE

Student Helper Award

Benoit Chevalier, FR
Imène-Sarah Henaoui, FR
Steven Molinski, CA
Laurie-Anne Payet, FR
Vinciane Saint-Criq, IE
John Sheridan, US

Free Registration Young Researchers Supported by FFC

Valentino Bezzerri, IT
Paul Eckford, CA
Mathieu Kerbirou, FR
Maela Tebon, IT
Valeria Tomati, IT

Vaincre La Mucoviscidose Travel Award

Nathalie Benz, FR
Julie Beucher, FR
Clément Boinot, FR
Anna Kupiniewska, FR
Manon Ruffin, FR
Laura Vachel, FR

UK CF Trust Young Investigator Travel Award

Information not available at the time of printing

28 March – 18:00-19:00 **Room: Provence**

Opening Keynote Lecture

Seeking CFTR Stability

John R Riordan, Andrei A Aleksandrov, Pradeep Kota, Lihua He, Nikolay V Dokholyan

Department of Biochemistry/Biophysics and Cystic Fibrosis Center, University of North Carolina, Chapel Hill, NC 27599, USA

CFTR is a highly dynamic protein with channel gating transitions at thermal equilibrium and influenced by allosteric interactions with distant sites throughout the multi-domain protein. As such CFTR may function optimally on the edge of thermodynamic stability. The ΔF508 mutation pushes it over the edge as has long been known. This thermal instability has been quantitatively described more recently for both NBD1 and the full-length channel. The mutation compromises the folding of NBD1 and the assembly of the entire protein that is a cooperative process in which there is an interdependence between domains. Reduced temperature and some small molecule "correctors" counteract the instability, particularly in combination, but these effects are short lived at physiological temperature. Cellular maturation of ΔF508 is promoted by second site modifications of NBD1 including the Teem suppressors, the solubilizing mutations discovered during crystallization trials, Regulatory Insertion (RI) deletion, proline insertions into mobile segments of NBD1 as well as mutagenic "patching" of interfaces between domains. It has been argued recently that both NBD1 stabilization and interface patching is required for effective correction and that the need for both maneuvers may be the reason why correctors studied so far are quite limited in their efficacy. However, in fact, these compounds may act at neither of these levels because they have strong incremental effects in addition to either and even when both types of manipulations are combined. Recently we found that the ΔF508 protein with NBD1 strongly stabilized by the introduction of proline residues at specific mobile sites in NBD1, together with the I539T substitution to form ΔF508/4PT" variant, had channel activity similar to the wild-type at physiological temperature, suggesting that either the native NBD1-CL4 interface is not important for function or that it is adequately restored by NBD1 stabilization. The fact that several CF associated missense mutations on the CL4 side of the interface impair either assembly or channel function, or both, argues against the former alternative and favors the latter. Similarly, the fact that sequences on both the NBD1 and CL4 sides of the interface are identical in human (ΔF sensitive) and chicken (ΔF insensitive) CFTR suggests that the interface is likely to be formed even in the absence of F508 (F509 in the case of chicken). The near wild-type behavior of 'ΔF508/4PT" human CFTR suggests that although the interface may be slightly rearranged, the connection between the stabilized NBD1 and CL4 is adequate for the essential gating-supporting signal transduction across it. Additional evidence that the interface could be restored by NBD1 stabilization was provided by the cross linking of cysteine residues placed on either side. Thus current evidence shows that NBD1 stabilization alone can restore ΔF508 CFTR maturation, lifetime and function whereas maturation and global assembly can be promoted without extending lifetime or thermal stability.

(Supported by the NIH and CFF.)

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Thursday 29 March – 08:45-10:30 **Room: Provence**

SYMPOSIUM 1
Exploring CFTR Structure and Function
Chairs: J. Riordan (US) / D. Sheppard (UK)

S1.1 Full-length CFTR structure at 9 Angstrom resolution: New insights into function

Rosenberg, M.F[1]., O'Ryan, L.[1], Hughes, G[1]., Zhao, Z.[2], Aleksandrov, L.A.[2], Riordan, J.R.[2] & Ford, R.C [1]

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Cystic fibrosis is an inherited disease, affecting about 1 in 2500 live births. The underlying defect involves loss of transmembrane chloride flux that is associated with the lack of a membrane protein channel termed the cystic fibrosis transmembrane conductance regulator (CFTR). We have studied its structure by expressing the protein in BHK cells, purifying it in the detergent dodecyl maltoside, and then crystallising it on a thin carbon support film of a gold electron microscope grid. The resulting 2D crystals were then studied by electron crystallography using a Polara FEG 300kV transmission electron microscope. The crystallographic data was interpreted using electron tomography which revealed that the crystals were composed of two layers of CFTR molecules packed on top of each other. The envelopes of the top and bottom CFTR molecules in the Coulomb density map were compared with existing structures of inward- and outward-facing conformations of other ATP-binding cassette transporters. Both were in the outward-facing state and resembled the well-characterised Sav1866 transporter, desipite being crystallised in the absence of nucleotide. The transmembrane domains in the lower molecular layer in the 2D crystals were somewhat distorted versus the expected positions and versus the upper molecule's transmembrane regions, which may have been due to their contact with the carbon support film. Combination by real-space alignment of the structural data for the two layers gives a significant improvement in the fitting of the Sav1866 model, with a final correlation coefficient of 0.7 between the CFTR map and a map of Sav1866 simulated at the same resolution (~1/9 per Angstrom). We identified regions in the CFTR map, not accounted for by Sav1866, which were potential locations for the regulatory (R) region as well as the channel gate. The R-region, which is likely to be at least partly disordered, may occupy average positions around the cytoplasmic nucleotide-binding domains. Additional density at the centre of the CFTR pore was close to residues in the Sav1866 fitted model that, when aligned with CFTR correspond to residues that have been associated with channel gating. We also identified regions in the fitted Sav1866 model that were missing from the map, hence regions that were either disordered in CFTR or differently organised compared to Sav1866. Apart from the N- and C-terminii, this indicated that in CFTR, the cytoplasmic end of transmembrane helix 5/11 and its associated loop could be partly disordered (or alternatively located). Hence the conformational fragility of CFTR, its Achilles heel, could be hypothesised to be an important part of its channel function.

S1.2 – Coupling between catalytic cycle and pore gating in the CFTR channel

László Csanády

Semmelweis University

CFTR pore blockers are negatively charged compounds which, by binding in the intracellular vestibule of the pore, occlude Cl- ion flow through the open pore. In single-channel records blocker binding causes rapid flickery closures which, when heavily filtered, appear as a reduction in unitary current amplitude. Besides this effect on ion flow, blockers may also affect gating: if conformational changes that normally occur during closing transitions affect the structure of the blocker binding site, blocker binding will alter the rates of these transitions, altering closing rate. A CFTR channel spends most (~90%) of its bursting time in the prehydrolytic open state O1, and only briefly transits through the posthydrolytic open state O2 on its way to close. Therefore, altering the rate of step O1->O2 is predicted to have a much larger impact on mean burst duration (Tb) than affecting the rate of the actual closing step O2->C. If during the O1->O2 conformational transition the blocker binding site is perturbed in a way which is incompatible with blocker binding, then the presence of the blocker should prolong the apparent lifetime of the O1 state and thereby increase Tb and the fraction of time spent in the bursting state (Pburst). In contrast, if the conformation of the blocker binding site changes only during step O2->C, then the presence of the blocker might only affect the life time of short-lived state O2, and have little effect on gating. Similarly, blocker effects on the rate of closure of ATP-hydrolysis-deficient mutants report on conformational rearrangements in the pore vestibule during transition O1->C.

We have studied the effects on CFTR gating of two intracellular pore blockers, NPPB and MOPS. Upon rapid application of 250 uM NPPB (Kd~20 uM at -120mV) macroscopic WT CFTR current is rapidly blocked followed by a partial recovery, and rapid removal of NPPB results in a large current overshoot followed by a relaxation to pre-application levels. These results indicate that NPPB profoundly affects gating by greatly increasing Pburst. In contrast, none of these effects is apparent upon application/removal of 20 mM MOPS- (Kd~8 mM at -120mV), suggesting that MOPS has no major effect on gating. We compared macroscopic closing rate of WT CFTR, following ATP removal, in the presence or absence of blocker. NPPB slowed closure by ~4-fold, while MOPS did not affect it. Correspondingly, in steady-state single-channel experiments NPPB, but not MOPS, prolonged Tb; fitting of the distributions of burst durations in NPPB confirmed slowing of rate O1->O2. Intriguingly, neither MOPS nor NPPB slowed non-hydrolytic closure of ATP-hydrolysis deficient mutants, suggesting that binding of neither blocker prevents the O1->C transition.

Thus, the smaller sulfate head-group of MOPS binds just as well in states O1 and O2, while the O1->O2 transition is prevented by the binding of the larger nitrobenzoate NPPB headgroup. This suggests a substantial conformational rearrangement of the intracellular vestibule between O1 and O2. In contrast, during transition C<->O1, conformational rearrangements of this vestibule appear to be more subtle, since both blockers bind equally well in O1 and C.

S1.3 – On the structure of the regulatory domain of the CFTR

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CFTR is an anion channel activated upon the cAMP-dependent phosphorylation, and gated by ATP binding to the nucleotide binding domains (NBDs). The regulatory domain (RD) is an intrinsically disordered region with multiple phosphorylation sites that regulates the CFTR activity. We have studied the molecular structure of the native and phosphorylated RD using small-angle X-ray scattering (SAXS). Recombinant proteins are produced in *E. coli*, and successively purified to homogeneity (>98%) by affinity, size exclusion and ion exchange chromatography. RD was phosphorylated in the presence of the catalytic subunit of PKA. Phosphorylated and dephosphorylated RD were characterized by gel filtration chromatography, circular dichroism and fluorescence spectroscopy. We measured the scattering of the proteins in solution at the synchrotron light. Together with the standard SAXS approaches based on the analysis of overall parameters, we applied an Ensemble Optimisation Method to identify the multiple protein conformations in solution compatible with the scattering data. The gyration radius of RD is 3.25 nm, that is bigger than expected for a globular protein of the same molecular mass. Accordingly, Kratky plot of the scattering data is compatible with a flexible protein. Phosphorylation causes a significant reduction of the gyration radius, to 2.92 nm. For from each, phosphorylated and dephosphorylated RD SAXS pattern, we selected a set of conformers using a genetic algorithm from a pool containing 10^4 conformers randomly generated models covering the protein configurational space. Successively, we fitted the experimental scattering curve to find the volume fractions of each conformer of the ensemble, to minimise the discrepancy between the model and the data. After discarding all data with low probability, it resulted on two ensemble of 6 different conformers each representing the experimental scattering obtained from the two RD phosphorylation states. This is the first experiment-based model of the CFTR regulatory domain. This data will be useful to understand the molecular mechanisms of normal and pathological CFTR functioning, and the action of potential CFTR drugs.

This work was supported by the Italian Cystic Fibrosis Research Foundation grant #7/2010, with the collaboration of Delegazione FFC di Cosenza 2, Work in Progress Communication "Sapore di Sale 2010", Gruppo di Sostegno di Monterotondo (RM), Delegazione FFC di Genova, Delegazione FFC "Il Sorriso di Jenny", LIFC Comitato provinciale di Livorno.

S1.4 Structure-Function Analysis of Interhelical Interfaces Comprised of the Intracellular Loops of CFTR

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CFTR is a plasma membrane phosphoglycoprotein, belonging to the ATP-Binding Cassette (ABC) transporter superfamily. CFTR functions as an ATP- and PKA-dependent chloride channel, regulating chloride ion flux across apical membranes of polarized epithelial cells. Mutations in CFTR cause Cystic Fibrosis disease; the major disease-causing mutation, F508del, is located in the first nucleotide binding domain (NBD1). This and many other (>1800) mutations affect protein folding and channel gating, presumably by destabilizing interdomain interfaces; the NBD1:intracellular loop 4 (ICL4) interface is implicated in F508del. Current small molecule 'correctors' lead to partial rescue of the processing defect with some functional (but inefficient) expression at the cell surface, and although the mechanism of action is poorly understood, the NBD1:ICL4 interface is likely repaired to some extent. Consequently, there is a need to understand the roles of this and other interdomain interactions in the biogenesis and functional expression of CFTR.

Functionally important interdomain interfaces include: NBD1:ICL4, NBD1:2, NBD1:2:ICL1:2:4, ICL3:Regulatory-domain, and membrane spanning domain (MSD) 1:2. Identification of other interfaces along the pore axis of CFTR is desired. Upon inspection of Sav1866-based atomic homology models of CFTR, and using HOLE software, previously unexplored ICL interfaces (ICL1:2, ICL1:3, ICL2:4) were predicted to play important roles in biosynthesis and gating. Further analysis identified a cluster of hydrophobic residues comprised of six ICL amino acids (all of which are located in the helical extensions, two of which are associated with disease) that may be involved in gating. These residues include: Ile^{177} (ICL1), Val^{181} (ICL1), Leu^{259} (ICL2), Val^{260} (ICL2), Leu^{973} (ICL3) and Val^{1056} (ICL4). Individually mutating Ile^{177} and Leu^{259}, two residues which form the narrowest constriction in the putative permeation pathway, did not affect maturation (as shown by immunoblots), but the double substitution of $Ile^{177}Ala/Leu^{259}Ala$ decreased the rate of functional activation by 40%, presumably by altering the ICL1:2 interface, as measured by a cell-based iodide efflux assay using the halide-sensitive fluorophore 6-methoxy-N-(3-sulfopropyl)quinolinium. Substitution of the ICL3 residue Leu^{973} with disease-causing Pro, but not disease-causing Phe or His, affected CFTR processing, suggesting that a conserved residue is required at this position for proper assembly and maintenance of alpha-helical structure. Interestingly, disruption of the putative ICL1:3 interface by double Ala-substitution of Ile^{177} (ICL1) and Leu^{973} (ICL3) created a mutant protein that was EndoglycosidaseH-sensitive, suggesting that it is synthesized and core-, but not complex-glycosylated at steady-state; therefore this interface is important for CFTR maturation. Further characterization of these and other ICL mutants, as well as cysteine cross-linking of residues within putative ICL:ICL interfaces is currently in progress. By understanding how the ICLs transmit signals between the MSDs and NBDs via interdomain interfaces, gating mechanisms will become more apparent, and could potentially allow for the design of next generation correctors to treat Cystic Fibrosis by improving the folding/assembly of mutant CFTR. Elucidation of such molecular events would contribute not only to our understanding of CFTR activity, but also to our understanding of channels and transporters in general.

This work is supported by the Canadian Institutes of Health Research and Cystic Fibrosis Canada.

S1.5 Conserved NBD-TMD Interactions Regulate the Biosynthesis of ABC Transporters

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ATP-binding cassette transporters are multi-domain proteins that facilitate solute movement across biological membranes. ATP binding and hydrolysis within the cytoplasmic nucleotide binding domains (NBDs) provide the energy for solute movement through the transmembrane domains (TMDs). Functional interactions between the NBDs and TMDs are facilitated by the intracellular loops (ICLs) of the TMDs. Unlike the structurally well-defined NBD-NBD interface that is associated with ATP binding and hydrolysis, the NBD-ICL interface is less well characterized, in part due to the insolubility of the TMDs. In mammalian ABC transporters, each NBD contacts two ICLs. For full-length transporters, NBD1 putatively interacts with ICL1 and ICL4 while NBD2 interacts with ICL2 and ICL3. In CFTR, alteration of NBD structures or disruption of the putative NBD-TMD interface results in altered maturation or function of full-length protein. Similarly, disease-associated mutations in other human ABC transporters lie at this conserved NBD-TMD interface, consistent with the hypothesis that native domain-domain assembly is crucial for transporter biosynthesis.

To further probe the hierarchy of ABC transporter biosynthesis, domain folding and assembly events were probed using isolated domains, a novel protein chimera and full-length transporters. Characterization of domain folding and full-length protein maturation suggests that native NBD folding is crucial for the biosynthesis of multiple ABC transporter systems. Disruption of NBD folding or the NBD-ICL interface adversely impacts multiple ABC transporters. To further characterize this domain-domain interaction, a soluble chimeric protein was engineered to contain the ICL1 and ICL4 sequences in conformations that putatively mimic those found in the full-length proteins. Using this system, the ICL-NBD interface is assessed directly and sites critical to the native interface are evaluated, including disease-causing mutations found within this interface. *In vitro* studies with the purified chimeras and their cognate NBD proteins provide a means to study the ICL-NBD interface directly. From these data we show that the hierarchical folding and assembly of human ABC transporters are conserved. Specifically, both domain folding and domain-domain interactions are critical for proper biosynthesis. In addition, the development of a soluble system to evaluate ICL-NBD interactions facilitates biochemical characterization of this crucial biosynthetic and functional interface.

Thursday 29 March – 11:00-12:45

SYMPOSIUM 2
The Wild and Mutant CFTR Network
Chairs: M. Amaral (PT) / P. Barbry (FR)

S2.1 – Correction of CFTR Misfolding Via Modulation of Protein Quality Control Machines

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CFTR is a polytopic membrane protein that functions as a Cl- channel and consists of 2 membrane spanning domains (MSD), 2 cytosolic nucleotide binding domains (NBD) and a cytosolic regulatory domain. The mechanism for CFTR folding is complex and the mode by which disease related CFTR mutants are selected for premature degradation is not clear. To gain insight into these issues and develop drugs to treat Cystic Fibrosis we are identifying the steps in CFTR folding that are facilitated by cytosolic and ER localized chaperones. In addition, we identified the Hsc70/CHIP complex and the Rma1/Derlin complex as cytosolic and ER membrane associated E3 ubiquitin ligases that cooperate to select misfolded CFTR for degradation. The RMA1 E3 ubiquitin ligase detects folding defects resultant from failed interaction of NBD1 with ICl4 ,and thereby recognizes a major folding defect in CFTRΔF508. Where as, the Hsc70/CHIP E3 acts in recognition of misfolded NBDII. Chemical correctors were observed to alter the conformation of specific sub-domains of CFTR and enable it to selectively pass through either the Rma1 and CHIP quality control checkpoints. Combined attenuation of RMA1 E3 function and chemical corrector treatment permited CFTRΔF508 to function at 50% of wild-type. Models that describe the mechanism by which different chemical correctors alter the conformation of CFTRΔF508 and thereby escape it to escape ER quality control and function at the cell surface will be discussed. This work is supported by the NIH and NACFF.

S2.2 - Intermediate filaments and CFTR/F508delCFTR: K8/K18/CFTR functional interaction.

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Misfolding of cystic fibrosis transmembrane conductance regulator (CFTR) due to deletion of phenylalanine 508 (F508del-CFTR) underlies CF pathology in most patients. We have recently shown that 2 intermediate filaments, namely keratin 8 and 18 (K8, K18), form a complex with F508del-CFTR and may participate in both its retention in ER and its subsequent degradation [Colas et al. Hum Mol Gen 2012; 21(3):623, Lipecka et al. J Pharmacol Exp Ther. 2006 317(2):500]. We also observed that K8 but not K18 directly interacts with NBD1 of mouse CFTR. Functional tests for CFTR activity show that either K8 or K18 silencing leads to the delivery of functional F508del-CFTR to the plasma membrane in CF cell lines, primary epithelial lung cells from CF patients, and in F508del-CFTR nasal epithelium. We have analyzed the interaction between K8 and CFTR/F508del-CFTR and between K18 and CFTR by proximity ligation in primary human epithelial cells. Our results indicate that the number of K8-F508del-CFTR interactions is diminished in cells derived from F508del patients, whereas those between K18 CFTR and F508delCFTR are unchanged. These results suggest that abnormal trafficking of F508delCFTR may be concomitant with increased dimerization of K8-K18. In parallel experiments based on the assumption that unwanted interactions of housekeeping with F508del-NBD1 favours increased degradation of F508del-CFTR, we have developed an in-silico NBD1/F508del-NBD1 dynamic model, which unmasks 2 cavities in F508del-NBD1. Using virtual screening technique. We have identified four new potential correctors. These molecules corrected F508del-CFTR function in several cell lines, primary human nasal epithelial cells and in nasal epithelium of F508del-expressing mice. Our most recent experiments show that these correctors diminish the number of K8/F508del-CFTR interactions in F508del cells. We conclude that K8/F508del-CFTR interaction represents a target for therapy in F508del-CFTR patients.

Acknowledgments: CE grant NEUPROCF (FP6), Associations Vaincre la Mucoviscidose et ABC Protéines, French Agence Nationale de la Recherche grant 'EICOCF'

S2.3 - ER Stress, GRASP, and Unconventional Trafficking of CFTR

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Aberrant anion transport caused by genetic defects in cystic fibrosis transmembrane conductance regulator (CFTR) protein is associated with wide spectrum of respiratory, pancreatic and fertility disorders including classical type of cystic fibrosis. CFTR is a glycoprotein that undergoes complex glycosylation as it passes through the Golgi-mediated conventional exocytosis. Therefore, the fully glycosylated mature CFTR, also known as band C, is expressed at the apical membrane of epithelial tissues. Many of the disease-causing mutations in the CFTR, such as the deletion of phenylalanine at position 508 (ΔF508), result in protein misfolding, retention in the endoplasmic reticulum (ER), and finally degradation by the ER-associated degradation pathway. As a result, negligible amounts of ΔF508-CFTR reach the plasma membrane, and ΔF508-CFTR remains in the ER core-glycosylated immature form, also known as band B, in cells. However, we found that the core-glycosylated CFTR (band B) can also reach the plasma membrane under certain conditions. For example, when ER-to-Golgi trafficking is blocked by ectopic expression of dominant negative mutants of Sar1 and Arf1, or by STX5 overexpression, the core-glycosylated wild-type and ΔF508 CFTRs can nonetheless reach the plasma membrane, which then exhibited substantial chloride channel activities. Therefore, we searched for the molecular mechanism mediating this unusual CFTR trafficking to the plasma membrane. An integrated molecular and physiological analysis indicates that mechanisms associated with ER stress induce cell surface trafficking of the ER core-glycosylated wild-type and ΔF508 CFTR via the GRASP-dependent pathway. Phosphorylation at a specific site of GRASP and the PDZ-based interaction between GRASP and CFTR are critical for this unconventional surface trafficking. Remarkably, up-regulation of GRASP in ΔF508-CFTR mice restores CFTR function and rescues mouse survival without apparent toxicity. These findings suggest that ER stress may induce unconventional protein secretion to relieve protein burden in the ER lumen and selective activation of this pathway would be a novel therapeutic strategy for diseases stemming from misfolded proteins.

S2.4 - Pharmacological Rescue of Mutant CFTR Detected Using a Novel Fluorescence Platform

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Numerous human diseases arise due to defects in protein folding and lead to degradation of those proteins in the ER. Among them is Cystic Fibrosis (CF), caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR), an epithelial anion channel. The most common mutation, F508del, disrupts CFTR folding, which blocks its trafficking to the plasma membrane.

To date, most F508del-CFTR corrector high throughput screening assays have relied on measurements of restored CFTR function. These methods require multiple wash steps and are susceptible to signal saturation. In addition, they rely on the recruitment of a functional CFTR to the cell surface. Immunofluorescence labeling methods to detect an epitope tagged version of CFTR also require multiple wash and binding steps, which adds variability and reduces throughput for drug screening. Direct detection of F508del-CFTR at the cell surface with a single labeling step would improve throughput and increase the dynamic range of corrector screening assays.

To address these issues, we tagged CFTR with a protein module that provides a unique and selective fluorescence assay of CFTR's abundance at the cell surface. The protein tag is comprised of a genetically encoded fluorogen activating protein (FAP) that has been fused to the N-terminus or inserted into the 4th extracellular loop (EL4) of CFTR. They faithfully report the behavior of WT and F508del-CFTR, and FAP-wt CFTR retains functional activity. The presence of FAP-CFTR at cell surface can be selectively detected by a cell impermeant fluorogen, while total CFTR is detectable by a cell permeant fluorogen.

Using this approach, we determined the efficacy of corrector compounds from the CFFT panel (www.cftrfolding.org) , both alone and in combination, to rescue F508del-CFTR to the plasma membrane. Combinations of correctors produced additive or synergistic effects, improving the density of mutant CFTR at the cell surface up to 9-fold over single compound treatment. The results correlated closely with functional assays of stimulated anion transport performed in polarized human bronchial epithelia that endogenously express F508del-CFTR. These findings indicate that the FAP-tagged construct quantitatively reports mutant CFTR correction activity, and that this approach should be useful as a screening assay in diseases that impair protein trafficking to the cell surface.

S2.5 – Decoding Proteostasis in Cystic Fibrosis

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Departments of Cell Biology[1], Molecular Biology[2] and Chemical Physiology[3], The Skaggs Institute for Chemical Biology[4], The Institute for Childhood and Neglected Disease[5], The Scripps Research Institute (TSRI), La Jolla, CA 92130 and The Salk Institute, Razavi-Neuman Center for Bioinformatics[7], La Jolla, CA. Proteostasis Therapeutics Inc[7], Boston, MA.

The cell exploits the emergent properties of proteostasis, a biological program directing the protein fold, to manage the health of the human proteome. The proteostasis system includes a multitude of folding chaperones, trafficking pathways and degradation systems that respond to folding stress responsive signaling pathways of high clinical relevance to promote human health. (Science (2008) 319:916; Curr. Opin Cell Biol (2011) 23:126)). Proteome/interactome maintenance by the proteostasis network (the PN) is very dynamic- promoting/protecting proteome health both inside and outside diverse cell, tissue and organ environments in response to stress and inherited disease (Science (2010) 367:766; Nature (2011) 471:42). Physical, pathological and inherited challenges to the basic biophysics (the kinetics and energetics) of the biological folds comprising the unique cellular and subcellular proteomes of each cell/tissue/patient can compromise proteome balance and alter the proteostasis code to protect the host from folding disease. By use of proteome-oriented mass spectrometry and bioinformatic tools we are building a multi-layered view of healthy biological protein folding and the changes that occur in response to energetically compromised folding stress that alters the protein fold found in disease such as cystic fibrosis, potentially pointing towards new therapeutic approaches to CF.

Thursday 29 March – 14:30-16:15

SYMPOSIUM 3
Potentiators and Correctors; Drugs for a Sick Channel
Chairs: C. Bear (CA) / W. Skach (US)

S3.1 – Drug discovery : integrating epigenomics, pharmacogenomics and diagnostics for future personalized medicines for cystic fibrosis patients

Mathilde Jollivet, Caroline Norez & Frédéric Becq

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The promise of personalized medicine is to recommend drug treatment tailored to an individual based on its genetic makeup. The genetic disease cystic fibrosis (CF) is a good example of a human pathology that could benefit from the development of personalized medicine. The reason for this is that CF is a progressive and multiform disease with different states ranging from mild to severe respiratory failure and/or to pancreatic insufficiency. All manifestations are nevertheless caused by at least one of the numerous mutations (about 2000 have been reported worldwide) in the CF transmembrane conductance regulator (CFTR) gene encoding the CFTR protein. The CF mutations can be classified into six classes and the possibility of classification resulted in research being directed toward mutation-specific therapeutic agents. To reverse the mistrafficking of F508del-CFTR, a number of approaches have been employed including the use of proteostasis regulators; sarcoplasmic-reticulum Ca2+-ATPase inhibitors such as thapsigargin, the nitric oxide donors S-nitrosoglutathione (GSNO), the heat chock protein HSP modulator sodium 4-phenylbutyrate, the glucosidase inhibitor miglustat, the type V phosphodiesterase inhibitors sildenafil and vardenafil and HDAC inhibitors such as SAHA. However, proteostasis regulator response is likely a multi-genic trait with numerous genes playing a role in drug response and toxicity. A second strategy is to use pharmacological chaperones, i.e. small molecules directly interacting with the CFTR protein that promote it folding (corr-4a), with less risk of adverse effects if the drug-receptor interaction is specific. Mutation-specific protein repair therapy with small molecules constitutes an attractive therapeutic approach for CF. For example PTC therapeutics is developing Ataluren (PTC124) for class I CF mutations, Vertex Pharmaceuticals is developing VX-809 and VX-661 two F508del-CFTR correctors for class II CF mutations and ivacaftor (VX-770, Kalydeco), a CFTR potentiator targeting the class III CF mutation G551D-CFTR. Among proteostasis regulators targeting the mistrafficking of F508del-CFTR, the HDAC7 inhibitor SAHA is a valuable candidate (hutt et al., 2010). We will show data comparing the pharmacological effects of several correctors, including SAHA, VX809, corr-4a, vardenafil and iminosugars on the functional rescue of F508del-CFTR in epithelial cells. We will discuss the hypothesis that the development of new class II therapeutic CFTR correctors could probably depend on our ability to unravel the complexities of the epigenome in non-CF and CF states, and to integrate pharmacogenomic informations. Therefore, we believe that combination of epigenomic, pharmacogenomic, diagnostics and mutation-specific therapeutics will progressively revolutionized our vision of drug discovery and patient care.

FB's lab is supported by Vaincre La Mucoviscidose and Mucovie

S3.2 - **Differential effects of CFTR correctors and potentiators on the CF mutants F508del and A561E**

Jia Liu, Yiting Wang, Zhiwei Cai, David N. Sheppard and Hongyu Li.

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The most common cystic fibrosis (CF) mutation, F508del, disrupts cystic fibrosis transmembrane conductance regulator (CFTR) delivery to the plasma membrane, attenuates protein stability and impedes channel gating. In the search for therapies that target the root cause of CF, small molecules have been identified that rescue the plasma membrane expression and channel function of F508del-CFTR. However, relatively little is known about the action of these small molecules, especially CFTR correctors, on other CF mutants. To address this issue, we have studied A561E, a common CF mutation in Portugal and Spain. Like F508del-CFTR, A561E is a temperature-sensitive processing mutant located in the first nucleotide-binding domain (Mendes et al. Biochem Biophys Res Commun 2003; 331:665-71). Moreover, single-channel recording revealed that low temperature rescued A561E-CFTR Cl^- channels exhibited a severe gating defect and limited stability, running down promptly in excised inside-out membrane patches studied at 37 °C. Thus, F508del and A561E have closely comparable effects on the expression and function of CFTR.

To learn whether CFTR potentiators abrogate the channel gating defect of A561E-CFTR, we tested potentiators on low temperature rescued A561E-CFTR Cl^- channels using the iodide efflux technique. We studied the potentiators PG-01 (P2), SF-03 (P3), UC_{CF}-853 (P4), $\Delta F508_{act}$-02 (P5) and UC_{CF}-180 (P9) (all from Cystic Fibrosis Foundation Therapeutics (CFFT) Compound Collection, and used at 10 µM in the presence of forskolin, 10 µM); genistein (50 µM) was used as a reference potentiator. All potentiators tested enhanced iodide efflux from both A561E- and F508del-CFTR expressing cells, but none restored iodide efflux to wild-type CFTR levels. For A561E-CFTR cells, the rank order of potentiation was P4 ≥ P9 ≥ P2 > P5 > P3, whereas for F508del-CFTR, it was P2 ∽ P5 ≥ P4 > P3 > P9.

To investigate whether CFTR correctors traffic A561E-CFTR to the plasma membrane, we incubated A561E-CFTR expressing cells with the corrector CF-106951 (C18; 5 µM) (CFFT Compound Collection) for 24 h at 37 °C; as a control we grew cells at low temperature (27 °C) for 48-72 h. When compared with low temperature rescued F508del-CFTR, C18 correction caused a three-fold increase in open probability (P_o) by reducing the interburst interval (IBI) by 44% and prolonging mean burst duration (MBD) by 47%. However, pretreatment of A561E-CFTR expressing cells with C18 did not enhance channel activity above that observed following low temperature correction, suggesting that the drug improves the function of F508del-, but not A561E-CFTR.

In conclusion, all of the CFTR potentiators tested enhanced both F508del- and A561E-CFTR channel function, whereas the CFTR corrector, CF-106951 augmented the function of only F508del-CFTR. Our data also suggest that F508del and A561E disrupt CFTR function by different mechanisms. Future studies should test the effects of CFTR correctors on other CF mutants to establish whether these agents are mutation-specific.

We thank RJ Bridges and CFFT for small molecules and MD Amaral for BHK cells. Supported by the CF Trust, Universities UK and University of Bristol.

S3.3 – Probing the interaction of small molecule modulatory compounds with full length reconstituted CFTR protein.

Paul D.W. Eckford, Mohabir Ramjeesingh, Canhui Li, Russell D.Viirre[1] and Christine E. Bear.

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The FDA approval of Ivacaftor (VX-770) this year heralds the first intervention that directly targets the primary defect caused by a CF gene mutation: G551D-CFTR. We are developing novel research methods which utilize purified and reconstituted, full length CFTR protein to determine the mechanism of action of VX-770 and other potentiators. We are also applying these tools to determine the site on full length F508del-CFTR to which certain corrector compounds directly bind to promote a more stable protein structure.

We completed the first detailed study of the mechanism of action of VX-770 using our novel functional assays of purified and reconstituted Wt-CFTR, F508del-CFTR and G551D-CFTR. We showed that VX-770 mediates its channel activity by binding directly to the "highly-PKA phosphorylated" form of the purified proteins to mediate channel gating via a non-canonical mechanism. We are well positioned to use our reconstitution system together with novel chemical probes (currently in development) to determine where modulators such as VX-770 bind to CFTR to mediate potentiation. Identification of the VX-770 binding site will reveal the molecular basis for gating, reveal the defects inherent in mutants with gating defects (such as G551D-CFTR and F508del-CFTR) and inform future drug discovery efforts. We recently published our generation of novel chemical probes derived from VRT-532, a dual acting: corrector-potentiator compound (Alkhouri, B. et al. J.Med.Chem. 2011). We determined which structural elements of VRT-532 could be modified without impacting its activity and then generated a fluorescent derivative and a photolabel for studies of its binding sites on normal and mutant CFTR protein. These compounds represent the first chemical tools designed for probing modulator binding sites on CFTR.

The sites to which corrector compounds bind on F508del-CFTR are currently unknown and this is limiting to development of the next generation of more effective compounds. We have defined those correctors which bind and "protect" the full length mutant protein from aggregation. We are currently using a chemical-biology approach as discussed in the preceding paragraph to define the binding sites for these compounds on CFTR. Insight into the molecular basis for their corrector activity will inform the development of new, more effective corrector compounds.

These studies were supported by an Operating grant to CEB from Cystic Fibrosis Canada (CFC) and operating grants to CEB and RDV from The Canadian Institutes of Health Research. P.D.W.E. is a recipient of the CFC and the CIHR Postdoctoral Award.

S3.4 - Keratin 8 - NBD1 of CFTR Complex: Structural Studies

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Misfolding of cystic fibrosis transmembrane conductance regulator (CFTR) with a deletion of phenyloalanine 508 underlies pathology in most CF patients (F508delCFTR). The deletion of F508 residue results in ubiquitin - dependent degradation of CFTRF508del by proteasome. Mutated CFTR (F508delCFTR) may be addressed to the plasma membrane and functions as CFTR-like Cl^- channel. We have recently shown that 2 intermediary filaments, keratin 8 and 18 (K8, K18), form a complex with F508delCFTR and participate in its retention in ER, and subsequent degradation. We observed that K8 directly interacts with NBD1 of CFTR and that disruption of these interaction leads to the delivery of functional F508delCFTR to the plasma membrane in CF cell lines, primary epithelial lung cells from CF patients, and in nasal epithelium of F508 mouse [Colas et al. Hum Mol Gen 2012; 21: 623]. The site of interaction between K8 and F508delCFTR may be considered as a potential targets for a new CF pharmacotherapy. However, to rational design new efficient inhibitors of this interaction it is necessary to resolve the structure at the molecular level of K8 complex with at least domain bearing CF causative deletion NBD1 in WT and F508del version. The aim of this work is to investigate the K8 structure in order to precisely determined the oligomerization state of K8 and identify which species interacts with NBD1, and determine structural properties of K8. Following experimental approaches were used: small angle X - ray scattering (SAXS), size exclusion chromatography (SEC) and hydrogen - deuterium exchange (HDex) coupled with mass spectrometry (MS).

Recombinant K8 was purified according to Herrmann H. et al (J Struct Biol 2002; 137: 82). Using SAXS and SEC we have found that even in low ionic strength full K8 forms still soluble but higher - order species probably so called unit - length filaments. Using HDex - MS, on K8 separated. We noticed that HDex pattern in K8 is in agreement with K8 division for characteristic regions (head, coils, linkers and tail) according to the protein data bank. We have revealed that the beginning of the head domain is well structured because it is characterized by slow HDex rate. We can conclude that this region is crucial for intermolecular interactions what is in agreement with the literature (Herrmann H. et al 2002). We have also noticed that regions of K8, which correspond to a - coils are more protected from HDex. In contrast flexible linkers are totally exposed for HDex. So despite of formation high - ordered species by K8 its structure is correctly folded.

Based of these data, we will try to discriminate interaction surface between K8 and NBD1 using HDex-MS and SAXS. In the future this finding will facilitate the designing a new drugs based on inhibiting this pathological interaction.

S3.5 - CFTR Corrector C18 Targets the First Membrane-Spanning Domain During Synthesis

Bertrand Kleizen[1], Floor Peters[1], Adrien Melquiond[2], Alexandre Bonvin[2], Ineke Braakman[1]

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Large efforts are in progress to find and design drugs that cure Cystic Fibrosis and correct F508del CFTR folding and its transport to the cell surface.
The multidomain protein CFTR consists of two membrane-spanning domains (MSDs), two nucleotide-binding domains (NBDs), and a regulatory region (R-region). Its domains fold cotranslationally, which is followed by posttranslational domain assembly. We previously found that the primary folding defect already emerges during translation of F508del NBD1, and can be restored by suppressor mutation I539T in the same subdomain as F508. From the defect in NBD1 the misfolding trickles through the entire protein, affecting various domain assembly steps. A drug that cures Cystic Fibrosis should correct primary as well as secondary folding defects.
We use both in vivo and in vitro radiolabeling approaches to determine when (during biosynthesis) and where (in the protein) corrector compounds act in repairing the F508del CFTR folding defect. We found that the CFF C18 corrector compound has a strong synergistic rescue effect on F508del CFTR with the I539T and G550E suppressor mutants. Adding the corrector compound only during pulse-labeling (synthesis) virtually maximized the rescue effect implying that C18 repaired F508del CFTR cotranslationally. Using C-terminally truncated molecules in combination with limited proteolysis we demonstrated that C18 affected MSD1 structure, irrespective of wild-type or F508del background. We currently employ in silico drug-to-protein docking studies to identify the precise MSD1 target location of the C18 corrector drug.

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S3.6 - Proteostasis Modulation as a Novel Therapeutic Approach in Cystic Fibrosis

Nicoletta Pedemonte[1], Valeria Tomati[1], Elvira Sondo[1], Emanuela Pesce[1], Emanuela Caci[1], Luis J. V. Galietta[1]

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The most frequent mutation in cystic fibrosis, F508del, causes the arrest of the CFTR protein in the ER and its degradation. Drug-like molecules called correctors are able to improve F508del-CFTR trafficking. However, correctors are characterized by a relatively low efficacy, particularly in primary airway epithelial cells from CF patients. Indeed, the activity of F508del-CFTR correctors is largely influenced by cell background. In addition, the additivity/synergy observed between correctors, indicates the existence of different mechanisms to rescue F508del-CFTR trafficking. The development of new drugs requires a better understanding of the cellular processes responsible for the fate of the mutant protein and its possible rescue.

To identify key proteins involved in the processing of F508del-CFTR, we have adopted a functional genomics approach based on the screening of a siRNA library. Since the composition of the CFTR interactome and the responsiveness to rescue maneuvers may differ from one cell type to the other, we have utilized a human cell line (CFBE41o-) that is as close as possible to the original native epithelium, as the use of primary bronchial cells for screenings is not feasible.

The established conditions for high-throughput siRNA transfection in 96-well format were as follow: CFBE41o- cells were reverse transfected using 10 nM siRNAs and lipofectamine 2000. After 48 hours, the functional assay was performed to determine the extent of F508del-CFTR activity in the plasma membrane.

The results have highlighted RMA1, AHA1 and Derlin-1 as targets whose silencing causes a significant F508del-CFTR rescue in CFBE41o- cells (85-95% increase relative to control-transfected cells). RMA1 is an ubiquitin E3 ligase that is responsible for F508del-CFTR labeling for degradation (Grove et al., 2009). Derlin-1 recognizes misfolded, non-ubiquitylated CFTR to initiate its dislocation and degradation early during CFTR biogenesis, perhaps by detecting structural instability within the first transmembrane domain (Sun et al., 2006). In addition, silencing of Ubc9 (the main SUMO E2 conjugating enzyme), CBX4 and PIAS3 (SUMO E3 ligases) was also effective (75-90% increase relative to control-transfected cells). These data suggest that sumoylation has a role in F508del-CFTR processing. However, we do not know at the moment whether sumoylation affects CFTR directly or through another protein. Interestingly, we also found that silencing of NHERF1 prevents CFTR rescue in CFBE41o- cells incubated at low temperature.

To maximize F508del-CFTR rescue, we have also tested combinations of active siRNAs and combinations of single siRNAs with small molecule correctors and low temperature. Indeed, it is expected that maneuvers having different mechanisms of action generate additive/synergistic effects when added together. Our results show that RMA-1 silencing can rescue F508del-CFTR at 37°C, but has no additive effect with respect to low temperature rescue, suggesting that low temperature acts at very early steps during CFTR biogenesis.

Our studies are now continuing with an unbiased siRNA screening of a genome-wide siRNA library to identify novel targets for F508del-CFTR rescue.

Supported by CFFT, Italian Ministry of Health, Telethon Foundation, and Italian Foundation for Cystic Fibrosis

Thursday 29 March – 16:45-18:15

SPECIAL GROUP DISCUSSION I
CFTR Biogenesis, Quality Control, and Drug Targets
Moderators: A. Harris (US) / W. Skach (US)

Discussion leaders:

Margarida Amaral
Bill Balch
Raymond Frizzell
Ann Harris
William Skach
Phil Thomas

This workshop will cover three topics relevant to the CF problem. Short talks will provide an introduction that are intended to promote active general discussion by the audience. The first topic will focus on transcriptional regulation using new methodology to map genome-wide open chromatin conformations and the resulting transcriptional networks that coordinate gene expression in airway epithelial cells. The importance of this pathway in controlling differentiated functions of airway cells will be highlighted. This will be followed by a discussion of interacting partners that control the fate of newly synthesized CFTR, highlighting both specific protein-protein interactions and the role of the global proteostatic environment. Finally, we will conclude with discussion of small molecules that correct CFTR trafficking as either a pharmacological chaperone or proteostasis modulator and ongoing microscopy-based siRNA screens of more than 6,000 independent genes to identify genes/proteins affecting the traffic/function of ENaC and CFTR.

Thursday 29 March – 16:45-18:15

SPECIAL GROUP DISCUSSION II
Understanding the CF Airway Milieu and Host Defence
Moderators: J. Stutts (US) / G. Pier (US)

Questions:

1) Is *Pseudomonas aeruginosa* still the predominant pathogen causing most of the morbidity and mortality or do we need to consider other organisms?
2) How does the CF airway milieu promote infection and compromise host defense?
   A) Is the historical/traditional succession of pathogens still relevant? If so, why do early colonizers not persist or become major individual causes of lung disease
   B) What are the leading mechanisms accounting for lung infection in CF
      I) Dehydrated ASL/poor transport (Figures and data to support)
      II) CFTR as receptor for *P. aeruginosa* (Figures and data to support)
      III) Non-specific, ineffective inflammation (Figures and data to support)
      IV) Others???
3) Why does the CF patient fail to mount sterilizing/effective immunity to CF microbial pathogens
   A) What do we know about molecular mechanisms for persistence of *S. aureus, P. aeruginosa, Burkholderia* and perhaps others or elaboration of antigens that fail to elicit protective immune response-(Figures and data to support)
   B) Why do some typical lung pathogens found in other setting of lung pathology rarely or never show up in CF: *K. pneumoniae*, *S. pneumoniae, persistent non-typable H. influenzae*.

Friday 30 March – 08:45-10:30 **Room: Provence**

SYMPOSIUM 4
Epithelial Ion Transport Mechanisms
Chairs: R. Frizzell (US) / J. Stutts (US)

S4.1 - Analysing the contribution of ENaC and CFTR to airway surface fluid homeostasis

Ambra Gianotti, Raffaella Melani, Emanuela Caci, Luis J.V. Galietta, Olga Zegarra-Moran

Laboratorio di Genetica Molecolare, Istituto Giannina Gaslini

The airway surface fluid (ASF) layer, maintained through a fine balance between secretion and absorption, is essential to preserve the epithelial hydration and the viscoelastic characteristics of mucus, permit cilia beating and an efficient mucociliary clearance. The perturbation of this system in cystic fibrosis (CF) results in chronic bacterial infection and severe lung damage. The dehydration of CF airways is probably a direct consequence of the incapacity of mutated CFTR to secrete Cl^- and water. In fact, this apically located channel represents the most important pathway for Cl^- secretion. So, the best approach to treat the ASF dehydration would be to recover the activity of mutated CFTR. In the last years, a large number of compounds able to increase the activity of some types of CFTR mutations, and are therefore called CFTR potentiators, have been identified. The efficacy of these compounds has been recently corroborated by the results of a phase 3 clinical trial with Vx770 that reports that patients who carry gating mutations showed a sustained improvement in lung function. However, potentiators are not expected to be effective for patients expressing the most common F508del-CFTR mutation and good correctors for this mutation have not been identified so far. A mutation-independent treatment to restore airway hydration of CF lungs may be the reduction of epithelial sodium channel (ENaC) activity. Decreasing Na^+ absorption will directly expand the ASF layer and will also hyperpolarize the apical membrane increasing in this way the driving force for Cl^- secretion across mutated CFTR channels that keep a residual function as well as through alternative Cl^- channels. We reported some time ago that short-interferring RNA (siRNA) against ENaC subunits produces a long term reduction of transepithelial Na^+ currents and of fluid absorption. Our goal here is two-fold. First, we aim to establish whether reducing ENaC expression leads to expansion of the ASF layer of CF and non-CF primary bronchial epithelia. Second, we aim to compare this effect with that produced by maximal CFTR activity. A siRNA approach targeting ENaC subunits was used to reduce Na^+ absorption, while to increase Cl^- secretion we utilized CFTR potentiators. To evaluate the effect of these procedures, we measured ENaC subunit expression, Na^+ and Cl^- short-circuit currents and the ASF height with confocal microscopy. Low concentration (10 nM) of siRNA sequences complementary to any of the ENaC subunit were able to reduce ENaC transcripts and Na^+ channel activity but only silencing alpha and beta ENaC subunits at the same time increased the ASF layer. The ASF expansion obtained in this way was similar to that measured after maximal CFTR stimulation on non-CF epithelia. CFTR potentiators increased airway hydration of corrected F508del epithelia. In conclusion, both the increased Cl^- secretion obtained with CFTR potentiators and the reduced Na^+ absorption resulting from ENaC silencing produced a significant increase of the ASF layer *in vitro*.

Supported by the Italian CF Foundation grant FFC#7/2009 with contributions from Work in progress Communication and Brooks Brothers Retail Brand Alliance Europe. R.M. and A.G. are Italian CFF contract holders.

S4.2 - Bicarbonate-Dependent Chloride Transport Drives Fluid Secretion by the Human Airway Epithelial Cell Line Calu-3

Jiajie Shan[1], Junwei Huang[1], Dusik Kim[1], Jie Liao[1], Renaud Robert[1], Alexandra Evagelidis[1], Seth L. Alper[2], Melissa L. Palmer[3], Scott C. Fahrenkrug[3], Scott M. O'Grady[3] & John W. Hanrahan [1,4]

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Epithelial secretion is defective in cystic fibrosis (CF) airways however the anion transport mechanisms and their roles in driving fluid secretion are not well understood. We have studied the secretion of Cl-, HCO3- and fluid by genetically matched control, CFTR knockdown (CFTR-KD), and AE2 knockdown (AE2-KD) cell lines derived from the human airway cell line Calu-3. Forskolin increased the short-circuit current (Isc) and equivalent short-circuit current calculated under open-circuit conditions (Ieq). Isc was equivalent to the HCO3- flux measured by pH-stat whereas Ieq equaled the sum of the HCO3- flux and net Cl- flux measured using 36Cl-. Ieq and HCO3- flux were both increased by bafilomycin and ZnCl2, suggesting that about one third of the HCO3- secretion was neutralized by parallel electrogenic H+ secretion. Ieq and fluid secretion depended on both Na+ and HCO3- , nevertheless the carbonic anhydrase inhibitor acetazolamide abolished forskolin-stimulated Ieq and HCO3- secretion, indicating that HCO3- transport requires carbonic acid synthesis. While the HCO3- concentration of secreted fluid increased ~20 mM during forskolin stimulation, Cl- remained the predominant anion and was therefore the primary anion driving fluid transport. Most basolateral Cl- loading was bumetanide-insensitive and mediated by basolateral AE2, as demonstrated using an AE2-KD cell line. HCO3- secretion was independent of mucosal Cl- and was increased slightly by bilateral Cl- removal, therefore it did not involve apical Cl-/HCO3- exchange. Basal secretion was blocked by CFTR inhibitors, reduced ~75% by the PKA inhibitor Rp-cAMPS, and sensitive to antagonists of membrane-bound adenylyl cyclases but not the soluble, HCO3--stimulated isoform. 2-APB, an inhibitor of store-operated Ca2+ entry, also inhibited basal secretion. The results suggest a model in which most HCO3- is recycled basolaterally by exchange for Cl-, and transepithelial Cl- flux through CFTR provides most of the osmotic force that drives fluid secretion. CFTR channels are partially stimulated by PKA under basal conditions due to cAMP which is generated locally by Ca2+-stimulated adenylyl cyclase as a result of constitutive, store-operated Ca2+ entry.

Supported by Cystic Fibrosis Canada, Ministère du Développement Économique, Innovation et Exportation (Québec) and the Canada Foundation for Innovation.

S4.3 – Why mouse submucosal gland serous acinar cells secrete so poorly in response to cAMP

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Airway submucosal exocrine glands are major sites of fluid secretion in the lung and are likely important to the pathology of cystic fibrosis (CF). Gland fluid secretion in response to intracellular cAMP ([cAMP]i)-elevating agonists is severely reduced in glands from CF patients and cftrtm1Unc-/- knock-out (cftr-/-) mice. We previously observed that agonist-evoked elevations of cAMP in porcine and human serous acinar cells causes PKA-mediated activation of CFTR, which functions as an apical membrane secretory Cl- channel. cAMP-activated CFTR-dependent fluid secretion in porcine bronchial and human nasal serous acinar cells requires cAMP-activated Ca2+ signals, which activate Ca2+-sensitive K+ channels required for counterion permeability. Elimination of these Ca2+ signals abolishes secretion. Interestingly, in mouse nasal submucosal gland serous acinar cells, neither cAMP-activated Ca2+ signals nor optically detectible cAMP-activated fluid secretion were observed. These observations agree with data demonstrating that intact murine submucosal glands exhibit a much smaller rate of cAMP-activated CFTR-dependent fluid secretion (normalized to the maximum rate of cholinergic-activated fluid secretion) compared with either human or porcine submucosal glands. We therefore hypothesized that [cAMP]i-elevating agonists are less efficient at stimulating fluid secretion from murine glands because of a lack of cAMP-activated Ca2+ signaling and subsequent failure to activate robust counterion permeability in the secretory serous acinar cells. The focus of the current study was to test this hypothesis as well as to more thoroughly examine the role of CFTR in fluid secretion by murine serous acinar cells. We utilized optical methods developed in rat salivary gland acinar cells and utilized in murine, human, and porcine airway submucosal gland serous acinar cells to track fluid secretion in freshly-isolated intact living cells with intact fluid secretion mechanisms. Fluorescence imaging Ca2+ and Cl- was combined with simultaneous differential interference contrast (DIC) imaging of cell volume. Cell volume changes in serous acinar cells track changes in cell solute content reflecting changes in the secretory state of the cells, with a shrinking or shrunken cell reflecting an actively secreting state and a swelling or swollen cell reflecting a minimally or non-secreting state. The results suggest that, while human, procine, and murine serous acinar cells share many similar mechanisms of fluid secretion, differences exist in the secretagogue-induced second messenger pathways that activate the secretion mechanisms. These data have important implications for explaining the differences observed between murine and human submucosal glands. Additionally, they also suggest that, while differences between human and mouse serous cells exist, mouse serous cells remain a useful model in which to study CFTR-dependent secretion.

S4.4 - HCO_3^- Permeability of ANO1/TMEM16A

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Bicarbonate is secreted by calcium activated chloride channel (CaCC) in salivary gland acinar cells. However, CaCC is known to be poorly permeable to HCO_3^-. Here, we report that ANO1/TMEM16A, one of the strong candidates for CaCC, is highly permeable to HCO_3^- when intracellular calcium level is high. To elucidate the possibility of ANO1 as a HCO_3^- channel, anion currents were measured in HEK 293T cells transfected with human ANO1 (ac isoform) using the whole-cell patch clamp techniques. Relative permeabilities of not only HCO_3^- but also other halide anions were altered by intracellular calcium level in whole cell patch clamp. ATP depletion in pipette solution, the treatment of broad spectrum kinase inhibitor (K292a) in bath solution, and cotransfection with WNK1/SPAK kinase or siWNK1/siSPAK did not affect the relative permeability change induced by calcium alteration, which suggest that high calcium effect on anion selectivity is not caused by phosphorylation signaling. The high $[Ca^{2+}]_i$-induced increase in HCO_3^- permeability was reproduced in mouse submandibular gland acinar cells where ANO1 is natively expressed. In summary, halide anion selectivity in ANO1 can be regulated by the intracellular calcium concentration.

S4.5 - 17β-Estradiol Acts Upon the C-terminal Domain of STIM1 to Inhibit Calcium Influx

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Cystic Fibrosis is a genetic disease that affects multiple organ systems, including the airways, and is known to affect women more severely than men. We have previously shown that 17β estradiol (E2), acting through estrogen receptor α (ESR1), inhibits Ca^{2+} influx in airway epithelia and have proposed that this leads to a reduction in Cl^- secretion. Ca^{2+} influx is regulated by STIM1, an endoplasmic reticulum (ER) Ca^{2+}-sensing protein. Depletion of ER-Ca^{2+}, which can be induced experimentally by thapsigargin, initiates translocation of STIM1 to the ER-plasma membrane junction where it aggregates and interacts with the store-operated Ca^{2+} channel Orai1, to induce Ca^{2+} influx. To better understand this process, we tested the hypothesis that E2/ESR1 prevents Ca^{2+} influx by inhibiting STIM1 function. Immunofluorescence studies were performed on polarized airway epithelial cells to determine STIM1 localization. Diffuse STIM1 staining was visible under basal conditions and upon ER-Ca^{2+} depletion STIM1 moved closer to and condensed at the apical membrane. Pretreatment with E2 alone did not affect STIM1 localization. However, after ER-Ca^{2+} depletion, STIM1 failed to move closer to the apical surface, suggesting STIM1 motility was inhibited by E2. Next, we transfected HEK293 cells with YFP-STIM1 and ESR1-CFP to better understand E2's inhibitory actions. We found that STIM1 puncta formation was inhibited by 50% when pretreated with E2 for 20 min. Additionally, E2 pretreatment reduced STIM1-STIM1 FRET to basal levels after ER-Ca^{2+} depletion. Together, these data suggest that E2 inhibits normal aggregation of STIM1. Fluorescent recovery after photobleaching (FRAP) studies revealed that E2 pre-treatment significantly decreased STIM1 motility, without inducing puncta formation, and addition of thapsigargin had no further affect. Because STIM1 is associated with microtubules through the plus-end tracking protein EB1, FRAP studies were performed on EB1-GFP expressing HEK293 cells. E2 exposure had no effect on EB1 motility, suggesting that the decrease in STIM1 motility by E2 is due to a direct effect on STIM1 and is not mediated through altered microtubule dynamics. FRAP studies were also performed on a STIM1 truncation mutant (STIM1-570STOP), which removes 115 amino acids from the C-terminal region of STIM1. When STIM1-570STOP was pretreated with E2, there was no difference in motility unlike WT STIM1, suggesting that STIM1's C-terminus is required for the inhibitory effect of E2. We next probed the phosphorylation status of STIM1 ± E2 and observed no increase in phosphorylation with E2. Therefore, we chose to probe upstream signaling proteins that may be activated in response to E2. FRAP was performed on cells that were pretreated with Ly294002 (10 μM) or Wortmannin (10 nM) to inhibit PI3K or with vehicle before E2 exposure. Both Ly294002 and Wortmannin exposure abolished the E2 effect, suggesting that E2 may activate PI3K to inhibit STIM1. Together these data suggest that E2/ESR1 inhibit Ca^{2+}-activated Cl^- secretion by alternatively mediating Ca^{2+} influx. We propose that this interaction may contribute to the gender gap observed in CF patients.

S4.6 - Causative Role of SLC26A9 in Pathogenesis of Bronchiectasis?

Naziha Bakouh[1], Thierry Bienvenue[2], Jordi Ehrenfeld[3], Delphine Roussel[1], Philippe Duquesnoy[4], Huguette Liote[5], Nicolette Farman[6], Annick Thomas[3], Aleksander Edelman[1], Gabrielle Planelles[1], Isabelle Sermet-Gaudelus[1,7]

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Objectives: One of the main cause of bronchiectasis is mucociliary clearance defects, such as chanellopathies, the most frequent being Cystic Fibrosis (CF). However, nearly 30 % of the non CF bronchiectasis remain without any aetiology. Recent research has unlighted the interaction between the Cystic Fibrosis Transmembrane Regulator (CFTR) protein and some members of the conserved SLC26 (solute carrier family 26) anion transporters, with possible suppression of CFTR activation in case of mutations of SLC26T gene. The identification of the lung-specific SLC26A9 isoform has raised the question of a possible regulation of CFTR by SLC26A9 in the airways.

Methodology and Results: We extensively analysed all exons and flanking introns of the SLC26A9 gene in a cohort of 96 patients with disseminated bronchiectasis of unknown origin without two *CFTR* mutations. We identified one novel missense mutation in SLC26A9, a p.Arg575Trp (c.1725C>T in exon 16), located in the β3 loop of the STAS domain of the protein. This missense mutation was not found in an ethnically matched control and CF population. The patient carrying the Arg575 also carried a p.Phe508del mutation in *CFTR* at the heterozygous state. This patient presented an abnormally elevated sweat chloride concentration. PCR analysis revealed that SLC26A9 was expressed in sweat glands and respiratory epithelium.

To characterize the function and the expression of the mutated SLC26A9-575 protein, RNAs (5ng) coding for SLC26A9 Wild type (WT) and its 575 mutant were injected in Xenopus oocytes, co-expressing or not CFTR. A similar expression of SLC26A9WT and SLC26A9-575 was detected in the membrane extracts of the oocytes. Current/voltage analysis in control and low chloride conditions showed that the expression of SLC26A9-575 was associated with a significant reduction in membrane Cl^- conductance compared to oocytes expressing SLC26A9 WT. In oocytes co-expressing both SLC26A9 and CFTR, PKA (forskolin/ IBMX)-stimulated currents were 3 fold larger than the sum of the currents resulting from CFTR and SLC26A9 expression alone suggesting a functional potentiation of CFTR channel function by SLC26A9 WT. At variance, there was no significant difference between the PKA-stimulated currents from oocytes expressing CFTR alone and oocytes co-expressing CFTR and SLC26A9-575. This suggests that the functional potentiation of CFTR by SLC26A9 WT is abolished by the mutation 575.

Co-immunoprecipitation experiments confirmed the specific interaction between CFTR and both SLC26A9 WT and SLC26A9-575, and suggest that the inhibition of the functional potentiation of CFTR by SLC26A9-575 mutants was not related to a loss of CFTR-SLC26A9 protein interaction.

Modelling SLC26A9 STAS domain revealed a potential interaction site with other proteins located between the aa 567 and 585, and containing p575. We designed a peptide corresponding to the 568-588 hot site. Peptide expression in oocytes was associated with a significantly increased response of the PKA-stimulated Cl^- current mediated by CFTR whereas the random peptide was without effect. **Conclusion:** We present a novel mutation in the SLC26A9 gene, which abolishes the functional potentiation of CFTR by SLC26A9 WT and is associated with bronchiectasis. Mutation in SLC26A9 might be a causing factor for bronchiectasis.

Friday 30 March – 11:00-12:45

SYMPOSIUM 5
The Airway Surface Liquid Battleground
Chairs: K. Foskett (US) / L. Galietta (IT)

S5.1 - The ASL Battleground

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Mucus clearance plays a major role in innate host defense. During normal mucus clearance, inhaled pathogens become trapped in the mucus layer and are expelled before they can colonize the airways. The mucus layer is kept away from the predominantly ciliated airway epithelia by the presence of a ~7 □m periciliary liquid layer (PCL), which acts as a lubricant for the mucus. Together, these layers make up the airway surface liquid (ASL) and the rate of mucociliary clearance is strongly influenced by the hydration state of the ASL. ASL volume is largely determined by the rate of isotonic Na+ absorption through the epithelial Na+ channel (ENaC). The extracellular loops of α and γ ENaC can be proteolytically cleaved at multiple sites by furin-type convertases, serine proteases and neutrophil elastase, leading to activation of the channel and increased Na+ absorption. Using a proteomic screen, we recently identified SPLUNC1 as an ASL volume-sensing molecule which acts as a potent inhibitor of ENaC cleavage, preventing excessive Na+ absorption when ASL volume is at optimum levels. Based upon studies of well-differentiated nasal and bronchial epithelial cultures grown at the air-liquid interface, we conclude that restraint of proteolytic cleavage of ENaC is vital for limiting Na+ and ASL absorption. However, the contribution of ENaC disregulation to chronic lung disease is controversial. Our data from CF nasal and bronchial epithelial cultures show that the disregulation of ENaC, is caused in part by a failure of appropriate regulation via SPLUNC1, resulting in a collapse of the PCL height. These PCL height measurements correlate well with the measured increases in Na+ flux, short circuit current measurements, and the increased proteolytic cleavage of ENaC seen in CF airway epithelia. In CF airways, mucin secretion continues in the presence of PCL depletion, leading to an increase in the ASL mucin concentration. This in turn leads to mucus stasis and an increased incidence of airway infections. In addition, we predict that increased mucin secretion in the absence of a functional PCL, will with time, lead to a greater ASL height in CF compared to normal airways, but with significantly increased mucin concentrations, decreased water availability (e.g. dehydration) and reduced ability to be cleared.

S5.2 – Host Defense in the CF airways – how disease impairs innate immunity

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Loss of CFTR function progressively impairs host defense in the respiratory tract, resulting in chronic infection and inflammation. The sequence of events leading from loss of CFTR anion channel function to lung disease pathology is complex, likely multifactorial, and a subject of intense investigation. Thus, no single hypothesis or pathologic scheme may suffice to explain the progressive lung disease associated with CF. Here are briefly summarized some facets of the antimicrobial defenses of the airways and how they may be altered in CF and contribute to lung disease.

The surface and submucosal gland epithelia elaborate a multilayered, constitutive and inducible array of antimicrobials. This antimicrobial shield includes a number of peptide and protein components. In addition to these polypeptides, secreted lipids including cholesteryl esters contribute directly to broad spectrum antimicrobial activity. A lactoperoxidase based oxidative system also contributes to host defense through the production of the potent antimicrobial hypothiocyanite. In concert, this polyfunctional system is remarkably effective, yet in CF this arm of innate mucosal immunity breaks down.

CFTR is an anion channel. In addition to playing an important role in regulating the airway surface liquid (ASL) volume and composition through Cl- transport, CFTR also conducts other anions including HCO3-, SCN-, and glutathione. Therefore impaired CFTR channel activity can have several effects on the ASL environment. Impaired bicarbonate secretion may alter ASL pH and characteristics of secreted mucins. The combined effect of subtle changes in ASL composition and volume create an unfavorable environment for antimicrobials. Furthermore, changes in mucin composition and viscosity may further hamper their effects by altering mucin charge properties and by reducing mucociliary clearance.

CFTR-dependent secretion by surface and submucosal gland epithelia is defective in CF and therefore the net abundance of antimicrobials may be reduced. Early evidence in a porcine model indicates that tracheal submucosal gland tissue mass is reduced in CF, a finding that if confirmed in humans, may provide an additional anatomical contribution to the host defense defect early in life.

In addition to the many direct links between loss of CFTR function and antimicrobial defenses, as the disease progresses and inflammation and infection persist additional secondary effects of disease further compromise airway innate immunity. A number or proteases including neutrophil elastase, cathepsins S and G, and bacterial products, can directly cleave host defense molecules and further reduce or destroy their activities. Chronic infection and inflammation also trigger extensive remodeling of the epithelium, changing the proportions of cell types present, causing goblet cell metaplasia, and further changing the secretory profile of the cells.

Recently, new models of CF were developed using gene targeting in pigs and ferrets. These new models reproduce several features of CF disease. These models present new opportunities for investigating the links between loss of CFTR function and the onset of lung disease.

S5.3 - Role of the HVCN1 proton channel in acid secretion by the airway epithelium

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The airway epithelium secretes considerable amounts of both acid and base into the airway surface liquid (ASL) to adjust the pH of the ASL. In disease conditions, the ASL pH varies widely and most studies found an acidic ASL pH in CF. We investigate a model of regulation of the ASL pH where bicarbonate secretion by CFTR alkalinizes and acid secretion by the HVCN1 proton channel re-acidifies ASL pH. In the current study we investigated the identity and the pH-dependent function of the apical proton channel in the airway epithelium. In pH stat recordings of confluent JME CF airway epithelia in Ussing chambers, Zn-sensitive acid secretion was activated at a mucosal threshold pH~7, above which it increased pH-dependently at a rate of 339 ± 34 nmole/h/cm2/pH unit. Similarly, proton currents measured in JME CF cells in patch clamp recordings were readily blocked by Zn and were activated by an alkaline outside pH. siRNA-mediated knock down of HVCN1 mRNA expression in JME CF cells resulted in a loss of proton currents in patch clamp recordings. To identify the proton channel in airways, we cloned the open reading frame of HVCN1 from primary human airway epithelia cultured from two sources. We found a wild type clone and a clone characterized by two sequential base exchanges (452T>C and 453G>A) resulting in the identification of a novel missense mutation M91T HVCN1. The activation of acid secretion in epithelia that natively expressed M91T HVCN1 required ~0.5 pH units more alkaline mucosal pH values compared to wild type epithelia. Similarly, activation of H+ currents across recombinantly expressed M91T HVCN1 required significantly larger pH gradients compared to wild type HVCN1. This study provides both functional and molecular indications that the HVCN1 H+ channel mediates pH-regulated acid secretion by the airway epithelium. These data indicate that apical HVCN1 represents a mechanism to re-acidify an alkaline airway surface liquid. Supported by NIH R21 HL089196 and CFF FISCHER10G0.

S5.4 - Rapid Effect of 17β-Estradiol on Airway Surface Liquid Hydration in Normal and Cystic Fibrosis Bronchial Epithelia

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Background: Male cystic fibrosis (CF) patients survive 9 years longer than females ("CF gender gap") and lung exacerbations in CF females vary during the estrous cycle. Estrogen has been reported to reduce the height of the airway surface liquid (ASL) in female CF bronchial epithelium. Here we investigated the effect of estrogen on ASL height and ion transport in normal (NuLi-1) and CF (CuFi-1) bronchial epithelium monolayers grown on permeable filters in an air-liquid interface.
Methods: Confocal fluorescence microscopy experiments were performed to measure ASL height in normal and CF bronchial epithelial cells treated with 17β-estradiol (E2 0.1 to 10nM). In order to determine the ion channel(s) involved in the effect of E2 on ASL height, different ion transporter modulators (amiloride, 10µM; bumetanide, 10µM; HMR1556, 1µM; ATP, 100µM, forskolin, 10µM) were used in ASL and Ussing chamber experiments. The nuclear-excluded Estrogen Dendrimer Conjugate and its negative control (empty dendrimer) were used in order to determine the involvement of the nuclear estrogen receptor pathway in the effect of E2 on ASL height.
Results: Confocal experiments revealed that ASL height was significantly higher in the non-CF cell line compared to the CF cells (NuLi 6.82 ± 0.33µm vs. CuFi 5.58 ± 0.14µm, n=20, $p< 0.001$). Physiological concentrations of E2 reduced the ASL height in both non-CF (25% decrease, n=5, $p< 0.05$, ANOVA) and CF (20% decrease, n=5, $p< 0.05$, ANOVA) cell lines after 30 min treatment. Treatment with the Cl^- transport inhibitor bumetanide or the KCNQ1 potassium channel blocker chromanol HMR1556 decreased ASL height significantly in both cell lines. However, E2 had no additive effect on ASL height in the presence of these ion transporter inhibitors. Moreover E2 decreased the bumetanide-sensitive Cl^- current in normal cells (E2: 6.47 ± 2.08 µA/cm^2, Control: 9.52 ± 2.08 µA/cm^2, n=3, $p< 0.05$, paired t-test) and produced an increase in amiloride sensitive current in CF cells (E2: 11.097 ± 1.805 µA/cm^2, Control: 8.801 ± 1.464 µA/cm^2, n=7, $p< 0.05$, paired t-test). Treatment with the nuclear-impeded Estrogen Dendrimer Conjugate (EDC 0.1 - 1nM E2 equivalent concentration) produced a significant reduction in ASL height in CF and non-CF cells (4.72 ± 0.25µm in NuLi-1 and 4.86 ± 0.42µm in CuFi-1, n=5, $p< 0.05$ compared to control condition, ANOVA) whereas the empty dendrimer had no effect.
Conclusion: These results demonstrate that E2 dehydrates both CF and normal ASL and these rapid responses to E2 are membrane-initiated rather than via the classical nuclear estrogen receptor signal transduction pathway. The ion transporter inhibitor data indicate that E2 acts on ASL by inhibiting Cl^- secretion in non-CF cells and increasing Na^+ absorption via ENaC in CF cells.

Acknowledgements: Supported by EU FP7 Marie Curie CEMP Fellowship (to VS-C), Higher Education Authority of Ireland (PRTLI4 NBIP to BJH) and the National Institutes of Health (R37 DK015556, to JAK.).

S5.5 - The ENaC Inhibitory Domain of SPLUNC1 Restores ASL Height in CF Airway Epithelia

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The epithelial Na^+ channel (ENaC) is responsible for Na^+ absorption in many epithelia, including the kidney, colon and lung. ENaC is made up of three subunits, α, β, and γ. Previous studies have shown that hyperactive ENaC in cystic fibrosis (CF) airways, due to a lack of CFTR which negatively regulates ENaC, leads to a decrease in airway surface liquid (ASL) volume which slows or abolishes mucus transport. The activation of ENaC is dependent on its proteolytic cleavage by enzymes such as neutrophil elastase (NE). The short palate lung and nasal epithelial clone(SPLUNC1) protein, is a potent regulator of ENaC hyperactivity and therefore ASL height regulation. This inhibition is achieved through the binding of SPLUNC1 to ENaC, preventing its cleavage. In this study we have identified the active site of SPLUNC1 and characterized its interaction and regulation of ENaC. The region of SPLUNC1 responsible for ENaC inhibition was identified by measuring the amiloride sensitive ENaC current, I_{NA}, in *Xenopus* oocytes co-injected with α,β, and γ ENaC in the presence of C- terminal SPLUNC1 truncants. Deletion of up to 85% of SPLUNC1 resulted in a similar inhibition of ENaC as seen with full-length SPLUNC1, which narrowed down the ENaC inhibitory domain of SPLUNC1 to residues 22-39. We synthesized a peptide corresponding to this region, named G22-A39, and measured the I_{NA} in the presence of 10 μM G22-A39 for 1h. A ~2.5 fold decrease in the I_{NA} was observed, indicating that we had identified the ENaC inhibitory domain of SPLUNC1. We have previously determined that SPLUNC1 co-immunoprecipitates with all three ENaC subunits, when these subunits are expressed simultaneously. Using a series of peptide pull-down assays we confirmed that G22-A39 was also able to interact in a similar fashion with all three subunits. This interaction was further explored by performing pull-down assays with individually expressed subunits and we determined that G22-A39 specifically interacts with the β-ENaC subunit. Furthermore, this interaction was dependent on the glycosylation state of β-ENaC. To determine if G22-A39 was also capable of inhibiting ASL hyperabsorption in CF human bronchial epithelial cultures(HBECs), the change in ASL height in both normal lung(NL) and CF HBECs was measured after treatment with G22-A39. Over a period of 24h, a single dose of 100 μM G22-A39 maintained the ASL height of CF HBECs to 8 μm, which is comparable to that observed in NL HBECs. In the diseased CF lung, high levels of proteolytic activity are typically present so the ability of G22-A39 to prevent ASL hyperabsorption in CF HBECs in biologically relevant conditions was tested. The HBECs were exposed to both purified NE or activated neutrophil supernatant(ANS), which is derived from human neutrophils and contains high NE and cathepsin activity, and the ASL height measured. G22-A39 prevented CF ASL hyperabsorption as a single dose over a 24h period in the presence of the purified NE and ANS. The ability of G22-A39 to function in this environment makes G22-A39 a strong therapeutic candidate for restoring ASL height and function in CF patients.

S5.6 - Role of CFTR and Bicarbonate in CF Mucus Secretion and Microrheology

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Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel cause CF, which is characterized by the accumulation of viscous, sticky mucus in multiple organs. How this anion channel defect leads to altered mucus properties remains a central question in CF research, although there is growing evidence that bicarbonate may play an important role. The sites of CFTR expression in airway epithelium remain controversial, with recent immunostaining detection predominantly in surface ciliated cells, and extensive functional studies indicating expression in the glands. Mucus-secreting airway goblet cells, which derive from a common precursor, do not express sufficient CFTR to be detected by conventional immunostaining in situ. Therefore we examined mucin granules that were isolated from human airway epithelial cell lines and well-differentiated primary cells cultured at the air-liquid interface. CFTR and MUC5AC co-immunostaining was clearly observed on single granules isolated from control Calu-3 cells, whereas only MUC5AC was detectable when granules were isolated from a stable CFTR knock-down Calu-3 cell line (1). Western blots obtained after electrophoresis of apical and basolateral samples in non-denaturing agarose gels showed that MUC5AC was secreted exclusively to the apical side and had a more homogeneous size distribution when produced by CFTR-deficient Calu-3 monolayers than by control cells. The total amount of high molecular weight glycoconjugates (HMGs) in secretions aspirated from CFTR-deficient cells was greatly reduced as measured by spectrocolorimetry and this was due, at least in part, to increased adhesion of the mucin to the cells. We compared the biophysical properties of mucus collected from control vs CFTR-deficient air-liquid interface (ALI) cultures using two point microrheology. Secretions from CFTR knock-down Calu-3 cells were more viscous than those from control cells. When mucus was collected from non-CF primary epithelial cells, mucus viscosity and elasticity were both reduced by activation of CFTR with forskolin, and this effect was abolished by removing bicarbonate. The results indicate that CFTR is expressed at low levels on mucin granules from Calu-3 cells and highly differentiated primary cells, and modulates the properties of secreted mucus.

1. Palmer ML, Lee SY, Carlson D, Fahrenkrug S and O´Grady SM. Stable knockdown of CFTR establishes a role for the channel in P2Y receptor-stimulated anion secretion. J Cell Physiol 206: 759-770, 2006.

Friday 30 March – 18:00-19:45

SYMPOSIUM 6
Inflammation in CF: the Chicken or the Egg?
Chairs: G. Döring (DE) / A. Edelman (FR)

S6.1 - Inflammation in CF: a walk on the lipid side

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Inflammation is considered a central subject in CF pathophysiology. Nevertheless, it constitutes an extremely complex ensemble of events and cellular and molecular actors. In an attempt to simplify the nature of questions and answers, most groups have focused their research in a particular aspect. In our case, we have set epithelial cells, devoid of pathogen infection, by targeting either CFTR function or expression, as the center of our research, and chosen the lipid environment as a potential source of information. Different strategies have been used: to consider lipids as (i) key components of structural and functional platforms for signaling events, (ii) a source of bioactive mediators, (iii) a source of oxidative stress. In the first case, we have unveiled an association between CFTR inhibition, cholesterol and sphingolipid depletion, and production of proinflammatory mediators, such as IL-8, and the eicosanoids prostaglandin E2 and leukotriene B4 by Calu3 cells (PLoS One. 2009 Oct 22;4(10):e7116). In the second case we have found in the same cell type that CFTR inhibition leads to a decrease in the synthesis of prostaglandins D2 and J2 in basal conditions, but to an upregultation when cells are stimulated by IL-1beta via PGD-synthase activation, probably leading to a dysregulation of inflammation-resolving mechanisms (unpublished results). In the third case, our results suggest phospholipid hydroperoxide detoxification by peroxiredoxin-6 as a mechanism impaired in CF mouse lung cells and a potential source of stress and subsequent exacerbation of the inflammatory response (PLoS One. 2009 Jun 29;4(6):e6075). In addition, using a global strategy we have also explored the hypothesis that lipid signatures obtained from biological fluids may be read as a source of information on the prognosis and evolution of the disease. The results showed significant associations between plasma signatures composed of free fatty acids, phospholipids, fatty acid amides and neutral lipids, and long term evolution of FEV1 and chronic infection by Pseudomonas aeruginosa (J Lipid Res. 2011 May;52(5):1011-22). Our works suggest a significant though minoritary contribution of non-pathogen related events to the general inflammation picture of CF, and a number of mechanisms involving lipid metabolism as key factors for CF evolution. We believe that the integration of global approaches in a systems biology perspective should provide an optimal way to address a problem of such complexity.

(Our work has been supported by Vaincre la Mucoviscidose and Agence Nationale de la Recherche).

S6.2 - TMEM16A or CFTR channel activity suppresses proinflammatory cytokines secretion of human cystic fibrosis respiratory epithelia

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Cystic fibrosis (CF) is caused by the functional expression defect of the CF transmembrane conductance regulator (CFTR), a cAMP-stimulated chloride channel at the apical plasma membrane. Impaired bacterial clearance of infected respiratory epithelia with hyperactive innate immune response are hallmarks of the lung disease and the primary cause of CF morbidity and mortality, yet the existence of and mechanism accounting for the innate immune defect that occurs prior to infection remain controversial. Here we show that inducible expression of both CFTR and the recently identified calcium-activated chloride channel TMEM16A attenuated the secretion of the pro-inflammatory cytokines; IL-6, IL-8, CXCL1 and CXCL2 in two human respiratory epithelial models (CFBE41o- and NCI-H441) under air-liquid, but not liquid-liquid interface culture. Expression of wild-type but not the dysfunctional G551D CFTR indicated that the secretion of neutrophil chemoattractant IL-8 was inversely proportional with channel activity in cftrΔF508/ΔF508 immortalized and primary human bronchial epithelia. These results were confirmed using CFTR specific small molecule inhibitors and activators. Likewise, direct but not P2Y-receptor mediated activation of TMEM16A attenuated the IL-8 secretion in CFBE41o- epithelia that may serve as a therapeutic intervention to rectify the hyperinflammatory phenotype of CF respiratory epithelia. Thus augmented proinflammatory cytokine secretion caused by defective anion transport at the apical membrane contributes to the excessive and persistent lung inflammation in CF and, perhaps, in other respiratory diseases associated with documented downregulation of CFTR.

S6.3 Evidence that Absence of Functioning CFTR Does Not Intrinsically Dysregulate Autonomous Epithelial Cell Inflammatory Responses

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Despite wide consensus that severe and sustained inflammation damages the CF lung, there are ongoing controversies about its etiology. From early reports of inflammation without infection, and cell autonomous NF-κB activation due to ER stress from mis-folded CFTR, through recent sophisticated studies of CFTR-dependent differences in Toll-like Receptor 4 function in endosomes, there have been many published reports supporting the "CF intrinsic hyper-inflammation hypothesis". Important decisions about research priorities in times of funding austerity require critical analysis of this issue. In this presentation, key studies by others and our own work will be concisely reviewed, focusing on the role of epithelial cells. Our initial comparison of CF and non-CF primary human bronchial epithelial (HBE) cells grown on plastic or at an air-liquid interface (ALI) showed minimal differences in baseline or acute bacterial product challenge-stimulated NF-κB activation or pro-inflammatory cytokine production (AJRCCM, 2004 Mar 1;169(5):645-53). We note that ALI HBE cell pro-inflammatory cytokine production varies substantially between cell donors and as a function of culture differentiation, requiring adequate sample sizes and careful attention to culture status to avoid type I errors. In recent studies of CF and non-CF primary ALI HBE cell cultures subjected to both acute and repeat bacterial product stimulation, we similarly found no differences in inflammatory cytokine production between CF and non-CF cells. To understand discrepancies, we analyzed the commonly used IB3-1, C38 and S9 cell lines. After minimal expansion, karyotyping revealed aneuploidy, with 30-60% abnormal chromosomes, >10% dicentric chromosomes, and distinct differences between IB3-1 and C38. Thus, the claim of "isogenic except for CFTR status" is likely incorrect, and becomes worse after multiple passages of these genetically unstable cells. We created 3 non-CF and 3 ΔF508 homozygous CF cell lines using a strategy in which the cells remain diploid, and like primary cells, these cells do not show CFTR-dependent differences in inflammatory responses (AJP LCMP. 2009 Jan;296(1):L82-91). Well-controlled studies of CFTR knockdown in non-CF cells, not involving chemical CFTR inhibitors, and correction in CF cells, not involving chemical CFTR modulators, are in progress. Published and evolving data suggests absence of intrinsic inflammation in newborn CF pigs, which we confirmed in routine histologic sections of lung tissue. However, recent data in neonatal and adult βENaC overexpressing transgenic mice reared in a sterile gnotobiotic environment indicated smoldering, low-level neutrophilic inflammation and activated macrophages, suggesting that impaired mucus clearance and luminal mucus accumulation per se create pro-inflammatory conditions. Collectively, the data argues against intrinsic, epithelial cell autonomous CF hyper-inflammation. The most plausible scenario is that absence of functioning CFTR, in the context of integrated mucosal physiology, impairs airway mucus clearance. Impaired mucus clearance and mucus accumulation may in itself be pro-inflammatory, but importantly predisposes the CF lung to stress, increases build-up of pro-inflammatory substances, and ultimately results in bacterial infection, after which the unique, viscous, static and hypoxic CF luminal environment exhibits particularly severe and sustained inflammation.

S6.4 - A Role of TLR9 in *Pseudomonas aeruginosa*-Induced Lung Inflammation

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Background: *Pseudomonas aeruginosa* (*PA*) is present in lung of cystic fibrosis (CF) patients. TLR9 is cleaved by asparagine endopeptidase (AEP) but its role in host response to *PA* is unknown.

Aims: Determine the roles of TLR9 in host defense during acute lung infection by *P.A*.

Methods: Wild type (WT) and TLR9$^{-/-}$ mice were infected i.n. with 10^7 CFU of a laboratory strain of *PA* (PAK). 24 h post-infection, we compared: mouse survival, pro-inflammatory cytokine levels in broncho-alveolar lavages (BALs), alveolar macrophages (AMs) and PMN recruitment and *P.A* loads in lungs, cytokine secretion by a macrophage cell line (MHS) and primary AMs.

Results**:** (*i*) TLR9$^{-/-}$ showed less mortality than WT mice, (*ii*) significantly lower number of AMs, PMN and bacteria were detected in BALs of TLR9$^{-/-}$ compared to WT mice; (*iii*) decreased levels of IL-6, KC and TNFα were detected in BALs of TLR9$^{-/-}$mice; (*iv*) stimulation of MHS by either PAK DNA or CpG triggered both TLR9 cleavage and cytokine secretion; (*v*) both AEP inhibitor and concanamycin B, a vacuolar H(+)-ATPase inhibitor that increases endosomal pH, reduced CpG- and PAK DNA-induced IL-6 and TNFα secretion by MHS; (*vii*) CFTR inhibitor attenuated CpG- and PAK DNA-induced IL-6 and TNF-a secretion, suggesting that CFTR can interfere with TLR9 signaling.

Conclusions: Signalling through TLR9 plays a role in inflammation induced by *PA* acute lung infection. This may help to better understand the mechanisms involved in *PA*-induced inflammation and to development of potential drugs for the treatment of CF.

Acknowledgments: “Vaincre la Mucoviscidose” (VLM) and the “Agence Nationale de la Recherche” (ANR) supported this work.

S6.5 - Targeting Enzymes Involved in the Metabolism of Glucosylceramide to Modulate Transcription of IL-8 Gene in CF Bronchial Epithelial Cells

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Accumulation of the sphingolipid (SL) ceramide is a peculiar feature in many respiratory disorders, including CF (Yang and Uhling, 2011), suggesting that CF lung pathology may be, at least partly, corrected by interfering with ceramide formation. The challenge therefore is to therapeutically adjust the pulmonary ceramide levels to their physiological range required to successfully fight infection with *P.aeruginosa*.
The iminosugar, N-butyl deoxynojirimycin (NB-DNJ, miglustat) is an inhibitor of the synthesis of glycosphingolipids (GSLs) which produces an anti-inflammatory effect *in vitro* and *in vivo* and reduces the *P.aeruginosa* induced immunoreactive ceramide expression (Dechecchi 2011). Also the galactose analogue *N*-butyldeoxygalactonojirimycin (NB-DGJ), has an anti-inflammatory effect in bronchial epithelial cells (Dechecchi, 2008). Considering that miglustat and NB-DGJ share two common enzyme targets: ceramide glucosyl-transferase (GlcCerT) and non-lysosomal glucosylceramidase (GBA2) both involved in the metabolism of glucosylceramide (GlcCer), we extended the investigation to other two more potent inhibitors of these pathways: Genz-123346 and Genz-529468. IB3-1 cells were incubated with ranging doses of Genz 123346 or solvent, 24 hrs before infection and the expression of IL-8 mRNA was measured 4 hrs later. We found that Genz-123346 significantly reduces the expression of IL-8 mRNA induced by *P.aeruginosa* (IC50=1.8 microM), similarly to miglustat and NB-DGJ. These results were confirmed also in CuFi-1 cells. Then, CF bronchial cells were treated with Genz-529468 before infection. Dose-response experiments showed that Genz-529468 is very potent in reducing the transcription of IL-8 (IC50=3 nM). Therefore, Miglustat, NB-DGJ, Genz 123346 and Genz-529468 reduce the immune response to *P.aeruginosa* in CF bronchial epithelial cells, being Genz-529468 the most powerful. Importantly, this inhibitor seems to be very selective for GBA2, suggesting that it may produce an anti-inflammatory effect by targeting the activity of GBA2. Considering that GBA2 is located close to the cell surface, a direct or indirect role in the SL metabolism linked to ceramide-mediated signaling processes might be envisioned. Our results further strengthen the hypothesis that the pharmacological modulation of SL metabolism, that can intercept the ceramide metabolic pathway at many levels, may be an effective approach for the treatment of CF lung inflammation. In particular GBA2 may be at least one of the targets to reduce the exaggerated inflammatory response in CF lungs.

Supported by: Italian Cystic Fibrosis Research Foundation (#16/2010) with the contribution of ”Assistgroup”, “Latteria Montello SpA” and “Delegazione FFC di Imola”.

S6.6 - Neutrophil Elastase Degrades CFTR *In Vitro* and *In Vivo* and Disables CFTR Channel Function In a P.aeruginosa Lung Infection Model

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Objective of the study: Cystic fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator (CFTR). Mutant CFTR (DF508) expression leads to ionic exchange imbalance, bacterial infections and chronic lung inflammation,and loss of function. Although lung stromal and inflammatory cells-derived proteases have well described maladaptive effects on lung tissue in this pathology, the direct effect of proteases on CFTR has not yet been addressed. The aim of the study was to characterize the effect of the neutrophil-derived protease elastase (NE) on CFTR protein and function as a Cl^- channel both *in vitro* and *in vivo*.

Methods:

1)In vitro: Epithelial cells (human bronchial NCI-H292 cell-line) were infected with adenovirus (Ad)- GFP-WT-CFTR or GFP-DF508 CFTR at a moi of 150 for 48 h. Cells were then incubated with either purified NE (10-100 nM) or human neutrophil lysates. After 7 h, GFP-CFTR expression (Western Blot analysis using anti-GFP antibodies) and function (patch-clamp analysis) were measured.

2) In vivo: C57Bl/6 mice were instilled i.n with PBS or 10^9 pfu of Ad-GFP-WTCFTR or Ad-null control. 4 days later, 50 ug of NE was instilled i.n and 6 hr later, mice were sacrificed, bronchoalveolar lavage fluid (BALF) was obtained, and lungs extracts prepared for Western Blot analysis. Alternatively, NE -/- mice or C57/Bl6 littermates were sequentially infected with Ad constructs (for 4 days, see above) and *P.a* bacteria (5.10^6 cfu/mouse). 20 hr later, lungs were processed as above. Part of lungs were embedded in O.C.T. compound (Tissue-Tek), sectioned at 5 µm thickness and stained with hematoxylin and eosin (H&E).

Results:

1) *In vitro:* we show here in bronchial epithelial cells that NE degrades both WT and DF508 CFTR. This cleavage was not dependent on intra-cellular NE signalling activity, since inhibitors of the ERK, p38, PI3K, EGF-R, NF-kB pathways did not prevent NE-induced CFTR degradation. NE main cleavage site occured in the CFTR NBD1-R region. Functionally, NE completely abolished forskolin-activated CFTR currents in cells infected with Ad-GFP-WT CFTR, while, expectedly, cells infected with Ad- GFP-DF508 CFTR alone or infected with Ad-GFP-DF508 CFTR and treated with NE yielded no current.

2) *In vivo:* we show that Ad-WT CFTR (and not Ad-controls) induced high amounts of CFTR expression without inducing *per se* significant cell inflammatory influx nor NE activity. When NE was instilled i.n, it induced very significant CFTR degradation, as assessed by Western Blot analysis, yielding a degradation product similar to that observed *in vitro* (110 kDa). This resulted in loss of channel function (npd measurement *in vivo*). When mice were infected with Ad-CFTR (or Ad controls) followed by *P.a* infection, a similar CFTR degradation product was produced, and this was NE-dependent (comparison between WT and NE -/- mice).

Conclusions: We demonstrate here that NE is instrumental in degrading CFTR *in vitro* and *in vivo* and is able to disable its channel function. Our data identify NE as an inflammatory mediator implicated in the down-regulation of CFTR expression and the potential re-inforcement of the inflammatory vicious circle observed in cystic fibrosis.

Saturday 31 March – 08:45-10:30

SYMPOSIUM 7
Models of CF disease
Chairs: J. Hanrahan (CA) / H. De Jonge (NL)

S7.1 - Use of A porcine Model of CF to Investigate Bacterial Erradication Defect

Joseph Zabner

University of Iowa

Lung disease causes most of the morbidity and mortality in cystic fibrosis (CF). Understanding the pathogenesis of this disease has been hindered, however, by the lack of an animal model with characteristic features of CF. To overcome this problem, we recently generated pigs with mutated CFTR genes. We now report that, within months of birth, CF pigs spontaneously developed hallmark features of CF lung disease, including airway inflammation, remodeling, mucus accumulation, and infection. Their lungs contained multiple bacterial species, suggesting that the lungs of CF pigs have a host defense defect against a wide spectrum of bacteria. In humans, the temporal and causal relations between inflammation and infection have remained uncertain. To investigate these processes, we studied newborn pigs. Their lungs showed no inflammation but were less often sterile than controls. Moreover, after introduction of bacteria into their lungs, pigs with CF failed to eradicate bacteria as effectively as wild-type pigs. These results suggest that impaired bacterial elimination is the pathogenic event that initiates a cascade of inflammation and pathology in CF lungs. Our finding that pigs with CF have a host defense defect against bacteria within hours of birth provides an opportunity to further investigate CF pathogenesis and to test therapeutic and preventive strategies that could be deployed before secondary consequences develop.

S7.2 – Early CFTR-Dependent Innate Immunity in the Ferret Lung

Nicholas W. Keiser, Xingshen Sun, Yulong Zhang, Elizabeth Stroebele, John T. Fisher, Xiaoming Liu, Hongshu Sui, Bo Liang, Diana C.M. Lei-Butters, and John F. Engelhardt

University of Iowa
Department of Anatomy and Cell Biology
Center for Gene Therapy of Cystic Fibrosis and Other Genetic Diseases

Abstract not authorised for publication

S7.3 – CF mouse models: similarities and differences with human CF

Hugo de Jonge, Pauline Ikpa, Marcel Bijvelds, Alice Bot

Department of Gastroenterology & Hepatology, Erasmus University Medical Center, Rotterdam, The Netherlands

The creation of mouse models of CF has provided new opportunities to elucidate disease pathogenesis, correlate genotype with phenotype, and evaluate the safety and efficacy of novel CF therapies aimed to correct the basal defect. Epithelial tissues from CF mice exhibit the loss of cAMP-and cGMP-stimulated Cl- secretion characteristic of the human disease, and CF mouse models display a range of abnormal intestinal, pulmonary and liver phenotypes dependent on the CFTR genotype, modifier genes, and environmental factors (diet, pathogens) (1). The option to selectively inactivate or restore Cftr in specific tissues and cell types is a unique feature of mouse models and has revealed an important role of CFTR in non-epithelial tissues (e.g. muscle, neurones, immune cells) in CF pathophysiology including intestinal obstruction, growth retardation and lung infection (2,3). F508del-CF mouse models have also proved valuable tools for pre-clinical testing of CFTR correctors including glafenin (4), sildenafil (5) and proteasome inhibitors (6). However whereas all hCFTR-F508del correctors tested sofar including VX-809 caused a moderate to strong dose-dependent rescue of mCFTR-F508del in both cultured cells and CF mouse intestine, most hCFTR-F508del potentiators,including VX-770, with the clear exception of genistein, failed to potentiate mCFTR-F508del. This differential response to potentiators is in line with the limited sequence homology between mouse and human CFTR (78%) and the pronounced differences in gating behaviour, and demonstrates the limitations of CF mice as a surrogate for human CF patients.

Among the numerous other phenotypic differences and similarities that exist between CF mice and CF patients, we will focus on 3 key aspects:
1. Reduced cGMP signaling in epithelial tissues from CF mice and CF patients, and its possible consequences for CFTR activation, CFTR trafficking and NHE3-mediated Na absorption. Real time qPCR studies in our lab and microarray studies in the Bill Colledge lab in Cambridge have demonstrated a strong down-regulation of the endogenous guanylyl cyclase C (GC-C) activating peptides guanylin (guca2) and uroguanylin in the intestine of CF mice (both F508del and KO) and CF patients. The reduced activity of the guanylin-GCC-cGMP signaling pathway, together with the reduction of inducible nitric oxide synthase (iNOS) expression in CF murine and human airway cells (7), indicates that the lack of epithelial cGMP signaling by guanylins and nitric oxide is a major defect in CF epithelia.
2. Human-mouse differences in cGMP-CFTR signaling in the intestine and its implications for pharmacological compensation of the cGMP signaling defect in CF.
3. Human-pig-mouse differences in the severity of CF liver and gall bladder disease and its relationship to the expression and function of compensatory calcium-activated chloride channels (CaCC). A possible role of the recently discovered failure of CF mouse liver to respond to hydrophobic bile salt exposure with an inflammatory and proliferative response (7) in the pathogenesis of CF liver disease will be discussed.

(1) Wilke M et al 2011 J Cystic Fibrosis 10 Suppl 2: S152-S171
(2) Hodges CA et al 2011 Am J Physiol 301: G528-G536
(3) Keiser NW and Engelhardt JF 2011 Curr Opin Pulm Med 17: 478-483
(4) Robert R et al 2010 Mol Pharmacol 77: 922-930
(5) Lumumba B et al 2008 Am J Respir Crit Care Med 177: 506-515
(6) Wilke M et al 2012, submitted
(7) Kelley TJ and Drumm ML 1998 J Clin Invest 102: 1200-1207
(8) Bodewes F et al 2012, submitted

S7.4 – A functional CFTR assay using primary cystic fibrosis intestinal organoids

J.F. Dekkers [1,2,3] , C.L. Wiegerinck [4], N.W.M. de Jong [1,3], H.R. de Jonge [5], M.J.C. Bijvelds [5], E.E.S Nieuwenhuis [4], S. van den Brink [6], H. Clevers [6], C.K. van der Ent [1], S. Middendorp [4], J.M. Beekman [1,2,3]

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[6] Hubrecht Institute for Developmental Biology and Stem Cell Research, and University Medical Center Utrecht, The Netherlands

We have recently established conditions allowing long-term expansion of epithelial organoids from human intestine, recapitulating essential features of the in vivo tissue architecture. Here, we apply this technology to study primary intestinal organoids of patients that suffer from cystic fibrosis (CF), a lethal genetic disease caused by mutation of the CFTR gene. Forskolin induces a rapid swelling of wild type organoids, but not of organoids derived from CFTR-deficient mice or a CF patient. This phenomenon is phenocopied by CFTR-specific inhibitors. Function of the common, temperature-sensitive CFTR-F508del mutant is restored upon incubation at low temperature, as well as by the corrector compounds VRT-325, Corr-4a, VX-809 and the potentiator VX-770. This relatively simple and robust assay will facilitate diagnosis, functional studies, drug development as well as personalized medicine approaches in CF.

7.5 - A Novel Approach to the Development of a Murine Model of CF Associated Chronic Pulmonary Bacterial Infection

Avril Monahan[1], Paul Mc Gurnaghan[2], Ronan Mc Mullan[2], Stuart Elborn[1], Rebecca Ingram[1]

[1]Queen's University Belfast, Centre for Infection and Immunity, Belfast, United Kingdom
[2]Department of Medical Microbiolgoy, Royal Victoria Hospital, Belfast, United Kingdom

Research into the immunopathogensis of chronic bacterial colonisation of cystic fibrosis patients has been significantly hampered by the lack of a biologically relevant animal model of chronic infection. Whilst several models of chronic bacterial infection have been described, these are mostly reliant on the use of artificial embedding materials to prevent rapid immune clearance of the bacteria. We hypothesised that a carefully selected, rational combination of bacterial isolate, mouse strain and infection regime, will result in a physiologically and clinically relevant murine model of chronic *S. aureus* and *P. aeruginosa* infection without the use of artificial embedding materials. Previously published models have largely utilised PA01 and lab *S. aureus* strains such as the Newman strain. We have screened panels of several hundred clinical isolates of S*. aureus* and *P. aeruginosa* and 5 strains of each were selected for murine infection experiments, based on genotypic and phenotypic characteristics. The selected *S. aureus* isolates include haemolytic and non-haemolytic strains originating from CF and non-CF sputum. The *P. aeruginosa* isolates originated from CF or ICU patients and were selected on the basis of phenotypic characteristics including ability to form biofilm *in vitro*. Significant differences in the *in vivo* virulence of these strains have been shown. Equally essential is the choice of mouse strain. Published work has almost exclusively been carried out using a C57BL/6 background however these mice are inherently resistant to bacterial infection We have performed survival experiment comparing the colonisation of C57BL/6, A/J, BALB/c, Biozzi, FVB/N, NIH, SJL/J, CD1, MF1 and NMRI female mice with *P. aeruginosa* and *S. aureus*. The overall aim is to find the combination of mouse and bacterial strain which results in the prolonged survival of the animal whilst maintaining chronic pulmonary infection resulting in a murine model of chronic infection which accurately replicates the *in vivo* processes during a natural infection. Once established, this model will allow investigation of the intricate immunological and physiological disease processes involved during chronic pulmonary infection of CF patients.

S7.5 - KCNN4 Potassium Channel Inactivation Decrease Lethality in a Cystic Fibrosis Animal Model

Carlos A Flores[1], Texia T Riquelme[1], Viviana Bustos[1], Pablo Cid[1], Francisco V Sepúlveda[1]

[1]Centro de Estudios Científicos, Valdivia, Chile

Cystic fibrosis (CF) animal models are affected of severe and lethal intestinal obstructive desease. The mouse model of CF showed poor hydration of luminal content, and increased levels of inflammatory markers are found on tissue isolated from the small intestine of those animals. The KCNN4 channel is widely expressed in cells of the inmmune system and its activity is known to modulate the function of T-lymphocytes, macrophages and mast cells. Given the known role of KCNN4 in the regulation of cells of the immune system we aim to study the role of KCNN4 pharmacological and genetic inactivation on CF intestinal disease.

We generated a double mutant animal carrying the deltaF508 CFTR mutation and Kcnn4 knocked-down gene expression and observed an almost complete reverse of lethal intestinal obstruction episodes compared to the deltaF508 animals from 43% to 3% lethality at 60 days of age. The reverse of lethality was not related to gain fo intestinal function since weight rate curves of deltaF508 and double mutant animals were equal and lower than control and KCNN4 null mice. Small intestine tissue samples of deltaF508 mice have a four-fold increase of Toluidine blue stained mast cells in the vecinity of Peyer´s patches (1.46 cells/field) compared to control mice (0.35 cells/field). In samples obtained from double mutant mice we observed a reduction of mast cell number to values similar to control mice (0.4 cells/field). The increase of mast cells seems to be restricted to the small intestine since tissue samples obtained from the skin of these animals showed to have similar numbers of mast cells. Bone marrow derived mast cells were used to study migration and proliferation of cells o in vitro assays. We observed that inhibition of KCNN4 channels by the specific blocker TRAM-34 reduce migration of mast cells on the Trasnwell assay. Proliferation of cells was not affected by KCNN4 genetic deletion or CFTR deltaF508 mutation. DeltaF508 animals injected with TRAM-34 for 7 days (daily subcutaneous dose) showed a reduction of intestinal mast cells (0.2 cells/field). Finally, serum samples from deltaF508 animals showed and increase of IgE levels (determined by ELISA) compared to control mice.

In summary, genetic deletion of KCNN4 reduced the lethality of the deltaF508 mice. We found that the increased number of mast cells present in the small intestine of deltaF508 mice are reduced by the genetic or pharmacological inactivation of KCNN4. In vitro assays indicates that KCNN4 is needed for mast cell response to chemotactic agents. The increased levels of IgE detected on DeltaF508 animals might be related to the increase of mast cells. The role of mast cells on intestinal CF disease and their role on inflammation remain to be solved.

Funded by FONDECYT 11100408

Saturday 31 March – 11:00-12:00

Keynote Lecture

Novel Immunological Aspects of Cystic Fibrosis

Gerd Döring

Institute of Medical Microbiology and Hygiene, University of Tübingen, Germany

Programme note: Abstract details are not authorized for inclusion

Saturday 31 March – 13:30-15:15

Symposium 8
Fighting Bacteria in CF
Chairs: P. McCray (US) / H. Fischer (US)

S8.1 – Vaccination strategies against CF pathogens

Gerald B. Pier

Channing Laboratory, Department of Medicine, Brigham & Women's Hospital, Harvard Medical School, Boston, MA USA

While numerous bacterial pathogens are isolated from the respiratory tract of CF patients, only one, mucoid *Pseudomonas aeruginosa*, remains significantly associated with the majority of the lung function decline and ultimate early mortality. Others, such as MRSA and Burkholderia spp. can have significant effects, but in the absence of mucoid *P. aeruginosa* infection, most CF patients have a more benign clinical course. Small studies have implicated other organisms as contributing to CF lung disease, and viral infections may precipitate or exacerbate pathology from established infections, but strong epidemiologic data to support a major role for pathogens other than mucoid *P. aeruginosa*, MRSA and Burkholderia are minimal.

Nonetheless, strategies to target both *P. aeruginosa* and other pathogens via active or passive vaccination represents an approach that, if successful, could markedly improve the course of CF lung disease. Given the chronic nature of infection in CF, an associated component of disease is the inability of patients to make protective immune responses. We have identified the alginate surface antigen of *P. aeruginosa,* and the surface polysaccharide poly-N-acetyl glucosamine (PNAG) of MRSA, Burkholderia and other pathogens, as prominent antigens that mostly fail to elicit antibodies capable of killing these organisms by opsonophagocytosis and thus providing protection from infection. Notably, surface or capsular polysaccharides are the most successful anti-bacterial vaccines developed to date. For *P. aeruginosa*, both active and passive therapies against the alginate capsule that is over-produced by mucoid strains has been validated in pre-clinical models as providing high levels of immunity to infection. A small minority of older (>12 years) CF patients fortuitously make opsonic/protective antibodies to PNAG and lack chronic infection, indicating an association between these antibodies and resistance to infection. A fully human IgG1 monoclonal antibody (MAb) to alginate is being developed for clinical evaluations for prophylactic or therapeutic benefits. Published studies also validate P. aeruginosa flagella as strong vaccine candidates and conjugate vaccines that target both alginate and flagella have strong potential for efficacy in CF patients and have been tested in animal models of infection. For MRSA and Burkholderia spp. the PNAG antigen expressed by these divergent organisms can be modified into a low-acetate glycoform that elicits opsonic and protective antibodies in animals. This defines an approach to target PNAG to control infection by PNAG-producing pathogens. Interestingly, CF patients chronically infected with *S. aureus* are among the few groups documented to make opsonic antibodies that are associated with lack of progression of infection. A fully human IgG1 MAb to PNAG has been through phase I safety and pharmacokinetic evaluation in healthy humans and conjugate vaccines are being developed for human evaluations. Overall, targeting flagella, alginate and PNAG represents a vaccination/passive therapy strategy to enhance immune resistance of CF patients to the most common pathogens causing significant respiratory decline due to infection.

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S8.2 - Current developments in anti-biofilm strategies and assessing their efficacy with an ex vivo sputum biofilm model

Mario Vaneechoutte & Pieter Deschaght

Laboratory Bacteriology Research (LBR), University of Ghent, Belgium

New insights into metabolism and gene regulation of bacterial cells, growing in biofilm modus, have lead to the development of several new strategies to interfere with biofilm formation and growth. These strategies and compounds may lead to biofilm degradation or reactivation of dormant persister cells, and may therefore improve our abilities to combat chronic infections, such as cystic fibrosis (CF) airway colonisation by Pseudomonas aeruginosa. Besides some classic antibiotics, shown to have high biofilm activity (azithromycin) or even act better on dormant cells (colimycine), alternative compounds have been proposed, such as ion and metal chelators (EDTA, DFO, lactoferrin), antisense oligonucleotides, which could block expression of any gene targeted, antimicrobial peptides, D-aminoacids, acidic nitric acid, and quorum sensing blockers (furanones). Moreover, these molecules could be combined with standard antibiotics, and/or with each other, and will have to be used in combination with other therapeutics, such as mucolytics.

Because it is impossible to test all of these combinations in clinical trials, and because susceptibility testing of planktonic cells has been shown to be irrelevant in predicting the in vivo susceptibility in the CF airwyas, biofilm models are used to evaluate the efficacy of these compounds. However, also here, each model leads to different predictions regarding their efficacy. The different current biofilm models and their advantages and limitations will be reviewed and an alternative model is proposed.

Indeed, new insights into the chronic colonization mode of the cystic fibrosis airways by P. aeruginosa lead us to propose an alternative biofilm model, which might have more predictive power regarding the susceptibility of P. aeruginosa CF airway biofilms for different antibacterial compounds. It can be considered as well-established that the P. aeruginosa biofilms are not located in the respiratory part of the airways (the lungs), are not adherent to the airway epithelia, but are predominantly present in the mucus in the lumen of the conductive airways (Bjarnsholt et al. 2009. Ped Pulmonol 44:547–558). This leads us to assume that sputum, especially when collected during intensive physiotherapy, contains i) the original biofilm structure, with ii) the original P. aeruginosa strains and iii) their different phenotypes, iv) with the other species present, v) within the original environment (patient sputum with DNA and leukocytes), and even vi) with compounds of the current therapies that the patient is receiving. The advantages and limitations of this ‘ex vivo sputum biofilm model’ (EVSM) will be discussed. A major additional advantage might be that the use of individual patient sputum may lead to personalized medicine, which may be important because it can be expected that no single approach will be equally efficient for treatment of P. aeruginosa colonization of all patients.
Results of testing some compounds with the EVSM will be presented, with special attention for the quantification of P. aeruginosa killing, because analysis by culture may overlook the noncultivable, dormant part of the biofilms, which is exactly the part that is responsible for the chronicity of the infection, whereas analysis by PCR will amplify also DNA from already killed bacteria.

S8.3 - Exposure of CFTRΔF508 airway epithelial cells to Pseudomonas aeruginosa biofilms, but not their non-CF counterparts, leads to neutrophil recruitment due to increased p38α MAPK activation

Trevor Beaudoin[1] , Shantelle Lafayette[2], Lucie Roussel[1], Julie Bérubé[1], Dao Nguyen[1,2] and Simon Rousseau[1,2]

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

P. aeruginosa chronically infects cystic fibrosis airways and fuels the ongoing destructive inflammation. P. aeruginosa is the most significant pathogen in CF with up to 80% of patients eventually being chronically infected with P. aeruginosa. In essence, lungs of CF patients form a specialized ecological niche exploited by P. aeruginosa. Multiple findings suggest that the chronic P. aeruginosa infections in the CF patients are biofilm infections, with the formation of large cellular aggregates of bacteria within the airways yet the impact of the switch from planktonic to biofilms growth on host responses is poorly understood. We report that in airway epithelial cells a threshold of p38α MAPK activation is required to trigger neutrophil recruitment, which is influenced by extrinsic and intrinsic factors. Planktonic P. aeruginosa diffusible material (PsaDM) induced a stronger p38α MAPK activation compared to biofilm PsaDM, via TLR5 and TLR2. The involvement of TLR2 is linked to mutations in the mucA gene, associated with mucoid strains of P. aeruginosa. In contrast, biofilm PsaDM activates p38α MAPK in a TLR-independent fashion via the lasI/lasR quorum-sensing system, but this activation is insufficient to recruit neutrophils. However, in airway epithelial cells from cystic fibrosis, hypersensitive to injurious stimuli, biofilm PsaDM activates p38α MAPK strongly enough to recruit neutrophils, which can contribute to lung injury. Therefore, our results suggest that as the molecular pathways driving inflammation changes through the course of the disease, strategies to decrease airway inflammation may require inhibition of different targets..

S8.4 - Lipoxin A_4 Delays the Invasion of Human Bronchial Epithelial and Human Cystic Fibrosis Bronchial Epithelial Cells by the Pathogen *Pseudomonas aeruginosa*

Gerard Higgins[1,2], Brian J Harvey[2], Paul McNally[1], Valerie Urbach[1]

[1]National Children's Research Centre, Dublin, Ireland,
[2]Royal College of Surgeons in Ireland, Dublin, Ireland

Cystic fibrosis (CF) is caused by a mutation of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene resulting in decreased Cl^- secretion and hyperabsorption of Na^+ in the airway leading to dehydration of the Airway Surface Liquid (ASL) layer. The reduction in ASL height impairs mucocillary clearance and favours lung infection and inflammation. The eicosanoid lipoxin A_4 (LXA_4) described as a signal for the resolution of inflammation, is decreased in the lungs of patients with CF (*Karp et al, 2004*). Our team have also discovered that LXA_4 stimulates Cl^- secretion (*Bonnans et al, 2003*), increases ASL height in Human Bronchial Epithelial (HBE) and Human Cystic Fibrosis Bronchial Epithelial (HCFBE) cells and stimulates ZO-1 expression and transepithelial electrical resistance (TER) in Human Airway Epithelial Cells (*Urbach et al, 2008*). We hypothesize that decreased levels of LXA_4 in CF airways could favour the invasion of HBE and HCFBE cells by microorganisms. A high percentage of CF lungs are chronically infected by *P. aeruginosa* by adolescence and it is strongly implicated in lung destruction. Using a gentamicin invasion assay, we investigated the role of LXA_4 on the epithelial integrity of HBE and HCFBE when infected with *P. aeruginosa*. Our results indicate that LXA_4 (1nM) alone did not affect *P. aeruginosa* growth but prevented the invasion of HBE and HCFBE cells by *P. aeruginosa* within the first 3 hours after inoculation. We report a novel role for LXA_4 in delaying the invasion of *P. aeruginosa*, which could lead to a new therapeutic route for CF patients.

Acknowledgements: Funded by the Children´s Medical and Research Foundation in Ireland, The Health Research Board of Ireland and INSERM

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S8.5 - Anti-Aspergillus Activity of Human Respiratory Epithelial Cells

Viviane Balloy[1,2], Jean-Paul Latgé[1], Michel Chignard[1,2]

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The innate immune response to fungi mainly involves macrophages and neutrophils. By contrast, the possible participation of respiratory epithelial cells (REC) has been poorly studied. In the present study we observed that REC display the property of inhibiting mycelium development of *Aspergillus fumigatus* (*A.f)* and that this activity is linked to PI3 kinase activation.
Bronchial cells (BEAS-2B cell line) were incubated with *A.f* and the growth of the fungus was estimated by optical microscopy observation and measurement of galactomannan concentrations in the extracellular media. The role of PI3 kinase was evidenced by the use of the specific inhibitor LY294002 (30 µM). Spore internalization in REC was followed by epifluorescence. FITC-labeled spores (green) when outside of cells were further labelled with an anti-FITC antibody labeled with a red fluorochrome (Alexafluor 568).
In the presence of REC, most of spores do not germinate as opposed to spores incubated in the absence of cells. The growth quantification by galactomamann concentration measurements display a 4.5 lower concentration in the presence of cells. The anti-aspergillus activity is inhibited when cells are incubated with LY294002, indicating the involvement of the PI3 kinase pathway. Of note, inhibitors of p38 MAP kinase and ERK1/2 are inactive. We evidenced that the activity is directed against the spores and not again the hyphae but is not linked to the spore internalization.
In conclusion, as macrophages and neutrophils, REC play a role in the anti-aspergillus activity. They are able to prevent the mycelium development and as such potentially prevent its dissemination. The next steps will be the analysis of i) the nature of this activity and, ii) the expression of this activity by cystic fibrosis REC.

Funding: This work was supported in part by a grant from Vaincre la Mucoviscidose

Saturday 31 March – 15:30-17:10

SPECIAL GROUP DISCUSSION – III
New Approaches for Exploring the CFTR Interactome
Moderators: M. Amaral (PT) / P. Fanen (FR)

The objective of this SGD is to discuss novel approaches to identify CFTR-interacting proteins CIPs and CFTR/ CF networks, in general. To this end, we've decided to put the focus of the SGD mostly on approaches involving "omics" and modelling how and how we can use this information for therapeutics.

Intro

1. Genomics and epigenetics
2. RNA networks

Discussion

3. Proteomics methods (co-IPs + mass spec) to identify CIPs
4. Interactomics

Discussion

5. siRNA screens to assess the functional relevance of CIPs on a large-scale
6. Bioinformatics approaches
7. Networks to therapeutics

Discussion

Saturday 31 March – 15:30-17:00

SPECIAL GROUP DISCUSSION – IV
Anti-Inflammatory and Antimicrobial Strategies
Moderators: A. Mehta (UK) / S. Rousseau (CA)

Overview: In CF patients, lung function declines and fails as a consequence of airway inflammation and chronic bacterial infections. Although many mechanisms have been proposed, the underlying reason why airway inflammation is deregulated in the CF lung remains unknown. Nevertheless, as infection and inflammation lead to lung tissue destruction, preventing either or both has been suggested as a mean to improve the health of CF patients. In this discussion, the merits and pitfalls of these strategies will be discussed. Here are a series of questions to be the focus of the debates:

Is there an intrinsic inflammatory defect in cells lacking CFTR?

What is the therapeutic window to decrease inflammation without fully impairing host defense responses in (i) infants that are not chronically infected or (ii) adults with chronic bacterial infection?

Can strategies aimed at decreasing the burden of oxidants be successful in significantly decreasing inflammation?

Should *Pseudomonas aeruginosa* be the focus of anti-microbial strategies or more scrutiny given to polymicrobial interactions?

Would strategies specifically aimed at disturbing microbial biofilms be more successful in decreasing the burden of infection in CF lungs?

Saturday 31 March – 17:30-19:15

SYMPOSIUM 9
Cellular Functions: CFTR and Beyond
Chairs: P. Fanen (FR) / G. Lukacs (CA)

S9.1 - Differential fragmentation of wild type, F508del and CK2-related CFTR mutants.

Kendra Tosoni*, Michelle Stobbart*, Diane M. Cassidy*, Mario A. Pagano†, Andrea Venerando†, Simão Luz‡, Margarida D Amaral‡,ǁ, Karl Kunzelmann§, Lorenzo A. Pinna†, Carlos M Farinha‡, Anil Mehta*

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Background: It is generally accepted that F508delCFTR misfolds and degrades in the ER en passant with its synthesis (but not all agree, Borthwick et al, 2011 PLoS One and references therein). Conceptually, degradation products can either be (proteasomally) generated as short peptides or cleaved into F508delCFTR-derived fragments. To test the latter idea, we investigated the fracture pattern of a number of CFTR mutants in a well characterised stable BHK cell line that we recently used to study the effects of the pleiotropic kinase CK2 on CFTR (Luz, S. et al 2011). In this, and related work (Venerando, A. et al 2011), investigators in Portugal, Italy, Germany and the UK demonstrated that F508delCFTR perturbs CK2 function finding that CK2 inhibition significantly attenuates CFTR synthesis, turnover and channel conductance. Luz et al also studied three CK2-related mutants (S511A/D, S422A/D and T1471A/D), each compared to wild type and F508delCFTR. They found that from a synthetic perspective, T1471D closely mirrored F508del as neither produced any mature band C of CFTR. This prompted a comparison of degradation patterns for different CFTR mutants.

Methods: Cells were harvested at 80% confluence, lysed into modified sample buffer (with 5 mM DTT) and subjected to SDS PAGE and Western blotting using a range of CFTR antibodies from the US CFF Consortium and from commercial sources that covered the N terminus, NBD1, R domain, NBD2 and the C terminus.

Results: Wild type CFTR is cleaved into two larger than expected fragments bearing NBD1 or NBD2 (but not both). These fragments were found in the absence of proteasomal inhibition and when cells were lysed directly into SDS PAGE buffer whilst on the culture dish. The NBD1 fragment also contained the N-terminus of CFTR and the NBD2 contained its cognate C-terminus (but not vice versa). The combined fragment molecular weights were about 170KDa suggesting that cleavage occurred somewhere in the R domain. In contrast, F508del CFTR and some of the CK2 mutants cleaved very differently. The F508del-NBD1 fragment was not observed and its cognate NBD2 fragment was much smaller than its equivalent fractured from wild type. The CK2 site mutants displayed their own characteristic patterns of mutation with the most striking effects seen with T1471D.

S9.2 - microRNA-based silencing of Delta/Notch signaling promotes multiple cilia formation

Pascal Barbry [1,2]; Benoît Chevalier [1,2]; Laurent Kodjabachian [3, 4]; Christelle Coraux [5, 6]; Brice Marcet [1,2]

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[2] Université de Nice Sophia Antipolis, France;
[3] CNRS; Institut de Biologie du Développement de Marseille-Luminy, France;
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Multiciliated cells lining the surface of some vertebrate epithelia are essential for various physiological processes, such as airway cleansing. Their apical surface is constituted by hundreds of motile cilia, which beat in a coordinated manner to generate directional fluid flow. MicroRNAs (miRNAs) are small non-protein-coding RNAs that induce post transcriptional mRNA repression. They have been implicated in the regulation of many cellular functions, including development, differentiation, response to stresses and cancer. We recently reported the identification of microRNAs of the miR-449 family as evolutionary conserved key regulators of vertebrate multiciliogenesis. This novel function of miR-449 was established using in vivo and in vitro antisense approaches in two distinct experimental models. miR-449 strongly accumulated in multiciliated cells in human airway epithelium and Xenopus laevis embryonic epidermis, where it triggered centriole multiplication and multiciliogenesis by directly repressing the Delta/Notch pathway. Our data complement previous reports that showed the blocking action of miR-449 on the cell cycle, and unraveled a novel conserved mechanism whereby Notch signaling must undergo microRNAmediated inhibition to permit differentiation of ciliated cell progenitors. Several important questions regarding the links between microRNAs, signaling pathways and the control of cell fate will be discussed, with possible links with cystic fibrosis physiopathology.
This work has been supported by the Association "Vaincre la Mucoviscidose".

S9.3 Stress Activated MAP KINASE Pathway(s) Regulates ER Quality Control Threshold

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Programme Note: Abstract details are not authorised for inclusion.

S9.4 - F508del-CFTR ER Retention, Hypoxia and Saturated Fatty Acid Accumulation: A Cross Road to Cystic Fibrosis Pathogenesis

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In the airways of cystic fibrosis (CF) patients, mutation in the CF transmembrane conductance regulator (CFTR) gene leads to an increased mucus viscosity and hypersecretion, impairing mucociliary clearance, which could also cause hypoxia.
CFTR dysfunction is also associated with an altered fatty acid metabolism, which results in low levels of Poly-Unsaturated Fatty Acids (PUFA) in cultured airway epithelial cells from CF patients (Anderson et al., 2008). This defect also appears to impact the overall fatty acyl content of phospholipids, since the amounts of five phosphatidylcholine (PC) species containing unsaturated chains are decreased in the plasma of CF patients.
In this study, we first investigated if such differences in the fatty acyl content of membrane phospholipids could be observed in freshly isolated epithelial cells from CF patient lungs bearing the F508del-CFTR mutation. Surprisingly, HPLC-MS (High-Performance Liquid Chromatography-Mass Spectrometry) analyses revealed an accumulation of PC species with two saturated fatty acyl chains (SFA), in the form of palmitic acid (C16:0), in CF samples, as compared to non-CF samples. This SFA accumulation within PC was not directly related to the presence of the F508del-CFTR mutation, since this phenomenon could not be observed in the CFBE cell line, a bronchial epithelial cell line from F508del-CFTR homozygote patients. We therefore concluded that the expression of the F508del-CFTR protein cannot account, by itself, for the observed palmitate accumulation within PC in lungs, and that another physiological side effect might be responsible for this phenomenon.
Among non-CF patients, two different populations with strikingly different PC profiles could be identified: smokers and non-smokers. Smokers presented a CF-like profile with an accumulation of palmitate within PC. This similar lipid signature between CF patients and non-CF smokers led us to postulate that SFA accumulation could be the result of hypoxia, related to alteration/obstruction of the upper airways, a physiological trait shared by both CF-patients and non-CF smokers. To test this hypothesis, we developed a cellular culture model for hypoxia and showed that oxygen scarcity resulted in palmitate accumulation within PC both in CFBE and wild-type (16HBE) cell lines. This observation reinforces the idea that SFA accumulation in CF is not-directly related to the expression of the F508del-CFTR protein, but could rather be a side-consequence of hypoxia.
Even if it is still a matter of debate for the F508del-CFTR protein, it is known that the accumulation of misfolded proteins in the ER induces a cellular stress response, known as the Unfolded Protein Response (UPR). Interestingly, SFA have a very similar impact on cells, likely because they impair protein folding/translocation in the ER and thus promote a misfolded protein overload in this compartment. Ultimately, under both circumstances, sustained UPR can lead to cell death by apoptosis. In this context, we are presently investigating the possible synergistic effects of F508del-CFTR retention in the ER and its side-related SFA accumulation in CF-patients on the induction of the UPR and cell survival. Preliminary data concerning these aspects will also be presented

S9.5 - The F508del Mutation in *Cftr* Gene Impacts Bone Formation

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The F508del mutation in the cystic fibrosis transmembrane conductance regulator (Cftr) gene is believed to be an independent risk factor for cystic fibrosis-related bone disease. We recently reported the expression of CFTR mRNA and protein in primary human osteoblasts and showed that reduction in CFTR-dependent chloride (Cl^-) activity affects the production of osteoprotegerin (OPG) and prostaglandin E2, two key regulators of bone formation/resorption process (Le Heron L et al. 2010). Here, we report both a defective CFTR-mediated Cl^- channel activity and a severe deficit of OPG production by cultured osteoblasts isolated from a 25-yr-old CF patient with the F508del/G542X mutation in CFTR. A total absence of CFTR-dependent (Cl_{CFTR}) chloride response in F508del-CFTR osteoblasts was observed compared to normal osteoblasts. In contrast, two other calcium-dependent (Cl_{Ca}) and volume-dependent (Cl_{swell}) chloride channels were found to be fully functional in both F508del-CFTR and normal osteoblasts. Interestingly, we found that both the basal and stimulated (TNF-α, 20 ng/ml, 4 hrs) OPG protein released by F508del-CFTR osteoblasts was considerably reduced (8-10%) compared to normal osteoblasts (Gimenez A. et al., Eur Respir J, 2012, in press).To evaluate whether the severe osteopenia in CF is directly linked to the F508del mutation, we measured the bone mineral density and histomorphometric parameters of bone formation and bone mass in F508del-CFTR homozygous mice (F508del *Cftr*^{tm1Eur}) and Cftr$^{+/+}$ littermate controls at 6 (prepubertal), 10 (pubertal) and 14 (young adult) weeks of age in two genders. The bone architecture of F508del *Cftr*^{tm1Eur} and wild type littermate mice was evaluated by bone densitometry, micro-CT and analysis of dynamic parameters of bone formation. Levels of serum insulin-like growth factor 1 (IGF-1) and osteocalcin were also determined. Reduced bone mineral density, lower femoral bone mass and altered trabecular bone architecture were observed in F508del *Cftr*^{tm1Eur} compared to controls at 6, 10, and 14 weeks of age. A decrease in bone formation rate in F508del *Cftr*^{tm1Eur} was evidenced compared to control mice, independently of age and sex. Interestingly, we found lower IGF-1 levels in F508del *Cftr*^{tm1Eur} mice compared to age-matched control mice whereas osteocalcin level was normal. Severe osteopenia and altered bone architecture were found in young and mature adult F508del *Cftr*^{tm1Eur} mice. Our findings demonstrate that the F508del mutation in CFTR impacts trabecular bone mass by reducing bone formation (Le Henaff C. et al, Am J Pathol, 2012, in press).

Supported by grants from Vaincre la Mucoviscidose and Inserm/Region Champagne Ardenne convention.

Room: La Villa Rose

Posters – CFTR Structure and Function

P. 1

Structure-Function Analysis of Interhelical Interfaces Comprised of the Intracellular Loops of CFTR

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CFTR is a plasma membrane phosphoglycoprotein, belonging to the ATP-Binding Cassette (ABC) transporter superfamily. CFTR functions as an ATP- and PKA-dependent chloride channel, regulating chloride ion flux across apical membranes of polarized epithelial cells. Mutations in CFTR cause Cystic Fibrosis disease; the major disease-causing mutation, F508del, is located in the first nucleotide binding domain (NBD1). This and many other (>1800) mutations affect protein folding and channel gating, presumably by destabilizing interdomain interfaces; the NBD1:intracellular loop 4 (ICL4) interface is implicated in F508del. Current small molecule 'correctors' lead to partial rescue of the processing defect with some functional (but inefficient) expression at the cell surface, and although the mechanism of action is poorly understood, the NBD1:ICL4 interface is likely repaired to some extent. Consequently, there is a need to understand the roles of this and other interdomain interactions in the biogenesis and functional expression of CFTR.

Functionally important interdomain interfaces include: NBD1:ICL4, NBD1:2, NBD1:2:ICL1:2:4, ICL3:Regulatory-domain, and membrane spanning domain (MSD) 1:2. Identification of other interfaces along the pore axis of CFTR is desired. Upon inspection of Sav1866-based atomic homology models of CFTR, and using HOLE software, previously unexplored ICL interfaces (ICL1:2, ICL1:3, ICL2:4) were predicted to play important roles in biosynthesis and gating. Further analysis identified a cluster of hydrophobic residues comprised of six ICL amino acids (all of which are located in the helical extensions, two of which are associated with disease) that may be involved in gating. These residues include: Ile^{177} (ICL1), Val^{181} (ICL1), Leu^{259} (ICL2), Val^{260} (ICL2), Leu^{973} (ICL3) and Val^{1056} (ICL4). Individually mutating Ile^{177} and Leu^{259}, two residues which form the narrowest constriction in the putative permeation pathway, did not affect maturation (as shown by immunoblots), but the double substitution of $Ile^{177}Ala/Leu^{259}Ala$ decreased the rate of functional activation by 40%, presumably by altering the ICL1:2 interface, as measured by a cell-based iodide efflux assay using the halide-sensitive fluorophore 6-methoxy-N-(3-sulfopropyl)quinolinium. Substitution of the ICL3 residue Leu^{973} with disease-causing Pro, but not disease-causing Phe or His, affected CFTR processing, suggesting that a conserved residue is required at this position for proper assembly and maintenance of alpha-helical structure. Interestingly, disruption of the putative ICL1:3 interface by double Ala-substitution of Ile^{177} (ICL1) and Leu^{973} (ICL3) created a mutant protein that was EndoglycosidaseH-sensitive, suggesting that it is synthesized and core-, but not complex-glycosylated at steady-state; therefore this interface is important for CFTR maturation. Further characterization of these and other ICL mutants, as well as cysteine cross-linking of residues within putative ICL:ICL interfaces is currently in progress. By understanding how the ICLs transmit signals between the MSDs and NBDs via interdomain interfaces, gating mechanisms will become more apparent, and could potentially allow for the design of next generation correctors to treat Cystic Fibrosis by improving the folding/assembly of mutant CFTR. Elucidation of such molecular events would contribute not only to our understanding of CFTR activity, but also to our understanding of channels and transporters in general.

This work is supported by the Canadian Institutes of Health Research and Cystic Fibrosis Canada.

Conserved NBD-TMD Interactions Regulate the Biosynthesis of ABC Transporters

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ATP-binding cassette transporters are multi-domain proteins that facilitate solute movement across biological membranes. ATP binding and hydrolysis within the cytoplasmic nucleotide binding domains (NBDs) provide the energy for solute movement through the transmembrane domains (TMDs). Functional interactions between the NBDs and TMDs are facilitated by the intracellular loops (ICLs) of the TMDs. Unlike the structurally well-defined NBD-NBD interface that is associated with ATP binding and hydrolysis, the NBD-ICL interface is less well characterized, in part due to the insolubility of the TMDs. In mammalian ABC transporters, each NBD contacts two ICLs. For full-length transporters, NBD1 putatively interacts with ICL1 and ICL4 while NBD2 interacts with ICL2 and ICL3. In CFTR, alteration of NBD structures or disruption of the putative NBD-TMD interface results in altered maturation or function of full-length protein. Similarly, disease-associated mutations in other human ABC transporters lie at this conserved NBD-TMD interface, consistent with the hypothesis that native domain-domain assembly is crucial for transporter biosynthesis.

To further probe the hierarchy of ABC transporter biosynthesis, domain folding and assembly events were probed using isolated domains, a novel protein chimera and full-length transporters. Characterization of domain folding and full-length protein maturation suggests that native NBD folding is crucial for the biosynthesis of multiple ABC transporter systems. Disruption of NBD folding or the NBD-ICL interface adversely impacts multiple ABC transporters. To further characterize this domain-domain interaction, a soluble chimeric protein was engineered to contain the ICL1 and ICL4 sequences in conformations that putatively mimic those found in the full-length proteins. Using this system, the ICL-NBD interface is assessed directly and sites critical to the native interface are evaluated, including disease-causing mutations found within this interface. *In vitro* studies with the purified chimeras and their cognate NBD proteins provide a means to study the ICL-NBD interface directly. From these data we show that the hierarchical folding and assembly of human ABC transporters are conserved. Specifically, both domain folding and domain-domain interactions are critical for proper biosynthesis. In addition, the development of a soluble system to evaluate ICL-NBD interactions facilitates biochemical characterization of this crucial biosynthetic and functional interface.

P. 3

Keratin 8 - NBD1 of CFTR Complex: Structural Studies

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Misfolding of cystic fibrosis transmembrane conductance regulator (CFTR) with a deletion of phenyloalanine 508 underlies pathology in most CF patients (F508delCFTR). The deletion of F508 residue results in ubiquitin - dependent degradation of CFTRF508del by proteasome. Mutated CFTR (F508delCFTR) may be addressed to the plasma membrane and functions as CFTR-like Cl^- channel. We have recently shown that 2 intermediary filaments, keratin 8 and 18 (K8, K18), form a complex with F508delCFTR and participate in its retention in ER, and subsequent degradation. We observed that K8 directly interacts with NBD1 of CFTR and that disruption of these interaction leads to the delivery of functional F508delCFTR to the plasma membrane in CF cell lines, primary epithelial lung cells from CF patients, and in nasal epithelium of F508 mouse [Colas et al. Hum Mol Gen 2012; 21: 623]. The site of interaction between K8 and F508delCFTR may be considered as a potential targets for a new CF pharmacotherapy. However, to rational design new efficient inhibitors of this interaction it is necessary to resolve the structure at the molecular level of K8 complex with at least domain bearing CF causative deletion NBD1 in WT and F508del version. The aim of this work is to investigate the K8 structure in order to precisely determined the oligomerization state of K8 and identify which species interacts with NBD1, and determine structural properties of K8. Following experimental approaches were used: small angle X - ray scattering (SAXS), size exclusion chromatography (SEC) and hydrogen - deuterium exchange (HDex) coupled with mass spectrometry (MS).

Recombinant K8 was purified according to Herrmann H. et al (J Struct Biol 2002; 137: 82). Using SAXS and SEC we have found that even in low ionic strength full K8 forms still soluble but higher - order species probably so called unit - length filaments. Using HDex - MS, on K8 separated. We noticed that HDex pattern in K8 is in agreement with K8 division for characteristic regions (head, coils, linkers and tail) according to the protein data bank. We have revealed that the beginning of the head domain is well structured because it is characterized by slow HDex rate. We can conclude that this region is crucial for intermolecular interactions what is in agreement with the literature (Herrmann H. et al 2002). We have also noticed that regions of K8, which correspond to a - coils are more protected from HDex. In contrast flexible linkers are totally exposed for HDex. So despite of formation high - ordered species by K8 its structure is correctly folded.

Based of these data, we will try to discriminate interaction surface between K8 and NBD1 using HDex-MS and SAXS. In the future this finding will facilitate the designing a new drugs based on inhibiting this pathological interaction.

Room: La Villa Rose

Posters – CFTR Biogenesis, Quality Control and Trafficking

P. 4

CFTR Corrector C18 Targets the First Membrane-Spanning Domain During Synthesis

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Large efforts are in progress to find and design drugs that cure Cystic Fibrosis and correct F508del CFTR folding and its transport to the cell surface.
The multidomain protein CFTR consists of two membrane-spanning domains (MSDs), two nucleotide-binding domains (NBDs), and a regulatory region (R-region). Its domains fold cotranslationally, which is followed by posttranslational domain assembly. We previously found that the primary folding defect already emerges during translation of F508del NBD1, and can be restored by suppressor mutation I539T in the same subdomain as F508. From the defect in NBD1 the misfolding trickles through the entire protein, affecting various domain assembly steps. A drug that cures Cystic Fibrosis should correct primary as well as secondary folding defects.
We use both in vivo and in vitro radiolabeling approaches to determine when (during biosynthesis) and where (in the protein) corrector compounds act in repairing the F508del CFTR folding defect. We found that the CFF C18 corrector compound has a strong synergistic rescue effect on F508del CFTR with the I539T and G550E suppressor mutants. Adding the corrector compound only during pulse-labeling (synthesis) virtually maximized the rescue effect implying that C18 repaired F508del CFTR cotranslationally. Using C-terminally truncated molecules in combination with limited proteolysis we demonstrated that C18 affected MSD1 structure, irrespective of wild-type or F508del background. We currently employ in silico drug-to-protein docking studies to identify the precise MSD1 target location of the C18 corrector drug.

Hsp27 Selectively Targets F508del CFTR for Degradation via the SUMO Pathway

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The mechanism of misfolding of the cystic fibrosis transmembrane conductance regulator (CFTR), and the pathways that drive its ER associated degradation (ERAD), remain incompletely understood. Like other eukaryotic ABC proteins, CFTR is comprised of two transmembrane domains (TMD1, TMD2), each linked to a cytoplasmic nucleotide-binding domain (NBD1, NBD2), but interrupted by a central regulatory domain. The common disease mutation of CFTR leading to protein misfolding, F508del, is located in the cytosol in NBD1, where it alters this domain's thermodynamic stability and subsequent domain-domain associations. As a consequence, the mutant protein is targeted for proteasome-dependent degradation.

Small heat shock proteins (sHsps) bind to destabilized proteins during cell stress and disease, but their physiological functions are less clear. Hsp27 is the predominant sHsp expressed in airway epithelial cells, and it interacted selectively with F508del CFTR, targeting the mutant protein for ERAD. Hsp27 was found to interact with Ubc9, the E2 conjugating enzyme for SUMOylation, and Hsp27 over-expression promoted preferential SUMOylation of F508del CFTR in vivo by the endogenous SUMO-2 or -3 isoforms. Expression of the SUMO pathway conjugating enzyme, Ubc9, also promoted the selective degradation of F508del CFTR. Conversely, knockdown of endogenous Hsp27 or the SUMO activating enzyme, SAE1, increased F508del expression, indicating that this pathway functions physiologically in mutant CFTR degradation.

To better understand the properties of F508del CFTR that lead to its SUMO-dependent modification and degradation, we developed an in vitro assay for SUMOylation of NBD1 utilizing purified components. In this assay, NBD1 SUMOylation was detected by either SUMO blot or from the molecular mass shift in NBD1 associated with the addition of SUMO. Purified WT and F508del NBD1 were found to be modified by the SUMO-1, -2, and -3 isoforms in vitro, and as found in vivo, greater modification was observed for F508del vs. WT NBD1, and Hsp27 augmented this NBD1 modification.

In vivo studies of NBD1 were performed by co-expressing Hsp27 with the membrane tethered fusion proteins, CD4T-NBD1 or CD4T-F508del-NBD1, in which the C-terminus of CD4T is fused to the WT or mutant NBD1 to link the NBDs to the ER membrane. As observed for full-length CFTR, we found that CD4T-F508del-NBD1 was selectively degraded relative to CD4T-NBD1. Co-IP experiments using lysates from transiently expressing cells showed that: a) Hsp27 preferentially promoted the SUMOylation and degradation of CD4T-F508del-NBD1, b) Hsp27 interacted preferentially with the F508del-NBD1-CD4T fusion protein, and c), Hsp27 associated with SUMO-1 and SUMO-2/3 in vivo.

These data suggest that the structural defect in F508del NBD1 is interpreted by Hsp27 in a manner similar to that observed for full-length F508del CFTR, and that this interaction makes the mutant NBD accessible to proteasome-mediated degradation by eliciting its SUMO modification. Recent over-expression and knockdown studies implicate the SUMO targeted ubiquitin ligase, RNF4, as a step leading to the ubiquitin-dependent, proteasomal degradation of F508del CFTR via this pathway. The recapitulation of this pathway for the NBD1 domain should expedite efforts to identify the site(s) of SUMO modification and further elucidate the mechanism.

Support: NIDDK and the US CF Fdn.

Study of Long-Range Regulatory Mechanisms of the CFTR Gene

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The cystic fibrosis transmembrane conductance regulator (CFTR) gene was identified in 1989, but more than 20 years later, the regulatory mechanisms controlling its complex expression are still not fully understood. Although, more then 1900 mutations have been identified, many cases of cystic fibrosis or CFTR Related Disorders remain still of unknown origin.

The promoter binds transcription factors and drives some aspects of *CFTR* gene expression, but it cannot alone account for tissue specific control. This implicates other distal cis- or trans-acting elements in cell-type-specific regulation of CFTR expression. Interestingly, the majority of the human genome is composed of non coding DNA whose function has not been thoroughly investigated. A significant number of conserved non-coding sequences (CNCs) are found in gene-poor regions, these large intergenic regions must have kept a function throughout evolution. As already described, some CNCs appear to be transcriptional enhancer *in vivo* [Pennacchio and al. 2006; Shin and al. 2005].

The aim of our project is to map potentials regulatory elements located within CNCs, which could interact specifically with the *CFTR* gene by tri-dimentional folding mechanism. These interactions can be detected by Chromosome Conformation Captures (3C) [Dekker and al. 2002]. The 3C technology is a high-resolution technique of molecular biology allowing the analysis of chromosome organization in cells. Thus we can measure the proximity of various chromatin areas of DNA in the *in vivo* nuclear space. Cellular lineages and primary cultures expressing or not the protein CFTR will be used.

We have already tested 53 CNCs located within the intergenic regions ASZ1/CFTR, MDFIC/TFEC and TFEC/TES. Unfortunately, no region appears to have a possible interaction with the *CFTR* promoter.

In the future we would like to set up the 4C technology [Göndor and al. 2008]. The Circular Chromosome Conformation Capture (4C) is a 3C-derived technique that is a high-throughput approach for performing a genome-wide screen of previously unknown interacting partners with a genomic point of interest, “the bait”.

Analyses will be pursued on patients affected by cystic fibrosis or by CFTR RD, in whom either a single mutation or none was found in the *CFTR* gene coding sequence.

Thanks to 3C analyses, we hope to discover new possible mutations at distance from the gene, which may lead to its dysfunction by modifying the chromatin conformation. These regions could be a target of small therapeutic molecules. Finally we could imagine that identified endogenous enhancers may be incorporated into therapeutic vectors to restore a stable expression of CFTR.

Room: La Villa Rose

Posters – Epithelial Transport

P. 7

A Role for in Cystic Fibrosis (CF) by Direct Interaction of Nucleoside Diphosphate Kinase (NDPK) with the First Nucleotide Binding Domain (NBD1) of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Protein

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Introduction: An unresolved issue is that the measured ATPase activity of the nucleotide binding domains of CFTR (NBD1 and NBD2) is too slow to account for the fast gating of CFTR. NDPK converts nucleosides diphosphates to nucleosides triphosphates, acts as a protein kinase and transfers phospho-histidine (>20 kJ/mol) directly onto ion channels. Our data suggest that the common isoforms NDPK-A and -B, that are required for transition to postnatal life, interact with CFTR but it is as yet unclear whether NDPK-A and -B directly interact and to what extent the CFTR activity is regulated by these interactions.
Aims: The aim of this work was to confirm or deny the direct interaction between NDPK-A or -B and CFTR and to establish how these interactions would occur by identifying the NDPKs binding sites on CFTR to better understand the role of these kinases on the regulation of the CFTR protein.
Methods: NDPK-A and -B and several CFTR domains were obtained in purified form and used for interaction studies by far-western blot and surface plasmon resonance (SPR) techniques.
Results:
In overlay assays, the CFTR domains were subjected to SDS/ PAGE and transferred to a membrane incubated with either NDPK-A or -B. The blots were then incubated with an anti-NDPK antibody and showed a signal at the size of NBD1 (28 kDa). To confirm this result, several quantities of NBD1 were subjected to electrophoresis and transferred onto a membrane incubated with either NDPK-A or -B. Using specific antibodies, the detection of NDPK-A and -B at 28 kDa confirmed the direct interaction between NBD1 and NDPKs. An irrelevant protein (BSA) was used and ruled out non-specific interactions. In SPR experiments, we immobilized NDPK-A and -B on a sensor chip and then injected increasing quantities of NBD1. The interaction was observed using computerized analysis as a curve (sensorgram) given as resonance units (RU) as a function of time (s). The binding of NBD1 domains was evaluated 20 s into the dissociation phase and we found that the RU values increased with the amount of injected NBD1. The specificity of the binding was checked by injection of an irrelevant protein (BSA). These data confirmed that both NDPK-A and -B bind the NBD1 domain of CFTR. We also used peptides from NBD1 region of CFTR, amino acids (aa) 350 to 747, to determine the site(s) of interaction with NDPK-B: we overlaid these peptides with membranes from human bronchial epithelial cells (16HBE14o-) and detected NDPK-B for the peptides corresponding to the aa 357 to 376 and 378 to 397.
Conclusions: In conclusion, we showed that NDPK-A and -B directly interact with CFTR at its NBD1 domain and highlighted the role of NDPKs on the regulation of the CFTR protein.
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P. 8

Study of the Chloride Channel ANO1 in Cystic Fibrosis Context

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Defective CFTR function in the airway epithelium is responsible for CF patient lung disease. This pathology is characterized by airways obstruction due to mucus hypersecretion, infection and inflammatory processes. CFTR represents the most important pathway for apical chloride (Cl^-) secretion in human bronchial epithelial cells. Calcium activated Cl^- channels (CaCCs) are also an important pathway of Cl^- secretion, although they seem to represent the main in mice airways. In 2008, three independent teams suggest that ANO1 (Anoctamine 1) also called TMEM16a (transmembrane protein 16a) could be a CaCC candidate. Indeed, ANO1 show similar properties to those observed for CaCC, notably regarding ion selection permeability and calcium activation. Furthermore, mice lacking ANO1 exhibit a defect in epithelial Cl^- transport and pathology similar to CF. ANO1 is an eight transmembrane protein expressed in various tissues and also in bronchial epithelium. Intracellular C termini of ANO1 contains two predicted sites for phosphorylation by extracellular regulated kinase 1,2 (Erk1,2). An alternative splicing mechanism has been described for ANO1 resulting in exclusion/inclusion of four segments (a, b, c and d) in the protein with significantly functional consequences.

The main aim of this study is to characterize ANO1 protein in CF vs non CF context. This aim is divided into three distinct parts that are intended to:

- Compare ANO1 expression, localization and activity in CF vs non CF context
- Understand the mechanisms regulating ANO1 activity
- Determine whether there are differences between CF and non CF alternative splicing

ANO1 expression, localization and activity were determined respectively by qPCR and western blot, immunofluorescence and halide sensor method. Transient transfections with a plasmid encoding ANO1-GFP have been performed to localize ANO1 in our cells. For ANO1 regulation study, we used different Mek1,2 inhibitors. ANO1 splicing was studied by PCR using specific probes for each exon studied. For this study, we used different CF and non CF models whose cell lines, mice and lung explants from CF and non CF patients.

Our results show that ANO1 expression and activity are significantly decreased in CF compared to non CF models. ANO1 is expressed at plasma membrane of bronchial epithelial cells and there is no difference in localization between CF and non CF cells. Preliminary results seems to show that ANO1 activity is reduced in presence of Mek1,2 inhibitors. Study of alternative splicing shows that it seems to have some differences in exons expression in CF vs non CF models.

We conclude that decreased ANO1 activity in CF cells could be explained by decreased ANO1 ARNm and protein expression and may contribute to the worsening of ionic imbalance and decrease lung function. Moreover, ANO1 seems to be regulated by Erk1,2 pathways although these results should be confirmed. First results obtained on alternative splicing seem to show that CF or non CF context induces different alternative splicing that could affect ANO1 function. All of these results lead us to think that this Cl^- channel could be a potential pharmacological target for the treatment of cystic fibrosis patients.

HCO_3^- Permeability of ANO1/TMEM16A

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Bicarbonate is secreted by calcium activated chloride channel (CaCC) in salivary gland acinar cells. However, CaCC is known to be poorly permeable to HCO_3^-. Here, we report that ANO1/TMEM16A, one of the strong candidates for CaCC, is highly permeable to HCO_3^- when intracellular calcium level is high. To elucidate the possibility of ANO1 as a HCO_3^- channel, anion currents were measured in HEK 293T cells transfected with human ANO1 (ac isoform) using the whole-cell patch clamp techniques. Relative permeabilities of not only HCO_3^- but also other halide anions were altered by intracellular calcium level in whole cell patch clamp. ATP depletion in pipette solution, the treatment of broad spectrum kinase inhibitor (K292a) in bath solution, and cotransfection with WNK1/SPAK kinase or siWNK1/siSPAK did not affect the relative permeability change induced by calcium alteration, which suggest that high calcium effect on anion selectivity is not caused by phosphorylation signaling. The high $[Ca^{2+}]_i$-induced increase in HCO_3^- permeability was reproduced in mouse submandibular gland acinar cells where ANO1 is natively expressed. In summary, halide anion selectivity in ANO1 can be regulated by the intracellular calcium concentration.

Lipoxin A_4 Stimulated Potassium Currents Increase Airway Epithalial Repair in Cystic Fibrosis

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Introduction: The main hallmarks of disease in cystic fibrosis are chronic bacterial infection and persistent inflammation. Overtime this leads to epithelial damage and airway remodeling, reducing lung function and resulting in patient morbidity and mortality. It has been well described that cystic fibrosis epithelial cells have reduced epithelial repair and differentiation capacity. Lipoxin A_4 is an anti-inflammatory lipid mediator known to have significantly reduced levels in bronchoalveolar lavage fluid of patients with cystic fibrosis. Furthermore, Lipoxin A_4 has been shown in other models to play a role in epithelial repair through increased cell proliferation and migration. We hypothesized that reduced repair in the cystic fibrosis epithelium may be rescued through exposure to lipoxin A_4 and we investigated the cellular mechanisms involved.
Material and methods: Healthy (NuLi-1) and cystic fibrosis (CuFi-1, homozygous F508del) bronchial epithelial cell lines and primary pediatric healthy (HBE) and cystic fibrosis (HCF) bronchial epithelial cells were grown either as monolayer on plastic or 3D differentiated cultures on permeable filters in air/liquid interface (ALI). Cell proliferation was measured using a MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Epithelial repair capacity was ascertained by scratch assay. Gene expression of potassium channels was investigated through use of RT PCR. Lipoxin A_4 receptor (FPR2) expression was measured using flow cytometry. Potassium currents were measured using whole-cell patch-clamp techniques.
Results: Under basal conditions, wound repair capacity was reduced in CF (CuFi-1) cells compared to control (NuLi-1). However, lipoxin A_4 (1nM) significantly stimulated epithelial repair in CuFi-1 cells to levels above that witnessed with NuLi-1. The levels of the lipoxin A_4 receptor, FPR2, found in ALI cultures were significantly reduced in CuFi-1 cells compared to Nuli-1. Lipoxin A_4 induced a significant increase in cell proliferation in Nuli-1 and CuFi-1 cells as well as in primary cultures of pediatric CF epithelial cells. Epithelial cell proliferation and repair induced by lipoxin A_4 were inhibited by the FPR2 receptor antagonist, Boc-2. Potassium channel inhibition, most significantly using K_{ATP} blocker glybenclamide, completely inhibited both basal and lipoxin A_4 induced cell proliferation. Treatment of airway epithelial cells with pinacidil, a K_{atp} channel opener, mimicked the effect of lipoxin A_4 on cell proliferation. Finally, the MAP kinase (ERK 1/2) inhibitor, PD98059 significantly inhibited the lipoxin A_4 induced cell proliferation. Potassium channel gene expression, including K_{ATP}, was measured in ALI cultures, and no significant difference was found between healthy and CF cells or between lipoxin A_4 stimulated and control groups. However, lipoxin A_4 increased whole-cell barium-sensitive (potassium) currents.
Conclusion: Taken together, these results show that lipoxin A_4 plays a key role in epithelial repair and proliferation in normal and CF airway epithelial cells, via stimulation of the FPR2 receptor leading to ERK phosphorylation and K_{ATP} potassium channel activation. These results highlight the potential therapeutic use of lipoxin A_4 to increase epithelial repair and thus lung function in patients with cystic fibrosis.

Rapid Effect of 17β-Estradiol on Airway Surface Liquid Hydration in Normal and Cystic Fibrosis Bronchial Epithelia

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Background: Male cystic fibrosis (CF) patients survive 9 years longer than females ("CF gender gap") and lung exacerbations in CF females vary during the estrous cycle. Estrogen has been reported to reduce the height of the airway surface liquid (ASL) in female CF bronchial epithelium. Here we investigated the effect of estrogen on ASL height and ion transport in normal (NuLi-1) and CF (CuFi-1) bronchial epithelium monolayers grown on permeable filters in an air-liquid interface.
Methods: Confocal fluorescence microscopy experiments were performed to measure ASL height in normal and CF bronchial epithelial cells treated with 17β-estradiol (E2 0.1 to 10nM). In order to determine the ion channel(s) involved in the effect of E2 on ASL height, different ion transporter modulators (amiloride, 10µM; bumetanide, 10µM; HMR1556, 1µM; ATP, 100µM, forskolin, 10µM) were used in ASL and Ussing chamber experiments. The nuclear-excluded Estrogen Dendrimer Conjugate and its negative control (empty dendrimer) were used in order to determine the involvement of the nuclear estrogen receptor pathway in the effect of E2 on ASL height.
Results: Confocal experiments revealed that ASL height was significantly higher in the non-CF cell line compared to the CF cells (NuLi 6.82 ± 0.33µm vs. CuFi 5.58 ± 0.14µm, n=20, $p< 0.001$). Physiological concentrations of E2 reduced the ASL height in both non-CF (25% decrease, n=5, $p< 0.05$, ANOVA) and CF (20% decrease, n=5, $p< 0.05$, ANOVA) cell lines after 30 min treatment. Treatment with the Cl^- transport inhibitor bumetanide or the KCNQ1 potassium channel blocker chromanol HMR1556 decreased ASL height significantly in both cell lines. However, E2 had no additive effect on ASL height in the presence of these ion transporter inhibitors. Moreover E2 decreased the bumetanide-sensitive Cl^- current in normal cells (E2: 6.47 ± 2.08 µA/cm^2, Control: 9.52 ± 2.08 µA/cm^2, n=3, $p< 0.05$, paired t-test) and produced an increase in amiloride sensitive current in CF cells (E2: 11.097 ± 1.805 µA/cm^2, Control: 8.801 ± 1.464 µA/cm^2, n=7, $p< 0.05$, paired t-test). Treatment with the nuclear-impeded Estrogen Dendrimer Conjugate (EDC 0.1 - 1nM E2 equivalent concentration) produced a significant reduction in ASL height in CF and non-CF cells (4.72 ± 0.25µm in NuLi-1 and 4.86 ± 0.42µm in CuFi-1, n=5, $p< 0.05$ compared to control condition, ANOVA) whereas the empty dendrimer had no effect.
Conclusion: These results demonstrate that E2 dehydrates both CF and normal ASL and these rapid responses to E2 are membrane-initiated rather than via the classical nuclear estrogen receptor signal transduction pathway. The ion transporter inhibitor data indicate that E2 acts on ASL by inhibiting Cl^- secretion in non-CF cells and increasing Na^+ absorption via ENaC in CF cells.

Acknowledgements: Supported by EU FP7 Marie Curie CEMP Fellowship (to VS-C), Higher Education Authority of Ireland (PRTLI4 NBIP to BJH) and the National Institutes of Health (R37 DK015556, to JAK.).

The ENaC Inhibitory Domain of SPLUNC1 Restores ASL Height in CF Airway Epithelia

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The epithelial Na^+ channel (ENaC) is responsible for Na^+ absorption in many epithelia, including the kidney, colon and lung. ENaC is made up of three subunits, α, β, and γ. Previous studies have shown that hyperactive ENaC in cystic fibrosis (CF) airways, due to a lack of CFTR which negatively regulates ENaC, leads to a decrease in airway surface liquid (ASL) volume which slows or abolishes mucus transport. The activation of ENaC is dependent on its proteolytic cleavage by enzymes such as neutrophil elastase (NE). The short palate lung and nasal epithelial clone(SPLUNC1) protein, is a potent regulator of ENaC hyperactivity and therefore ASL height regulation. This inhibition is achieved through the binding of SPLUNC1 to ENaC, preventing its cleavage. In this study we have identified the active site of SPLUNC1 and characterized its interaction and regulation of ENaC. The region of SPLUNC1 responsible for ENaC inhibition was identified by measuring the amiloride sensitive ENaC current, I_{NA}, in *Xenopus* oocytes co-injected with α,β, and γ ENaC in the presence of C- terminal SPLUNC1 truncants. Deletion of up to 85% of SPLUNC1 resulted in a similar inhibition of ENaC as seen with full-length SPLUNC1, which narrowed down the ENaC inhibitory domain of SPLUNC1 to residues 22-39. We synthesized a peptide corresponding to this region, named G22-A39, and measured the I_{NA} in the presence of 10 μM G22-A39 for 1h. A ~2.5 fold decrease in the I_{NA} was observed, indicating that we had identified the ENaC inhibitory domain of SPLUNC1. We have previously determined that SPLUNC1 co-immunoprecipitates with all three ENaC subunits, when these subunits are expressed simultaneously. Using a series of peptide pull-down assays we confirmed that G22-A39 was also able to interact in a similar fashion with all three subunits. This interaction was further explored by performing pull-down assays with individually expressed subunits and we determined that G22-A39 specifically interacts with the β-ENaC subunit. Furthermore, this interaction was dependent on the glycosylation state of β-ENaC. To determine if G22-A39 was also capable of inhibiting ASL hyperabsorption in CF human bronchial epithelial cultures(HBECs), the change in ASL height in both normal lung(NL) and CF HBECs was measured after treatment with G22-A39. Over a period of 24h, a single dose of 100 μM G22-A39 maintained the ASL height of CF HBECs to 8 μm, which is comparable to that observed in NL HBECs. In the diseased CF lung, high levels of proteolytic activity are typically present so the ability of G22-A39 to prevent ASL hyperabsorption in CF HBECs in biologically relevant conditions was tested. The HBECs were exposed to both purified NE or activated neutrophil supernatant(ANS), which is derived from human neutrophils and contains high NE and cathepsin activity, and the ASL height measured. G22-A39 prevented CF ASL hyperabsorption as a single dose over a 24h period in the presence of the purified NE and ANS. The ability of G22-A39 to function in this environment makes G22-A39 a strong therapeutic candidate for restoring ASL height and function in CF patients.

17β-Estradiol Acts Upon the C-terminal Domain of STIM1 to Inhibit Calcium Influx

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Cystic Fibrosis is a genetic disease that affects multiple organ systems, including the airways, and is known to affect women more severely than men. We have previously shown that 17β estradiol (E2), acting through estrogen receptor α (ESR1), inhibits Ca^{2+} influx in airway epithelia and have proposed that this leads to a reduction in Cl^- secretion. Ca^{2+} influx is regulated by STIM1, an endoplasmic reticulum (ER) Ca^{2+}-sensing protein. Depletion of ER-Ca^{2+}, which can be induced experimentally by thapsigargin, initiates translocation of STIM1 to the ER-plasma membrane junction where it aggregates and interacts with the store-operated Ca^{2+} channel Orai1, to induce Ca^{2+} influx. To better understand this process, we tested the hypothesis that E2/ESR1 prevents Ca^{2+} influx by inhibiting STIM1 function. Immunofluorescence studies were performed on polarized airway epithelial cells to determine STIM1 localization. Diffuse STIM1 staining was visible under basal conditions and upon ER-Ca^{2+} depletion STIM1 moved closer to and condensed at the apical membrane. Pretreatment with E2 alone did not affect STIM1 localization. However, after ER-Ca^{2+} depletion, STIM1 failed to move closer to the apical surface, suggesting STIM1 motility was inhibited by E2. Next, we transfected HEK293 cells with YFP-STIM1 and ESR1-CFP to better understand E2's inhibitory actions. We found that STIM1 puncta formation was inhibited by 50% when pretreated with E2 for 20 min. Additionally, E2 pretreatment reduced STIM1-STIM1 FRET to basal levels after ER-Ca^{2+} depletion. Together, these data suggest that E2 inhibits normal aggregation of STIM1. Fluorescent recovery after photobleaching (FRAP) studies revealed that E2 pre-treatment significantly decreased STIM1 motility, without inducing puncta formation, and addition of thapsigargin had no further affect. Because STIM1 is associated with microtubules through the plus-end tracking protein EB1, FRAP studies were performed on EB1-GFP expressing HEK293 cells. E2 exposure had no effect on EB1 motility, suggesting that the decrease in STIM1 motility by E2 is due to a direct effect on STIM1 and is not mediated through altered microtubule dynamics. FRAP studies were also performed on a STIM1 truncation mutant (STIM1-570STOP), which removes 115 amino acids from the C-terminal region of STIM1. When STIM1-570STOP was pretreated with E2, there was no difference in motility unlike WT STIM1, suggesting that STIM1's C-terminus is required for the inhibitory effect of E2. We next probed the phosphorylation status of STIM1 ± E2 and observed no increase in phosphorylation with E2. Therefore, we chose to probe upstream signaling proteins that may be activated in response to E2. FRAP was performed on cells that were pretreated with Ly294002 (10 μM) or Wortmannin (10 nM) to inhibit PI3K or with vehicle before E2 exposure. Both Ly294002 and Wortmannin exposure abolished the E2 effect, suggesting that E2 may activate PI3K to inhibit STIM1. Together these data suggest that E2/ESR1 inhibit Ca^{2+}-activated Cl^- secretion by alternatively mediating Ca^{2+} influx. We propose that this interaction may contribute to the gender gap observed in CF patients.

Regulation by CFTR Activity of OAG Activated Ca^{2+} Influx in Cystic Fibrosis Cells

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In the genetic disease cystic fibrosis (CF), the mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene leads to defective airway epithelial sodium (Na^+) and chloride (Cl^-) transports. In addition, abnormal calcium homeostasis has been measured in F508del cells. In that context, the transient receptor potential canonical channel 6 (TRPC6) has been proposed to be responsible for the abnormal OAG-dependent Ca^{2+} influx measured into the cytosol of epithelial cells. We have previously presented a model in which TRPC6 and CFTR ion channels are likely to interact within a multi-protein complex in which each ion channel is regulating the other (Antigny et al., 2011).
To further understand this functional coupling, our goal is now to determine whether the presence of CFTR proteins at the cell membrane or its presence and activity regulate OAG-Ca^{2+} influx.
To that end, we used chinese hamster ovary (CHO-K1) cells stably transfected with either pNut vector containing wild type CFTR (CHO-WT cells), F508del-CFTR (CHO-F508del) or G551D-CFTR (CHO-G551D). Cells were loaded with 3µM of Fluo-4 acetoxymethyl ester and Ca^{2+} influx was recorded following OAG (100 µM) stimulation. In addition, to study the effect of CFTR on the regulation of OAG-Ca^{2+} influx, we measured the activity of CFTR Cl^- channels on a cell population by the iodide (^{125}I) efflux technique in the presence of CFTR activators (forskolin, genistein) or inhibitor (GPinh5a).
Our first objective was to verify if the presence of CFTR at the cell membrane is required to modulate OAG-Ca^{2+} influx. Our results showed that, compared to CHO-WT cells, the OAG-Ca^{2+} influx was increased in CHO-F508del cells and normalized in the presence of the corrector Miglustat. Importantly, the abnormal increase in OAG-Ca^{2+} influx was reduced in CHO-G551D cells compared to CHO-F508del cells. In parallel, we measured the ion channel function of CFTR in these 4 cell lines and found activation by forskolin+genistein in CHO-F508del corrected by Miglustat, CHO-G551D and CHO-WT cells but, as expected, not in CHO-K1 cells. Taken together, these results suggest that CFTR at the cell membrane is needed to regulate OAG-Ca^{2+} influx.
Our second objective was to investigate in CHO-WT and CHO-G551D cells if the activity of CFTR at the cell membrane is required to modulate OAG-Ca^{2+} influx. Our preliminary results showed that in forskolin+genistein stimulated CHO-G551D cells the OAG- Ca^{2+} response was decreased. On the contrary, the inhibition of CFTR activity by GPinh5a up regulates the OAG-Ca^{2+} influx.
In conclusion, our results suggest that the activity of CFTR at the cell membrane regulates calcium responses in CF cells leading to near non-CF level of Ca^{2+} influx in pharmacologically corrected CF cells. This study begins also to explain how calcium homeostasis actors interact with CFTR, which could lead to the identification of new potential therapeutic target candidates.

Supported by Vaincre la mucoviscidose.

P. 15

Lipoxin A_4 Increases the Airway Surface Liquid Layer Height in Cystic Fibrosis Bronchial Epithelial Cells via an Apical ATP Release Activating a P2Y Receptor Pathway

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Cystic fibrosis (CF) is caused by a mutation of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene. One of the resulting abnormalities is dehydration of the Airway Surface Liquid (ASL) by a dysfunctional ion transport, leading to impaired mucocillary clearance, lung infection and inflammation. The eicosanoid LXA_4 described as a signal of the resolution of inflammation, is decreased in the lungs of patients with CF (*Karp et al, 2004*). This decrease in LXA_4 could participate to chronic airway inflammation in CF. However, the effects of LXA_4 on airway epithelial function in CF are not known. Using live cell imaging, we investigated the role of LXA_4 on ASL height in Human Bronchial Epithelial Cells (HBE) and Human Cystic Fibrosis Bronchial Epithelial (HCFBE) cells cultured in air/liquid interface. LXA_4 and its stable analogue TA39 increase the ASL height in a dose and time dependent manner in both the HBE and HCFBE cells. LXA_4 causes apical ATP release from HBE and HCFBE cells which was inhibited by the Panx1 channel inhibitor, carbenoxolone. Inhibition of the P2Y2 and P2Y11 receptors with reactive blue 2 and NF340 respectively, prevents the ASL height increase induced by LXA_4. The ASL height increase and ATP release induced by LXA_4 were both blocked by the FPR2 receptor inhibitor, Boc-2. We report a novel role for LXA_4 in restoring the ASL height in CF airway epithelium mediated by the FPR2 receptor ATP release and subsequent activation of P2Y receptors, which could lead to a new therapeutic route for CF patients.

Acknowledgements: Funded by the Children´s Medical and Research Foundation in Ireland, The Health Research Board of Ireland and INSERM.

Fusion Dependent Activation of Vesicular P2X4 in Alveolar Type II Cells Leads to a Cell Volume Increase-Coupling Secretion and Fluid Homeostasis in the Lung?

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The importance of proper fluid transport in the lungs is particularly obvious in Cystic Fibrosis and the various pathogenic states thought to arise from dehydration of the airways. While the role for extracellular ATP has been studied in relationship to ASL volume regulation, little has been determined regarding the effect of extracellular ATP on the alveolar epithelium and fluid homeostasis. The alveolar epithelium consists of type I and II (ATII) cells, with the ATII cells being responsible for surfactant secretion via exocytosis of lamellar bodies (LBs). Exocytic fusion of LBS with the plasma membrane results in a non-selective, transient and inward-rectifying cation current. Recently published data demonstrates that this current occurs across the purinergic receptor, P2X4, which is located on the membranes of fused LBs (PNAS 2011, 108(35):14503-8). This directed ion transport may result in fluid absorption or secretion from or into the pulmonary hypophase. To demonstrate that fluid transport across the alveolar epithelium is modulated by this fusion-activated cation influx following LB exocytosis, atomic force microscopy (AFM) and fluorescence experiments were performed. These experiments confirmed that exocytosis of LBs following extracellular ATP stimulation results in an instant increase in ATII cell volume that is regulated within minutes. In ATII cells stimulated with ATP and having LBs fusing with the plasma membrane, there was a 30% increase in cell height directly following fusion. However, this effect was not seen in ATII cells without fusion of the LBs with the plasma membrane. These data suggest that directed ion transport via fusion activated vesicular P2X4 and the resulting surfactant secretion is coupled to proper fluid transport in the lung.

Role of CFTR and Bicarbonate in CF Mucus Secretion and Microrheology

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Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel cause CF, which is characterized by the accumulation of viscous, sticky mucus in multiple organs. How this anion channel defect leads to altered mucus properties remains a central question in CF research, although there is growing evidence that bicarbonate may play an important role. The sites of CFTR expression in airway epithelium remain controversial, with recent immunostaining detection predominantly in surface ciliated cells, and extensive functional studies indicating expression in the glands. Mucus-secreting airway goblet cells, which derive from a common precursor, do not express sufficient CFTR to be detected by conventional immunostaining in situ. Therefore we examined mucin granules that were isolated from human airway epithelial cell lines and well-differentiated primary cells cultured at the air-liquid interface. CFTR and MUC5AC co-immunostaining was clearly observed on single granules isolated from control Calu-3 cells, whereas only MUC5AC was detectable when granules were isolated from a stable CFTR knock-down Calu-3 cell line (1). Western blots obtained after electrophoresis of apical and basolateral samples in non-denaturing agarose gels showed that MUC5AC was secreted exclusively to the apical side and had a more homogeneous size distribution when produced by CFTR-deficient Calu-3 monolayers than by control cells. The total amount of high molecular weight glycoconjugates (HMGs) in secretions aspirated from CFTR-deficient cells was greatly reduced as measured by spectrocolorimetry and this was due, at least in part, to increased adhesion of the mucin to the cells. We compared the biophysical properties of mucus collected from control vs CFTR-deficient air-liquid interface (ALI) cultures using two point microrheology. Secretions from CFTR knock-down Calu-3 cells were more viscous than those from control cells. When mucus was collected from non-CF primary epithelial cells, mucus viscosity and elasticity were both reduced by activation of CFTR with forskolin, and this effect was abolished by removing bicarbonate. The results indicate that CFTR is expressed at low levels on mucin granules from Calu-3 cells and highly differentiated primary cells, and modulates the properties of secreted mucus.

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Room: La Villa Rose

Posters – Lung Physiology and Pathophysiology

P. 18

MiR-449 MicroRNAs Control Biogenesis Of Motile Cilia In Human Airway Epithelium

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The airway epithelium is composed of basal, mucus-secretory and multiciliated cells. Multiciliated cells lining the surface of airway epithelium bear hundreds of motile cilia beating in coordinated waves, thus orchestrating the mucociliary clearance essential for airway cleansing.
Cystic Fibrosis (CF) is characterized by chronic infections leading to remodeling tissue with goblet cell hyperplasia and a loss of multiciliated cells which impair mucociliary clearance and ultimately lead to respiratory insufficiency. A better understanding of the mechanisms governing motile cilia biosynthesis appears mandatory for developing therapeutic strategies in CF for fastening the restoration of respiratory epithelium integrity and mucociliary clearance.
We have identified a functional link between microRNAs (miR or miRNAs: a class of small non coding regulatory RNA) and multiciliogenesis, using an in vitro model of human airway epithelium regeneration recapitulating the full differentiation process. First, we found that the miR-449 family of miRNAs were strongly up-regulated during multiciliogenesis and specifically accumulated in multiciliated cells. miR-449 knockdown using antagomiR dramatically reduced multiciliogenesis at an early step, before centriole multiplication. Then, we demonstrated that Notch1 and its ligand Delta-like 1 were specifically inhibited by miR-449. Human DLL1 and NOTCH1 protein levels were lower in multiciliated cells than in surrounding cells,
decreased after miR-449 overexpression and increased after miR-449 inhibition. Finally, multiciliogenesis was blocked by specifically disrupting Notch1-miR-449 interaction using protector oligonucleotides targeting miR-449 binding site.
Altogether our data demonstrate that miR-449 promote centriole multiplication and multiciliogenesis by directly repressing the Delta/Notch pathway. Our results unravel a novel mechanism in the Notch signaling system, whereby the signal-sending cell must undergo microRNAs-mediated clearance of the Notch pathway to execute its differentiation program. Our study paves the way for the identification of new relevant therapeutic targets in CF.

Causative Role of SLC26A9 in Pathogenesis of Bronchiectasis?

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Objectives: One of the main cause of bronchiectasis is mucociliary clearance defects, such as chanellopathies, the most frequent being Cystic Fibrosis (CF). However, nearly 30 % of the non CF bronchiectasis remain without any aetiology. Recent research has unlighted the interaction between the Cystic Fibrosis Transmembrane Regulator (CFTR) protein and some members of the conserved SLC26 (solute carrier family 26) anion transporters, with possible suppression of CFTR activation in case of mutations of SLC26T gene. The identification of the lung-specific SLC26A9 isoform has raised the question of a possible regulation of CFTR by SLC26A9 in the airways.

Methodology and Results: We extensively analysed all exons and flanking introns of the SLC26A9 gene in a cohort of 96 patients with disseminated bronchiectasis of unknown origin without two *CFTR* mutations. We identified one novel missense mutation in SLC26A9, a p.Arg575Trp (c.1725C>T in exon 16), located in the β3 loop of the STAS domain of the protein. This missense mutation was not found in an ethnically matched control and CF population. The patient carrying the Arg575 also carried a p.Phe508del mutation in *CFTR* at the heterozygous state. This patient presented an abnormally elevated sweat chloride concentration. PCR analysis revealed that SLC26A9 was expressed in sweat glands and respiratory epithelium.

To characterize the function and the expression of the mutated SLC26A9-575 protein, RNAs (5ng) coding for SLC26A9 Wild type (WT) and its 575 mutant were injected in Xenopus oocytes, co-expressing or not CFTR. A similar expression of SLC26A9WT and SLC26A9-575 was detected in the membrane extracts of the oocytes. Current/voltage analysis in control and low chloride conditions showed that the expression of SLC26A9-575 was associated with a significant reduction in membrane Cl^- conductance compared to oocytes expressing SLC26A9 WT. In oocytes co-expressing both SLC26A9 and CFTR, PKA (forskolin/ IBMX)-stimulated currents were 3 fold larger than the sum of the currents resulting from CFTR and SLC26A9 expression alone suggesting a functional potentiation of CFTR channel function by SLC26A9 WT. At variance, there was no significant difference between the PKA-stimulated currents from oocytes expressing CFTR alone and oocytes co-expressing CFTR and SLC26A9-575. This suggests that the functional potentiation of CFTR by SLC26A9 WT is abolished by the mutation 575.

Co-immunoprecipitation experiments confirmed the specific interaction between CFTR and both SLC26A9 WT and SLC26A9-575, and suggest that the inhibition of the functional potentiation of CFTR by SLC26A9-575 mutants was not related to a loss of CFTR-SLC26A9 protein interaction.

Modelling SLC26A9 STAS domain revealed a potential interaction site with other proteins located between the aa 567 and 585, and containing p575. We designed a peptide corresponding to the 568-588 hot site. Peptide expression in oocytes was associated with a significantly increased response of the PKA-stimulated Cl^- current mediated by CFTR whereas the random peptide was without effect.

Conclusion: We present a novel mutation in the SLC26A9 gene, which abolishes the functional potentiation of CFTR by SLC26A9 WT and is associated with bronchiectasis. Mutation in SLC26A9 might be a causing factor for bronchiectasis.

P. 21

Anti-Aspergillus Activity of Human Respiratory Epithelial Cells

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The innate immune response to fungi mainly involves macrophages and neutrophils. By contrast, the possible participation of respiratory epithelial cells (REC) has been poorly studied. In the present study we observed that REC display the property of inhibiting mycelium development of *Aspergillus fumigatus* (*A.f)* and that this activity is linked to PI3 kinase activation.
Bronchial cells (BEAS-2B cell line) were incubated with *A.f* and the growth of the fungus was estimated by optical microscopy observation and measurement of galactomannan concentrations in the extracellular media. The role of PI3 kinase was evidenced by the use of the specific inhibitor LY294002 (30 µM). Spore internalization in REC was followed by epifluorescence. FITC-labeled spores (green) when outside of cells were further labelled with an anti-FITC antibody labeled with a red fluorochrome (Alexafluor 568).
In the presence of REC, most of spores do not germinate as opposed to spores incubated in the absence of cells. The growth quantification by galactomamann concentration measurements display a 4.5 lower concentration in the presence of cells. The anti-aspergillus activity is inhibited when cells are incubated with LY294002, indicating the involvement of the PI3 kinase pathway. Of note, inhibitors of p38 MAP kinase and ERK1/2 are inactive. We evidenced that the activity is directed against the spores and not again the hyphae but is not linked to the spore internalization.
In conclusion, as macrophages and neutrophils, REC play a role in the anti-aspergillus activity. They are able to prevent the mycelium development and as such potentially prevent its dissemination. The next steps will be the analysis of i) the nature of this activity and, ii) the expression of this activity by cystic fibrosis REC.

Funding: This work was supported in part by a grant from Vaincre la Mucoviscidose

P. 22

P. aeruginosa Signaling Molecules Abolished Hypoxia-InducibleFactor-1 (HIF-1) Signaling Pathway in Human Cystic Fibrosis Airway Epithelial Cells

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Chronic pulmonary infection-inflammation cycles, as found in cystic fibrosis (CF) patients, are associated with hypoxic stress to lung tissue. The underlying molecular and cellular responses to hypoxia are regulated by the hypoxia-inducible factor-1 (HIF-1) transcription factor, composed of the oxygen-regulated subunit HIF-1alpha and the constitutively expressed HIF-1beta. In addition to oxygen limitation, HIF-1alpha has also been shown to be stabilized upon bacterial infection, eliciting activation of innate immunity, control of the pro-inflammatory response, and increased bacterial killing. Recently, we have shown that *Pseudomonas aeruginosa*, the primary pathogen associated with morbidity and mortality in CF patients, is also able to stabilize HIF-1alpha (Legendre *et al.*, 2011).
P. aeruginosa secretes multiple signaling factors, such as homoserine lactones, alkyl quinolones and phenazines, which are already known to interfere with host response. Therefore, in order to investigate if those secreted factors could influence HIF-1 signaling, *P. aeruginosa* cell-free supernatant extracts were prepared and tested for their ability to modulate HIF-1alpha levels.
Surprisingly, among *P. aeruginosa* factors tested, we found that a subset of these key signaling molecules were specifically able to completely suppress HIF-1alpha protein expression in human airway epithelial cells. The HIF-1alpha protein subunit is primarily regulated at a post-translational level by prolyl hydroxylase domain (PHD) proteins, through the hydroxylation of HIF-1alpha and subsequent 26S-proteasomal degradation. Using specific inhibitors targeting each step of this HIF-1alpha degradation cascade, we were able to demonstrate that these signaling molecules trigger degradation of HIF-1alpha protein via a 26S-proteasome-dependent mechanism but in PHD-independent manner. Moreover, the calpain pathway and ROS production, two other factors known to target HIF-1alpha to its proteasomal degradation, are not involved in our model. Currently, we are identifying precisely which *P. aeruginosa* signaling molecules are responsible for this degradation and are looking at the impact of these molecules under hypoxic conditions, where HIF-1alpha signaling is necessary to survive.
In conclusion, this is the first report showing that bacterial signaling molecules can repress HIF-1alpha protein level. This is highly significant in the CF context as many of *P. aeruginosa* molecules (i) have been found in the sputum of CF patients, and (ii) probably play a key role driving eukaryotic signalling since the thick mucus layers lead to reduction of the direct contact bacterial and epithelial cells. Finally, this study suggests that *P. aeruginosa* signaling molecules could be able to target directly HIF-1 signaling, potentially reducing the effectiveness of the airway response in the fight against infection, inflammation and hypoxia. All three interconnected factors are present in the CF lung environment and are responsible for the deterioration of pulmonary function.

Legendre C, Mooij MJ, Adams C and O'Gara F. Impaired expression of Hypoxia-Inducible Factor-1α in cystic fibrosis airway epithelial cells - A Role for HIF-1 in the pathophysiology of CF? *Journal of Cystic Fibrosis. 2011 Jul;10(4):286-90.*

A Role of TLR9 in *Pseudomonas aeruginosa*-Induced Lung Inflammation

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Background: *Pseudomonas aeruginosa* (*PA*) is present in lung of cystic fibrosis (CF) patients. TLR9 is cleaved by asparagine endopeptidase (AEP) but its role in host response to *PA* is unknown.

Aims: Determine the roles of TLR9 in host defense during acute lung infection by *P.A*.

Methods: Wild type (WT) and TLR9$^{-/-}$ mice were infected i.n. with 10^7 CFU of a laboratory strain of *PA* (PAK). 24 h post-infection, we compared: mouse survival, pro-inflammatory cytokine levels in broncho-alveolar lavages (BALs), alveolar macrophages (AMs) and PMN recruitment and *P.A* loads in lungs, cytokine secretion by a macrophage cell line (MHS) and primary AMs.

Results: (*i*) TLR9$^{-/-}$ showed less mortality than WT mice, (*ii*) significantly lower number of AMs, PMN and bacteria were detected in BALs of TLR9$^{-/-}$ compared to WT mice; (*iii*) decreased levels of IL-6, KC and TNFα were detected in BALs of TLR9$^{-/-}$mice; (*iv*) stimulation of MHS by either PAK DNA or CpG triggered both TLR9 cleavage and cytokine secretion; (*v*) both AEP inhibitor and concanamycin B, a vacuolar H(+)-ATPase inhibitor that increases endosomal pH, reduced CpG- and PAK DNA-induced IL-6 and TNFα secretion by MHS; (*vii*) CFTR inhibitor attenuated CpG- and PAK DNA-induced IL-6 and TNF-a secretion, suggesting that CFTR can interfere with TLR9 signaling.

Conclusions: Signalling through TLR9 plays a role in inflammation induced by *PA* acute lung infection. This may help to better understand the mechanisms involved in *PA*-induced inflammation and to development of potential drugs for the treatment of CF.

Acknowledgments : Vaincre la Mucoviscidose" (VLM) and the "Agence Nationale de la Recherche" (ANR) supported this work.

Investigating the Effects of Neutrophils on Biofilm Formation and Development of *Burkholderia cepacia* Complex (Bcc)

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The primary morbidity of Cystic fibrosis (CF) - the generation of unusually viscous pulmonary mucous - greatly diminishes mucociliary clearance from the lung. This engenders an environment well-suited to the proliferation of airborne microorganisms. CF patients' lungs become chronically-colonised with bacteria, despite the continuous accretion of large numbers of neutrophils into the alveolar lumen. Though less common colonisers of CF patients than *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex (Bcc) members are disproportionally associated with patient mortality owing to a unique morbidity they may induce, termed '*cepacia* syndrome', which is characterised by an acute-onset deterioration of lung function with associated septicaemia. Treatment is a challenge as most members of Bcc are multi-drug-resistant and display an ability to form biofilms, further reducing their antibiotic susceptibility.

In order to ascertain whether biofilm formation confers protection from phagocytosis by neutrophils on Bcc species, the nature of their interaction was examined using confocal laser-scanning microscopy (CLSM). In addition, the impact of neutrophils on biofilm development was investigated using *in vitro* static assays for biofilm density. CLSM confirmed that differentiated, neutrophil-like HL60 cells (dHL60s) remained at the exterior surface of established *B. multivorans* LMG 13010 biofilms (72 h) up to 2 hours post inoculation, impeding phagocytosis. This was despite limited migration and the appearance of pseudopodia. The CF-associated species *B. multivorans* LMG 13010, *B. cenocepacia* LMG 18826 and *B. dolosa* LMG 18941 were grown as static biofilms for 4, 24 or 48 hours, at which point dHL60 cells were added. Following 24 and 48 hours co-incubation, biofilm biomass was determined using crystal violet. There was a progressive dHL60 cell number-dependent enhancement of biofilm development for each species following addition of dHL60s, with respect to untreated biofilms. By contrast, inoculating planktonic LMG 13010 together with dHL60 cells resulted in less biofilm formation overall up to 72 hours ($p< 0.05$).

As neutrophils undergo apoptosis and disintegrate shortly after recruitment to the lung, this experiment was repeated with whole-cell lysates of dHL60 cells in order to investigate the effect of neutrophil components on biofilm development. Again, biofilm density increased after incubation with lysed dHL60s. These results suggest that Bcc members produce denser biofilms when incubated with dHL60 cells, either through interaction with the living cell or components thereof and that this impedes phagocytosis. Interestingly, dHL60s readily secreted significant quantities of IL-8 (2.5 ± 0.11 ng/$1x10^6$ dHL60 cells; $p< 0.05$) and extracellularly degranulated less myeloperoxidase enzyme when added to more mature biofilms (48-72 h).

Hence, biofilms facilitate Bcc species' persistence in the CF lung and the presence of neutrophils reinforces that biofilm. Therefore, strategies to improve neutrophil efficacy in clearing colonising Bcc must focus on overcoming biofilm-mediated resistance.

This work has been carried out with funding from Science Foundation Ireland (SFI)

Evaluation of an Aazithromycin Analog (CYS0073) in Lung Inflammatory Response

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Rationale: Azithromycin (AZM) is a macrolide with both antimicrobial and anti-inflammatory activities. Several clinical trials have ascertained that long-term use of AZM benefits cystic fibrosis (CF) patients and, recently, non-CF COPD patients. Although the side-effects of long-term use of AZM seem limited, increased antimicrobial resistances and emergence of hypermutable bacteria have been observed. In this context, a new drug, analog to AZM but lacking its antibiotic effects, namely CYS0073, has been developed. This molecule has been shown to correct the inflammation-driven immune dysfunction in inflammatory bowel and arthritis models. This study aimed at studying its effects in airway inflammatory models.

Methods: Human lung epithelial cells (A549) and murine primary alveolar macrophages were treated with CYS0073 or AZM. Pro-inflammatory cytokine production was determined by ELISA.
Results: We observed that CYS0073 (and AZM) was able to reduce the production of pro-inflammatory cytokines by macrophages (TNF-α and IL-6 measured) but not by epithelial cells (IL-8 measured).

Conclusion: Our preliminary results suggest that CYS0073 is as effective as AZM in reducing inflammation driven by macrophages. Further in vitro and in vivo studies will be performed to identify the CYS0073 molecular targets. Finally, in inflammatory respiratory diseases, it might be worth considering this analog as a new therapy, alternative to AZM, as it would not display the antibiotic resistance observed with AZM.

Lipoxin A_4 Delays the Invasion of Human Bronchial Epithelial and Human Cystic Fibrosis Bronchial Epithelial Cells by the Pathogen *Pseudomonas aeruginosa*

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Cystic fibrosis (CF) is caused by a mutation of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene resulting in decreased Cl^- secretion and hyperabsorption of Na^+ in the airway leading to dehydration of the Airway Surface Liquid (ASL) layer. The reduction in ASL height impairs mucocillary clearance and favours lung infection and inflammation. The eicosanoid lipoxin A_4 (LXA_4) described as a signal for the resolution of inflammation, is decreased in the lungs of patients with CF (*Karp et al, 2004*). Our team have also discovered that LXA_4 stimulates Cl^- secretion (*Bonnans et al, 2003*), increases ASL height in Human Bronchial Epithelial (HBE) and Human Cystic Fibrosis Bronchial Epithelial (HCFBE) cells and stimulates ZO-1 expression and transepithelial electrical resistance (TER) in Human Airway Epithelial Cells (*Urbach et al, 2008*). We hypothesize that decreased levels of LXA_4 in CF airways could favour the invasion of HBE and HCFBE cells by microorganisms. A high percentage of CF lungs are chronically infected by *P. aeruginosa* by adolescence and it is strongly implicated in lung destruction. Using a gentamicin invasion assay, we investigated the role of LXA_4 on the epithelial integrity of HBE and HCFBE when infected with *P. aeruginosa*. Our results indicate that LXA_4 (1nM) alone did not affect *P. aeruginosa* growth but prevented the invasion of HBE and HCFBE cells by *P. aeruginosa* within the first 3 hours after inoculation. We report a novel role for LXA_4 in delaying the invasion of *P. aeruginosa*, which could lead to a new therapeutic route for CF patients.

Acknowledgements: Funded by the *Children´s Medical and Research Foundation in Ireland, The Health Research Board of Ireland and INSERM*

P.aeruginosa-Dependent Kinase Signaling Network in Bronchial Epithelial Cells

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CF lung pathology is characterized by excessive release of pro-inflammatory cytokines and chemokines. Chronic infection by Pseudomonas aeruginosa amplifies the chemotaxis of neutrophils, which is mainly leaded by the expression and release of Interleukin-8 (IL-8) from bronchial epithelial cells. The persistence of a huge number of neutrophils in the bronchial lumen acts as a double-edged sword, as the redundant release of proteases contributes to the tissue damage and the release of neutrophil DNA, due to Neutrophil Extracellular Traps, apoptosis and hypoxic necrosis, further increases airway surface liquid viscosity. Identification of molecular targets amplifying this excessive pro-inflammatory signaling is important to test candidate therapeutic targets. We previously reported the cooperative role of the nuclear factors NF-kB, AP-1, NF-IL6, CREB and CHOP in the activation of transcription of IL-8 gene in human bronchial epithelial cells upon infection with P.aeruginosa. Protein kinases potentially activating these transcription factors, such as MAPK p38a, p38b, p38d, ERK1/2, JNK, RSK1/2, MSK1/2, HSP27, GSK3 and AKT have been investigated in human bronchial epithelial cells IB3-1 and CuFi-1 after infection with P.aeruginosa for 30 min. Short-time bacterial exposure increases the phosphorylation not only of p38 MAP kinase, but also of RSK1, MSK2, and HSP27. To understand their direct link to IL-8 expression, a panel of chemical inhibitors of protein kinases was tested in three different bronchial epithelial cell lines (CuFi-1, IB3-1 and Calu-3) stimulated with *P.aeruginosa* for 4 hours. IL-8 transcription and release was reduced by inhibitors of MAPK p38, ERK1/2 and JNK as indicated by using SB203580, U0126 and SP600125, respectively. Downstream effectors of p38 and ERK, respectively RSK and HSP27, seem to be also involved, as indicated by pre-incubating cells with SL0101 (for RSK) and KRIBB3 (for HSP27). These results consolidate previous findings and are consistent with parallel activator pathways, starting from MAPK p38, ERK and JNK, with their downstream sequential effectors RSK and HSP27. Since an interesting role has been recently reported on AKT in response to P.aeruginosa and TNF-a in bronchial epithelial cells (Cao J, 2010; Chattoraj SS, 2011), we investigated the link between AKT phosphorylation and IL-8 transcription. We observed both basal and P.aeruginosa-induced hyper-phosphorylation of AKT1/2/3 in IB3-1 and CuFi-1 cells. The pharmacological inhibitor of AKT triciribine (1 mM) and siRNA-mediated AKT3 silencing significantly reduced P.aeruginosa-dependent IL-8 transcription. Similar effect was obtained with wortmannin (100 nM), which inhibits the AKT-activator PI3K. The ligand/receptor coupling activating PI3K/AKT is under investigation. In conclusion, expanding our understanding on the pro-inflammatory kinase signaling network is relevant for CF, also considering the expansion of research on cancer and chronic inflammatory diseases aimed to develop pharmacological inhibitors of some of these transmembrane signaling kinases.

Supported by: Italian Cystic Fibrosis Research Foundation (#19/2011) with the contribution of ”Fondazione Cariverona”.

Targeting Enzymes Involved in the Metabolism of Glucosylceramide to Modulate Transcription of IL-8 Gene in CF Bronchial Epithelial Cells

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Accumulation of the sphingolipid (SL) ceramide is a peculiar feature in many respiratory disorders, including CF (Yang and Uhling, 2011), suggesting that CF lung pathology may be, at least partly, corrected by interfering with ceramide formation. The challenge therefore is to therapeutically adjust the pulmonary ceramide levels to their physiological range required to successfully fight infection with *P.aeruginosa*.
The iminosugar, N-butyl deoxynojirimycin (NB-DNJ, miglustat) is an inhibitor of the synthesis of glycosphingolipids (GSLs) which produces an anti-inflammatory effect *in vitro* and *in vivo* and reduces the *P.aeruginosa* induced immunoreactive ceramide expression (Dechecchi 2011). Also the galactose analogue *N*-butyldeoxygalactonojirimycin (NB-DGJ), has an anti-inflammatory effect in bronchial epithelial cells (Dechecchi, 2008). Considering that miglustat and NB-DGJ share two common enzyme targets: ceramide glucosyl-transferase (GlcCerT) and non-lysosomal glucosylceramidase (GBA2) both involved in the metabolism of glucosylceramide (GlcCer), we extended the investigation to other two more potent inhibitors of these pathways: Genz-123346 and Genz-529468. IB3-1 cells were incubated with ranging doses of Genz 123346 or solvent, 24 hrs before infection and the expression of IL-8 mRNA was measured 4 hrs later. We found that Genz-123346 significantly reduces the expression of IL-8 mRNA induced by *P.aeruginosa* (IC50=1.8 microM), similarly to miglustat and NB-DGJ. These results were confirmed also in CuFi-1 cells. Then, CF bronchial cells were treated with Genz-529468 before infection. Dose-response experiments showed that Genz-529468 is very potent in reducing the transcription of IL-8 (IC50=3 nM). Therefore, Miglustat, NB-DGJ, Genz 123346 and Genz-529468 reduce the immune response to *P.aeruginosa* in CF bronchial epithelial cells, being Genz-529468 the most powerful. Importantly, this inhibitor seems to be very selective for GBA2, suggesting that it may produce an anti-inflammatory effect by targeting the activity of GBA2. Considering that GBA2 is located close to the cell surface, a direct or indirect role in the SL metabolism linked to ceramide-mediated signaling processes might be envisioned. Our results further strengthen the hypothesis that the pharmacological modulation of SL metabolism, that can intercept the ceramide metabolic pathway at many levels, may be an effective approach for the treatment of CF lung inflammation. In particular GBA2 may be at least one of the targets to reduce the exaggerated inflammatory response in CF lungs.

Supported by: Italian Cystic Fibrosis Research Foundation (#16/2010) with the contribution of ”Assistgroup”, “Latteria Montello SpA” and “Delegazione FFC di Imola”.

Neutrophil Elastase Degrades CFTR *In Vitro* and *In Vivo* and Disables CFTR Channel Function In a P.aeruginosa Lung Infection Model

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Objective of the study: Cystic fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator (CFTR). Mutant CFTR (DF508) expression leads to ionic exchange imbalance, bacterial infections and chronic lung inflammation,and loss of function. Although lung stromal and inflammatory cells-derived proteases have well described maladaptive effects on lung tissue in this pathology, the direct effect of proteases on CFTR has not yet been addressed. The aim of the study was to characterize the effect of the neutrophil-derived protease elastase (NE) on CFTR protein and function as a Cl^- channel both *in vitro* and *in vivo*.

Methods:

1)In vitro: Epithelial cells (human bronchial NCI-H292 cell-line) were infected with adenovirus (Ad)- GFP-WT-CFTR or GFP-DF508 CFTR at a moi of 150 for 48 h. Cells were then incubated with either purified NE (10-100 nM) or human neutrophil lysates. After 7 h, GFP-CFTR expression (Western Blot analysis using anti-GFP antibodies) and function (patch-clamp analysis) were measured.

2) In vivo: C57Bl/6 mice were instilled i.n with PBS or 10^9 pfu of Ad-GFP-WTCFTR or Ad-null control. 4 days later, 50 ug of NE was instilled i.n and 6 hr later, mice were sacrificed, bronchoalveolar lavage fluid (BALF) was obtained, and lungs extracts prepared for Western Blot analysis. Alternatively, NE -/- mice or C57/Bl6 littermates were sequentially infected with Ad constructs (for 4 days, see above) and *P.a* bacteria (5.10^6 cfu/mouse). 20 hr later, lungs were processed as above. Part of lungs were embedded in O.C.T. compound (Tissue-Tek), sectioned at 5 µm thickness and stained with hematoxylin and eosin (H&E).

Results:

1) *In vitro:* we show here in bronchial epithelial cells that NE degrades both WT and DF508 CFTR. This cleavage was not dependent on intra-cellular NE signalling activity, since inhibitors of the ERK, p38, PI3K, EGF-R, NF-kB pathways did not prevent NE-induced CFTR degradation. NE main cleavage site occured in the CFTR NBD1-R region. Functionally, NE completely abolished forskolin-activated CFTR currents in cells infected with Ad-GFP-WT CFTR, while, expectedly, cells infected with Ad- GFP-DF508 CFTR alone or infected with Ad-GFP-DF508 CFTR and treated with NE yielded no current.

2) *In vivo:* we show that Ad-WT CFTR (and not Ad-controls) induced high amounts of CFTR expression without inducing *per se* significant cell inflammatory influx nor NE activity. When NE was instilled i.n, it induced very significant CFTR degradation, as assessed by Western Blot analysis, yielding a degradation product similar to that observed *in vitro* (110 kDa). This resulted in loss of channel function (npd measurement *in vivo*). When mice were infected with Ad-CFTR (or Ad controls) followed by *P.a* infection, a similar CFTR degradation product was produced, and this was NE-dependent (comparison between WT and NE -/- mice).

Conclusions: We demonstrate here that NE is instrumental in degrading CFTR *in vitro* and *in vivo* and is able to disable its channel function. Our data identify NE as an inflammatory mediator implicated in the down-regulation of CFTR expression and the potential re-inforcement of the inflammatory vicious circle observed in cystic fibrosis.

CF Epithelial Cells Lack the Anti-Inflammatory Zinc Finger Domain of A20 and the Adaptor Protein TAX1BP1

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Objectives: A20 inhibits TLR-induced NF-κB signalling at the level of TRAF6, but is reliant on complex formation with TAX1BP1, a TRAF6 binding protein[1]. We have previously shown reduced expression of A20 in LPS stimulated CF epithelial cells together with an inability to interact with TRAF6[2]. Gibberellin (GA) is a plant diterpenoid involved in the regulation of plant development via induction of A20/ZnF proteins[3]. GA has previously been shown to have anti-inflammatory properties in diabetic mice[4].
We hypothesise that induction of A20 would compensate for the loss of normal A20 function resulting in reduced inflammation in CF epithelium.

Methods: Cell lines (16HBE41o- , CFBE41o-) and nasal epithelial cells (NECs) from patients (F508del homozygous) and age-matched controls were stimulated with LPS (*P.aeruginosa*, Sigma, 10mg/ml) for 0-24h. Full length A20 and TAX1BP1 were assessed by Western Blotting. Additionally, expression of the C-terminus of A20 was determined. In an attempt to induce A20, cells were pre-incubated with Gibberellin (GA, 30 uM) 1h prior to addition of LPS and induction confirmed by qPCR.

Results and Conclusions: A20 mRNA was maximally induced in 16HBE41o- 1h after LPS stimulation. Thereafter, A20 expression fell back to basal levels. However, in 16HBE41o- pretreated with GA, A20 remained at maximal levels 24h after LPS stimulation. This increase in A20 expression was accompanied by reduced IL-8 release in GA treated cells. Reduced IL-8 secretion was confirmed in primary control NECs treated with LPS for 24h where a 34% reduction was observed with pretreatment with GA. However, this anti-inflammatory effect was not maintained in CF cells.
The C-terminal domain of A20 facilitates A20 interaction with TAX1BP1 and TRAF6[3], but CFBE41o- cells poorly expressed the C-terminus. Additionally, TAX1BP1 mRNA expression was abolished in CF epithelial cells (both cell lines and NECs) following 24h LPS stimulation. Overall, we report significant deviations in the expression of A20 (C-terminus) and TAX1BP1 in the CF epithelium, which cannot be reversed through treatment with GA. These deviations may explain the lack of association with TRAF6 and may contribute to chronic and persistent airways inflammation.

[1] Shembade *et al.* 2009, [2] Kelly *et al.* 2010, [3] Liu *et al.* 2011; [4] Davis *et al.* 1989, [5] Shembade *et al.* 2010.

Supported by CF Trust (PJ541)

Room: La Villa Rose

Posters – CF Microbiology

P. 32

Synthesis and Screening for Anti-Biofilm Activity of New Homoserine Lactones (HSL) Analogs of *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is a ubiquitous bacterium that can cause severe and chronic lung infections in patients with cystic fibrosis, particularly in establishing a resistant structured form referred to as biofilm, which is very difficult to eliminate. Biofilm formation is mainly regulated by the communication system of the Quorum Sensing (QS), which is controlled by the natural N-acyl homoserine lactones (HSL) molecules: C4-HSL and C12-HSL. *Pseudomonas aeruginosa* is an example of a human opportunistic pathogen for which HSL-related compound have been described as potent inhibitors of biofilm formation and virulence factors, given their similarity to the natural QS autoinducers. Thus, the aim of our study is to design potent analogs of natural HSL and to screen them for their anti-biofilm activity.

Eleven original compounds based on the structure of the C4-HSL were synthesized, and then screened for their ability to impair biofilm formation in an innovative *in vitro* model that allow to analyze the impact of compounds on different stages of biofilm formation. In this model, the analogs effectiveness was evaluated by CFU enumerations of adherent cells and confocal microscopy observations. Among this first series of analogs, a compound (N-pymimidyl butanamide) showed a significant inhibition of biofilm formation when added from the early stages of biofilm formation (i.e., adhesion and microcolony formation) in a dose-dependent manner, coupled with an absence of cytotoxicity. To demonstrate antagonism with C4-HSL, we also showed that anti-biofilm activity of this compound was impaired when C4-HSL was added. A structure-activity study was then performed on this compound to synthesize a new series of analogs, which are actually tested for their biofilm inhibition. In parallel, we developed the quantitative PCR method in order to increase the rapidity and efficiency of the quantification of the adherent biomass.

To improve these initial results, we propose to synthesize new analogs, based on the structure of the C4-HSL, and then on that of the C12-HSL, and to screen them for their anti-biofilm activity, in order to affect all regulatory mechanisms of the QS. We also propose to assess the screening model in anaerobic conditions in order to approximate the *in vivo* colonization conditions by the bacterium. Furthermore, mechanisms of action of selected compounds and their potential ability to enhance the activity of antipyocyanic antibiotics will be analyzed. Finally, analogs cytotoxicity will be tested on lung cells to select candidate molecules for testing in animal models that is the first phase required before any clinical development in humans.

Thus, this project should allow us to identify one or more active compounds against the *Pseudomonas aeruginosa* biofilm and to define optimal conditions for further *in vivo* investigations like test concentration, preventive and/or curative activity and establishment of new therapeutic protocols with or not antibiotics association.

Altered Virulence Features of Sequential Clonal Variants of *B. cenocepacia* Isolated from a CF Patient

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Burkholderia cepacia complex, (Bcc) comprises seventeen bacterial species that cause severe respiratory infections in Cystic Fibrosis (CF). These pathogens are clinically challenging as they are highly transmissible and antibiotic resistant. This study investigates the virulence features of 3 *B. cenocepacia* clonal variants isolated from a CF patient over four years. Isolate IST439 was the first bacterium recovered while the clonal variant IST4113 was obtained after 3 years of persistent infection and intravenous therapy with ceftazidime/gentamicin. The last isolate IST4134 was obtained prior to the death of the patient from cepacia syndrome.

The potential adaptation of *B. cenocepacia* to lung conditions during chronic infection was examined. The two later isolates opened epithelial tight junctions more rapidly than the initial isolate (2hrs Vs 6hrs), suggesting that during chronic infection these strains adapt to gain access to underlying tissue. Furthermore, the two later isolates were on average more than 4 times more invasive of epithelial cells than the first isolate and induced significantly greater levels of IL-6 and IL-8 from epithelial cells compared to the initial isolate ($P< 0.001$).

The ability of these clonal variants to utilize host proteins as an iron source was investigated. It was found that they can all utilize hemin and ferritin as an iron source in an iron deprived environment ($P< 0.001$). In response to *P. aeruginosa* exoproducts, gene expression of the siderophore ornibactin is significantly up-regulated in all isolates ($P< 0.001$), although their growth is inhibited by 50%, indicating that iron acquisition strategies are important for survival in the lung.

In summery the epithelial interactions of *B. cenocepacia* are altered under iron limiting conditions and possibly during chronic infection and the siderophore response of *B. cenocepacia* is up-regulated in the presence of *P. aeruginosa*. Ongoing studies are aimed at a greater understanding of the potential adaptation of *B. cenocepacia* in the CF lung and the role of iron availability in *B. cenocepacia* pathogenesis.

Funded by Technological Sector Research Programme Strand III

The *Pseudomonas aeruginosa* Density in Airways of Cystic Fibrosis Patients Treated for an Exacerbation is Not Linked to Clinical Improvement

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Objectives: Cystic fibrosis (CF) patients colonized with *Pseudomonas aeruginosa* suffer intermittently from pulmonary exacerbations. Evidence exists that these exacerbations are a risk factor for a more rapid decline of lung function. It was previously shown that there is no significant increase of the bacterial density prior to exacerbation. We investigated whether there is a linkage between the *P. aeruginosa* density and the clinical parameters during hospitalization combined with antibiotic exacerbation treatment and intensified physiotherapy.

Methods: The 15 included CF patients, attending the Ghent University Hospital all suffered from an exacerbation and were hospitalized during a 2-week period in which they received antibiotic treatment. At day 1, day 8 and day 15, the FEV_1, FCV, concentration of white blood cells, C-reactive protein (CRP) and sedimentation were determined. On the same days, the *P. aeruginosa* density in sputum samples (obtained after physiotherapy) was determined using bacterial culture, qPCR and propidium monoazide qPCR (PMA-qPCR). This latter technique allows to PCR amplify only the DNA from living *P. aeruginosa* bacteria.

Results and conclusion: For all patients, there is an improvement of the clinical parameters during the hospitalization. Quantitative PCR data revealed a significantly higher *P. aeruginosa* density (± 10^2 cfu/ml) in comparison with quantitative culture, while for PMA-qPCR, the density was 10 times higher compared with culture. All three techniques confirmed that for most of the patients, there was a decrease of the *P. aeruginosa* density after 15 days of treatment, i.e. by culture for 11 patients, by qPCR for 11 and by PMA-qPCR for 12 patients a decrease of the *P. aeruginosa* density was noticed after 15 days of treatment. However, out of this group of patients with a general density decrease (i.e. after 15 days), a substantial part of patients had an increased *P. aeruginosa* density comparing day 15 with day 8. More specifically, by culture 3 patients, by qPCR 2 patients and by PMA-qPCR 5 patients had a higher *P. aeruginosa* density at day 15.
In conclusion, we can state that a) the qPCR based techniques detect much higher (log1 - log2) *P. aeruginosa* densities in comparison with the culture technique; b) the antibiotic treatment is able to reduce the bacterial density for most patients, but for a substantial part of these patients, there is a renewed increase of the density between day 8 and day 15, and c) the change of the *P. aeruginosa* density does not correlate with the improvement of the clinical parameters during hospitalization, because all patients had improved clinical parameters at the end of the stay, despite a minimal decrease or even increase of the *P. aeruginosa* density for some patients.

Room: La Villa Rose

Posters – CF Models

P. 36

A Novel Approach to the Development of a Murine Model of CF Associated Chronic Pulmonary Bacterial Infection

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Research into the immunopathogensis of chronic bacterial colonisation of cystic fibrosis patients has been significantly hampered by the lack of a biologically relevant animal model of chronic infection. Whilst several models of chronic bacterial infection have been described, these are mostly reliant on the use of artificial embedding materials to prevent rapid immune clearance of the bacteria. We hypothesised that a carefully selected, rational combination of bacterial isolate, mouse strain and infection regime, will result in a physiologically and clinically relevant murine model of chronic *S. aureus* and *P. aeruginosa* infection without the use of artificial embedding materials. Previously published models have largely utilised PA01 and lab *S. aureus* strains such as the Newman strain. We have screened panels of several hundred clinical isolates of S*. aureus* and *P. aeruginosa* and 5 strains of each were selected for murine infection experiments, based on genotypic and phenotypic characteristics. The selected *S. aureus* isolates include haemolytic and non-haemolytic strains originating from CF and non-CF sputum. The *P. aeruginosa* isolates originated from CF or ICU patients and were selected on the basis of phenotypic characteristics including ability to form biofilm *in vitro*. Significant differences in the *in vivo* virulence of these strains have been shown. Equally essential is the choice of mouse strain. Published work has almost exclusively been carried out using a C57BL/6 background however these mice are inherently resistant to bacterial infection We have performed survival experiment comparing the colonisation of C57BL/6, A/J, BALB/c, Biozzi, FVB/N, NIH, SJL/J, CD1, MF1 and NMRI female mice with *P. aeruginosa* and *S. aureus*. The overall aim is to find the combination of mouse and bacterial strain which results in the prolonged survival of the animal whilst maintaining chronic pulmonary infection resulting in a murine model of chronic infection which accurately replicates the *in vivo* processes during a natural infection. Once established, this model will allow investigation of the intricate immunological and physiological disease processes involved during chronic pulmonary infection of CF patients.

P. 37

KCNN4 Potassium Channel Inactivation Decrease Lethality in a Cystic Fibrosis Animal Model

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Cystic fibrosis (CF) animal models are affected of severe and lethal intestinal obstructive desease. The mouse model of CF showed poor hydration of luminal content, and increased levels of inflammatory markers are found on tissue isolated from the small intestine of those animals. The KCNN4 channel is widely expressed in cells of the inmmune system and its activity is known to modulate the function of T-lymphocytes, macrophages and mast cells. Given the known role of KCNN4 in the regulation of cells of the immune system we aim to study the role of KCNN4 pharmacological and genetic inactivation on CF intestinal disease.

We generated a double mutant animal carrying the deltaF508 CFTR mutation and Kcnn4 knocked-down gene expression and observed an almost complete reverse of lethal intestinal obstruction episodes compared to the deltaF508 animals from 43% to 3% lethality at 60 days of age. The reverse of lethality was not related to gain fo intestinal function since weight rate curves of deltaF508 and double mutant animals were equal and lower than control and KCNN4 null mice. Small intestine tissue samples of deltaF508 mice have a four-fold increase of Toluidine blue stained mast cells in the vecinity of Peyer´s patches (1.46 cells/field) compared to control mice (0.35 cells/field). In samples obtained from double mutant mice we observed a reduction of mast cell number to values similar to control mice (0.4 cells/field). The increase of mast cells seems to be restricted to the small intestine since tissue samples obtained from the skin of these animals showed to have similar numbers of mast cells. Bone marrow derived mast cells were used to study migration and proliferation of cells o in vitro assays. We observed that inhibition of KCNN4 channels by the specific blocker TRAM-34 reduce migration of mast cells on the Trasnwell assay. Proliferation of cells was not affected by KCNN4 genetic deletion or CFTR deltaF508 mutation. DeltaF508 animals injected with TRAM-34 for 7 days (daily subcutaneous dose) showed a reduction of intestinal mast cells (0.2 cells/field). Finally, serum samples from deltaF508 animals showed and increase of IgE levels (determined by ELISA) compared to control mice.

In summary, genetic deletion of KCNN4 reduced the lethality of the deltaF508 mice. We found that the increased number of mast cells present in the small intestine of deltaF508 mice are reduced by the genetic or pharmacological inactivation of KCNN4. In vitro assays indicates that KCNN4 is needed for mast cell response to chemotactic agents. The increased levels of IgE detected on DeltaF508 animals might be related to the increase of mast cells. The role of mast cells on intestinal CF disease and their role on inflammation remain to be solved.

Funded by FONDECYT 11100408

P. 39

AGER -429T/C is Associated with an Increased Lung Cystic Fibrosis Disease Severity

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Background: The clinical course of cystic fibrosis (CF) lung disease varies between patients bearing identical *CFTR* mutations, suggesting the involvement of modifier genes besides environmental factors. In CF, the exuberant airway inflammation drives the decline of lung function which remains the main cause of morbidity and mortality. RAGE, a pro-inflammatory protein predominantly expressed in the respiratory tract, is encoded by the polymorphic gene *AGER*. We assessed the association of one of its promoter variant *AGER* -429 T/C with lung disease severity in CF patients from the French CF Gene Modifier Study. In addition, we tested whether this variant was associated with a modulation in RAGE expression in vitro.

Methods: We analyzed the lung function of 774 European CF patients p.Phe508del homozygous. Lung function data, specifically FEV_1, were analyzed as CF-specific percentile adjusted on age and height and further corrected for attrition due to mortality. *AGER* -429T/C polymorphism was genotyped by allelic discrimination by fluorescence. The function of this promoter SNP was evaluated *in vitro* by the measurement of the luciferase activity after transfection of bronchial and alveolar epithelial cells.

Results: The distribution of the genotype is similar to that found in the Caucasian population. The majority of patients have the genotype TT (73%), 24% are heterozygous TC and 3% are homozygous for the minor allele CC. We observed that *AGER* -429 minor allele (C) was associated with poorer lung function ($p = 0.04$). *In vitro*, the promoter activity, measured by the luciferase activity, was also significantly higher in the cells transfected with *AGER* -429C allele compared to the cells transfected with the *AGER* -429T allele ($p= 0.016$).

Conclusion: Our findings support the hypothesis that *AGER* can be a modifier gene of lung disease severity in CF. Increased RAGE expression had been already found on airway neutrophils in CF and could lead to an increased airway inflammation and more severe lung disease. Altogether, these results could suggest that RAGE could be an interesting modifier gene of CF lung disease.

Genetic Investigation of Cystic Fibrosis Transmembrane Regulator (CFTR) Mutations in a Cohort of Consecutive Patients Candidate for Assisted Reproductive Techniques

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Objectives: As shown in literature, an association between cystic fibrosis transmembrane regulator (CFTR) gene mutations and infertility may occur. The present study, investigated the frequency of mutations in the CFTR gene, in a group of consecutive patients candidate for assisted reproductive techniques with the aim of identify subjects carriers of the most severe ones.

Methods: Overall, 22,416 alleles were screened for 56 CFTR gene mutations and IVS8-poliT polymorphism utilizing the CFTR INNO-LiPA Amplification kit including both general and Italian regional strips. The frequency of mutations were separately calculated and the Chi-square test was used for comparisons.

Conclusions: CFTR mutations were detected in 6.2% of the screened alleles, a percentage similar to that reported in the general population. In the large group of alleles analyzed 93.4% were wt, 4.4% were characterized by mild mutations, and 1.7% by severe or severe/mild mutations. Indeed, the most common severe mutation was ΔF508/N observed in 192/22,416 (0.86%) of all alleles analyzed, followed by the N1303K mutation with the frequency of 36/22,416 (0.16%). Whereas regarding mild mutations, the most frequent was the 5T polymorphism present in 916/22,416 (4.1%). Our results together with previous studies, reinforce the importance of an accurate determination of mutations in the CFTR gene, including the 5T polymorphism, in order to inform the couple of their carrier risk and the possibility on having affected child. Moreover, our findings highlight the potential of genetic screening as a tool to identify possible compound heterozygous subjects without CF-like symptoms.

Room: La Villa Rose

Posters – Therapeutic Strategies to Correct Ion Transport Defects

P. 41

Mechanism of the CFTR-VX-770 Interaction: Direct Binding and ATP-Independent Modulation of Channel Activity

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The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) is an ATP-activated, phosphorylation-dependent chloride channel on the apical membrane of epithelial cells. Disease-causing mutations in the CF gene can lead to defects in CFTR protein folding, trafficking and/or channel function. The G551D mutant is the second most clinically relevant mutation and displays proper membrane trafficking but impaired channel activity at the cell surface. Recently, the small molecule VX-770 (Ivacaftor; Kalydeco), has shown efficacy in restoring lung function in patients bearing the G551D mutation and this has been linked to repair of the channel gating defect on the apical surface of the respiratory epithelium. However, these studies have shed little light on the detailed mechanism of action of VX-770.

As CFTR channel activity is regulated by phosphorylation and ATP binding, the mechanism of VX-770 activity should be interrogated in a system which permits their control. The current studies, employing purified and reconstituted normal and mutant CFTR protein, reveal that VX-770 binds directly to the PKA phosphorylated form of Wt, G551D and F508del-CFTR protein to modulate its activity. Further, we show that VX-770 likely opens CFTR channels by binding to an allosteric site, distinct from the canonical catalytic site to which the natural activating ligand ATP binds. Channel opening by VX-770 occurs in the absence of added ATP and appears to be independent but additive with ATP. Our findings explain why VX-770 is effective in patients bearing the G551D mutation, a mutation which disrupts the canonical catalytic site.

We have initiated studies to determine the structural basis for the potentiating activity of VX-770, including an examination of structural elements of the VX-770 molecule and the features of the binding site on CFTR. To determine structural features of VX-770 important for potentiation, we examined the effect of a VX-770 fragment and show this portion doesn't potentiate CFTR activity. This indicates that this particular region of the VX-770 structure alone is not sufficient for binding and potentiation of channel activity, and we are examining other portions of the molecule.

CFTR contains multiple Trp residues, at least some of which are sensitive to the polarity of their local environment. Addition of physiological concentrations of VX-770 to purified, PKA-phosphorylated full length CFTR quenches the intrinsic Trp fluorescence of the protein. The effect is progressive and saturable, suggesting direct binding to a discrete binding site on the protein. Addition of VX-770 to CFTR protein that had not been pre-phosphorylated with PKA did not induce progressive and saturable quenching of the fluorescence. This work shows that intrinsic Trp fluorescence measurements can be used to monitor the properties of the VX-770 binding site and we are working towards defining its features.

To summarize, we show that VX-770 binds directly to CFTR to modify channel opening through a non-canonical mechanism. Our studies set the stage for future work in which VX-770 can be used as an incisive probe with which to define the structural properties of the protein and of the small molecules which mediate the gating activity of CFTR.

The Iminosugar IsoLAB Corrects the Defective Trafficking of F508del-CFTR in Cystic Fibrosis Cells

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Cystic fibrosis (CF) is an autosomal and recessive disease due to mutations in Cystic Fibrosis Transmembrane conductance Regulator gene (*CFTR*). Around 90% of people with CF have at least one copy of the F508del mutation and over 70% are homozygous for this mutation. This mutation leads to the retention of CFTR protein in the endoplasmic reticulum (ER) to abnormal gating of CFTR channel and endocytosis. In previous study we identified several iminosugars as new correctors of the F508del-CFTR defective trafficking (Norez et al., 2006; Ardes-Guisot et al., 2011) and proposed that inhibition of the ER-α-glucosidase is part of the mechanism of correction. However, in a recent work we hypothesized that another iminosugar, IsoLAB, corrects the F508del-CFTR trafficking independently of the ER-α-glucosidase inhibition (Best et al., 2010). The aim of the present work was to study the pharmacological effect of IsoLab and its mechanism of action on different human CF cells.

To that end, the CFTR activity was recorded in response to a cocktail of activators (forskolin 10µM + genistein 30µM) ± the CFTR inhibitor (CFTRinh-172, 10µM) in different human CF cells: CF epithelial tracheal CF-KM4 cell line; CF epithelial nasal CF-15 cell line, Embryonic Kidney transiently transfected HEK293-F508del-CFTR cell line, epithelial carcinoma stably transfected Hela-F508del-CFTR cell line and airway epithelial cells freshly dissociated from CF lungs.

Using the iodide efflux technique, we demonstrated that IsoLAB restores a CFTR activity in CF15 cells with an EC_{50} of 25µM after 2h of treatment. The correcting effect of IsoLAB is time-dependent with a maximal effect obtained after 2-4h of treatment, and then progressively declined with a total loss of effect after 8h. The level of correction induced by IsoLAB (100µM, 2h) is similar to those observed in CF cells treated with miglustat (100µM, 2h) or exposed to a low temperature (27°C, 24h). Altogether, these results show that the rescue of F508del-CFTR function by IsoLAB is time- and concentration-dependent with no effect on the stabilization of F508del-CFTR at the cell membrane. Using single cell fluorescence imaging, we confirmed the corrector effect of IsoLAB (100µM, 2h) in several CF cell lines: CF15, CF-KM4, HEK293-F508del-CFTR, Hela-F508del-CFTR, and more interestingly in airway epithelial cells freshly dissociated from CF lungs. Finally using patch-clamp technique in whole-cell configuration on HEK293 transfected either by F508del-CFTR or by wt-CFTR, we demonstrated that the amplitude of the current densities recorded on IsoLAB-corrected CF cells corresponds to about 60% of wt-CFTR activity.

We conclude from this present work that IsoLAB is a potent corrector of F508del-CFTR trafficking. Further study will be conducted to determine the mechanism of action of IsoLab in CF cells.

Supported by « Fondation pour la Recherche Médicale » and « Vaincre la Mucoviscidose »

P. 43

Modulation of Annexin A5 Expression by Gonadotropin-Releasing Hormone (GnRH) in Human Bronchial Epithelial Cell Lines: Consequences on CFTR Protein

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Background: The Cystic Fibrosis Transmembrane conductance Regulator (CFTR) protein functions as a cAMP-activated chloride (Cl-) channel, regulated by protein-protein interactions. Changes in its interacting partners are implicated in the pathophysiology of cystic fibrosis but the extend of which CFTR is regulated by these interactions remains unknown. Nevertheless, we previously showed that annexin A5 (anxA5) binds to both normal and F508del-CFTR through the first nucleotide binding domain (NBD1). Moreover, in transfected epithelial cell lines with pcDNA3.1/anxA5, anxA5 overexpression increases CFTR levels in the plasma membrane and enhances CFTR-dependent Cl- secretion in both wild-type (wt-CFTR) and F508del-CFTR expressing cells.

Objectives: Thus, one therapeutic approach would be to find a drug to modulate anxA5 expression and subsequently to improve CFTR-dependent Cl- secretion in F508del-CFTR expressing cells. Because GnRH (type I) is known to stimulate anxA5 expression in the anterior pituitary cells, this hormone is a potential candidate.

Materials and methods: Using RT-PCR techniques and western blotting, expression of GnRH receptors was studied in human bronchial epithelial cell lines expressing either wt-CFTR or F508del-CFTR (16HBE14o-, CFBE41o-/wt, CFBE41o-/F508del). We further treated these cells with GnRH (10-9M) and anxA5 expression was assessed by western blotting. Effects of GnRH treatment on CFTR cell surface expression is now under investigation by membrane biotinylation techniques. Its impact on CFTR-dependent Cl- secretion will be studied by iodide effluxes and patch-clamp experiments. Finally, RT-PCR, western blotting and immunohistochemistry studies of GnRH receptor will be performed in human airways and primary cultures of human bronchial epithelial cells to check potential clinical use of the hormone.

Results: Beside the GnRH receptors expression in the different epithelial cell lines, we also showed that anxA5 expression is increased after 1h incubation with the hormone. Moreover, biotinylation experiments after GnRH exposure revealed an increased surface expression of CFTR channels in wt-CFTR expressing cells. Experiments for F508del-CFTR expressing cells are under investigation. Preliminary RT-PCR experiments show that GnRH receptors are present in primary cultures of human bronchial epithelial cells.

Conclusion-Perspectives: GnRH treatment leads to an anxA5 overexpression in both wt and F508del-CFTR expressing cells. We have to perform iodide effluxes and patch-clamp experiments in order to assess the impact of anxA5 overexpression on CFTR-dependent Cl- secretion. Biotinylation assays show an increased surface expression of wt-CFTR channels after GnRH exposure. The remaining question is to know wether it is due to a regulation of traffic events to the cell surface or to a stabilization in the plasma membrane. Finally, GnRH analogs such as Enantone (leuprorelin) or Decapeptyl (triptorelin) can be found on market. One perspective of this project would be to study the effects of these molecules on primary cultures of human bronchial epithelial cells.

Evaluation of F508del-CFTR Correctors Efficacy in CFBE41o- Cells

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Great efforts are being conducted to find “correctors”, drug-like molecules able to reduce the folding and trafficking defect of the CFTR protein caused by F508del, the most frequent mutation in cystic fibrosis (CF). In most cases, correctors are found by cell-based high-throughput screenings of chemical libraries. However, most correctors identified so far show low efficacy, particularly in primary airway epithelial cells. In particular, their activity is strongly dependent on cell background, probably because they act mostly by modulating the CFTR interactome and not CFTR itself (Pedemonte et al., Am J Physiol Cell Physiol 298:C866, 2010). Some compounds, such as corr-4a, are effective in different cell lines and, to a lesser extent, in primary cells. In contrast, other putative correctors are effective only in one cell line and totally ineffective in primary cultures. These findings suggest that counterscreening on a second cell line expressing F508del-CFTR may be useful to categorize compounds having a more general mechanism of action versus those with more cell-specific activity. We have recently generated a version of bronchial CFBE41o- cells with co-expression of F508del-CFTR and the halide-sensitive yellow fluorescent protein. These cells have been used to assess the relative efficacy of known correctors. We have confirmed activity for corr-4a, vx-325, the aminoarylthiazole EN277I, SAHA, and VX-809 but not for other many other correctors described previously. When these compounds were tested in primary bronchial epithelial cells, only VX-809, corr-4a, and VRT-325 elicited a significant F508del-CFTR rescue. However, the maximal effect for these compounds, including VX-809, reached only 10-20% of the total CFTR activity measured in non-CF bronchial cells.

To investigate the mechanism of action of correctors, we have utilized specific assays to monitor their activity on the proteasome and on histone deacetylases (HDACs). Indeed, inhibition of these targets has been shown previously to induce F508del-CFTR rescue (Vij et al., J Biol Chem 281:17369, 2006; Hutt et al., Nat Chem Biol 6:25, 2010).

We measured the chymotrypsin-like, trypsin-like and caspase-like activities of the proteasome complex in cells under resting conditions or upon treatment with correctors, using a specific assay and bortezomib/MG-132 as positive controls. We found that VX-809, but not the other correctors, inhibits partially proteasome activity.

We also used an assay for HDACs. In contrast to SAHA, a known HDAC inhibitor, all other correctors were ineffective.

Our studies indicate that CFBE41o- cells are a useful model to identify and study F508del correctors. However, they are not entirely predictive of activity in primary airway epithelial cells as shown by the example of SAHA. Our results also show that even the best correctors such as VX-809 cause only a partial rescue of F508del-CFTR in CFBE41o- and primary airway epithelial cells. A larger correction could be obtained by combination of active compounds. In this respect, CFBE41o- cells could be useful to rapidly test these combinations using high-throughput assays.

Supported by Telethon-ITALY, Cystic Fibrosis Foundation, and Fondazione Italiana Fibrosi Cistica.

Proteostasis Modulation as a Novel Therapeutic Approach in Cystic Fibrosis

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The most frequent mutation in cystic fibrosis, F508del, causes the arrest of the CFTR protein in the ER and its degradation. Drug-like molecules called correctors are able to improve F508del-CFTR trafficking. However, correctors are characterized by a relatively low efficacy, particularly in primary airway epithelial cells from CF patients. Indeed, the activity of F508del-CFTR correctors is largely influenced by cell background. In addition, the additivity/synergy observed between correctors, indicates the existence of different mechanisms to rescue F508del-CFTR trafficking. The development of new drugs requires a better understanding of the cellular processes responsible for the fate of the mutant protein and its possible rescue.

To identify key proteins involved in the processing of F508del-CFTR, we have adopted a functional genomics approach based on the screening of a siRNA library. Since the composition of the CFTR interactome and the responsiveness to rescue maneuvers may differ from one cell type to the other, we have utilized a human cell line (CFBE41o-) that is as close as possible to the original native epithelium, as the use of primary bronchial cells for screenings is not feasible.

The established conditions for high-throughput siRNA transfection in 96-well format were as follow: CFBE41o- cells were reverse transfected using 10 nM siRNAs and lipofectamine 2000. After 48 hours, the functional assay was performed to determine the extent of F508del-CFTR activity in the plasma membrane.

The results have highlighted RMA1, AHA1 and Derlin-1 as targets whose silencing causes a significant F508del-CFTR rescue in CFBE41o- cells (85-95% increase relative to control-transfected cells). RMA1 is an ubiquitin E3 ligase that is responsible for F508del-CFTR labeling for degradation (Grove et al., 2009). Derlin-1 recognizes misfolded, non-ubiquitylated CFTR to initiate its dislocation and degradation early during CFTR biogenesis, perhaps by detecting structural instability within the first transmembrane domain (Sun et al., 2006). In addition, silencing of Ubc9 (the main SUMO E2 conjugating enzyme), CBX4 and PIAS3 (SUMO E3 ligases) was also effective (75-90% increase relative to control-transfected cells). These data suggest that sumoylation has a role in F508del-CFTR processing. However, we do not know at the moment whether sumoylation affects CFTR directly or through another protein. Interestingly, we also found that silencing of NHERF1 prevents CFTR rescue in CFBE41o- cells incubated at low temperature.

To maximize F508del-CFTR rescue, we have also tested combinations of active siRNAs and combinations of single siRNAs with small molecule correctors and low temperature. Indeed, it is expected that maneuvers having different mechanisms of action generate additive/synergistic effects when added together. Our results show that RMA-1 silencing can rescue F508del-CFTR at 37°C, but has no additive effect with respect to low temperature rescue, suggesting that low temperature acts at very early steps during CFTR biogenesis.

Our studies are now continuing with an unbiased siRNA screening of a genome-wide siRNA library to identify novel targets for F508del-CFTR rescue.

Supported by CFFT, Italian Ministry of Health, Telethon Foundation, and Italian Foundation for Cystic Fibrosis

Rescuing Both Trafficking and Chloride Channel Function of ΔF508 CFTR

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ΔF508 CFTR is recognized as a mutant protein by the ER quality control mechanism and retained in the ER. ΔF508 CFTR also has a gating defect which is manifested by a very low open probability when rescued by low temperature. This combination of defects lead to the proposal that a therapy would require a combination of corrector and potentiator. In previous studies we showed that transcomplementation of ΔF508 CFTR by a truncated CFTR overcomes defective processing. Here we study whether Δ27-264 which is missing the first 4 transmembrane segments of CFTR can rescue DF508 CFTR chloride channel function. Whole cell currents were recorded from CHO cells over expressing wt, ΔF508, Δ27-264, or both Δ27-264 and ΔF508 CFTR. Currents were activated by cpt-cAMP and Forskolin.
Importantly no current was generated from unrescued ΔF508, or from 27-264 CFTR alone. Only when both were added did we observe significant whole currents which reached near to that of wt-CFTR. Our data show that transcomplementatation with Δ27-264 CFTR can rescue both trafficking and chloride channel function of ΔF508 CFTR. We next asked whether a small molecule corrector could compare with what we found with transcomplementation. To accomplish this, we treated cells with 20 uM of the corrector CFFT 106951 for 36-48 hours. Corrector 106951 increased whole cell currents to a level about 1/3 of wt CFTR, less than what we observed with transcomplementation. In single channel experiments, 106951 restored the open probability to 0.4, near to wild type CFTR. These data suggest that both transcomplementation and the corrector 106951 correct the channel properties of CFTR.

ΔF508 Correction in CF Cells Using ZFN Homology-Directed Repair

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The finding that CFTR cDNA could correct the CF phenotype in CF cells triggered a search for an efficient and effective way to deliver cDNA to patients; a phase I trial is currently underway to evaluate a regular repeat dosing regimen. An alternative to cDNA addition, which can only transiently restore activity, is to permanently correct the gene defect using zinc finger nuclease (ZFN) homology-directed repair (HDR). This approach can permanently correct mutations in genes in ~20% of treated cells[1], and has been used extensively *in vitro*, *in vivo*, and also in a phase I trial[2].

ZFNs create a double strand break (DSB) which promotes HDR using a donor DNA molecule containing the correct sequence. Previous reported CFTR-specific ZFNs were relatively unsuccessful suggesting that CFTR may be inherently difficult to target due to chromatin effects on DNA accessibility or target site methylation[3]. Here we describe a pair of ZFNs that create a DSB in the human CFTR gene 203 bp upstream of DF508 in ~20% of cells suggesting there is no intrinsic limitation. Moreover, we show that the CTT deletion which causes the DF508 mutation can be repaired using these ZFNs with a donor plasmid containing 4.3 kb of wild type sequence, albeit at a lower level of efficiency. As HDR efficiency decreases with distance from the DSB, we will evaluate other nucleases which create a DSB at or nearer the CTT deletion[4] with our donor, and explore the use of the existing ZFNs to introduce a CFTR partial cDNA (exons 10-24) with appropriate splice acceptor and poly A sites to allow full length corrected CFTR mRNA production, an approach successfully used *in vivo* in a model of haemophilia[5].

References:

[1]PMID:21182467
[2] PMID:21242897
[3] PMID:18657511
[4] PMID:21493687
[5] PMID:21706032

S2.4 - Pharmacological Rescue of Mutant CFTR Detected Using a Novel Fluorescence Platform

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Numerous human diseases arise due to defects in protein folding and lead to degradation of those proteins in the ER. Among them is Cystic Fibrosis (CF), caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR), an epithelial anion channel. The most common mutation, F508del, disrupts CFTR folding, which blocks its trafficking to the plasma membrane.

To date, most F508del-CFTR corrector high throughput screening assays have relied on measurements of restored CFTR function. These methods require multiple wash steps and are susceptible to signal saturation. In addition, they rely on the recruitment of a functional CFTR to the cell surface. Immunofluorescence labeling methods to detect an epitope tagged version of CFTR also require multiple wash and binding steps, which adds variability and reduces throughput for drug screening. Direct detection of F508del-CFTR at the cell surface with a single labeling step would improve throughput and increase the dynamic range of corrector screening assays.

To address these issues, we tagged CFTR with a protein module that provides a unique and selective fluorescence assay of CFTR's abundance at the cell surface. The protein tag is comprised of a genetically encoded fluorogen activating protein (FAP) that has been fused to the N-terminus or inserted into the 4th extracellular loop (EL4) of CFTR. They faithfully report the behavior of WT and F508del-CFTR, and FAP-wt CFTR retains functional activity. The presence of FAP-CFTR at cell surface can be selectively detected by a cell impermeant fluorogen, while total CFTR is detectable by a cell permeant fluorogen.

Using this approach, we determined the efficacy of corrector compounds from the CFFT panel (www.cftrfolding.org) , both alone and in combination, to rescue F508del-CFTR to the plasma membrane. Combinations of correctors produced additive or synergistic effects, improving the density of mutant CFTR at the cell surface up to 9-fold over single compound treatment. The results correlated closely with functional assays of stimulated anion transport performed in polarized human bronchial epithelia that endogenously express F508del-CFTR. These findings indicate that the FAP-tagged construct quantitatively reports mutant CFTR correction activity, and that this approach should be useful as a screening assay in diseases that impair protein trafficking to the cell surface.

Room: La Villa Rose

Posters – Others

P. 49

The F508del Mutation in *Cftr* Gene Impacts Bone Formation

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The F508del mutation in the cystic fibrosis transmembrane conductance regulator (Cftr) gene is believed to be an independent risk factor for cystic fibrosis-related bone disease. We recently reported the expression of CFTR mRNA and protein in primary human osteoblasts and showed that reduction in CFTR-dependent chloride (Cl^-) activity affects the production of osteoprotegerin (OPG) and prostaglandin E2, two key regulators of bone formation/resorption process (Le Heron L et al. 2010). Here, we report both a defective CFTR-mediated Cl^- channel activity and a severe deficit of OPG production by cultured osteoblasts isolated from a 25-yr-old CF patient with the F508del/G542X mutation in CFTR. A total absence of CFTR-dependent (Cl_{CFTR}) chloride response in F508del-CFTR osteoblasts was observed compared to normal osteoblasts. In contrast, two other calcium-dependent (Cl_{Ca}) and volume-dependent (Cl_{swell}) chloride channels were found to be fully functional in both F508del-CFTR and normal osteoblasts. Interestingly, we found that both the basal and stimulated (TNF-α, 20 ng/ml, 4 hrs) OPG protein released by F508del-CFTR osteoblasts was considerably reduced (8-10%) compared to normal osteoblasts (Gimenez A. et al., Eur Respir J, 2012, in press).To evaluate whether the severe osteopenia in CF is directly linked to the F508del mutation, we measured the bone mineral density and histomorphometric parameters of bone formation and bone mass in F508del-CFTR homozygous mice (F508del *Cftr*^{tm1Eur}) and Cftr$^{+/+}$ littermate controls at 6 (prepubertal), 10 (pubertal) and 14 (young adult) weeks of age in two genders. The bone architecture of F508del *Cftr*^{tm1Eur} and wild type littermate mice was evaluated by bone densitometry, micro-CT and analysis of dynamic parameters of bone formation. Levels of serum insulin-like growth factor 1 (IGF-1) and osteocalcin were also determined. Reduced bone mineral density, lower femoral bone mass and altered trabecular bone architecture were observed in F508del *Cftr*^{tm1Eur} compared to controls at 6, 10, and 14 weeks of age. A decrease in bone formation rate in F508del *Cftr*^{tm1Eur} was evidenced compared to control mice, independently of age and sex. Interestingly, we found lower IGF-1 levels in F508del *Cftr*^{tm1Eur} mice compared to age-matched control mice whereas osteocalcin level was normal. Severe osteopenia and altered bone architecture were found in young and mature adult F508del *Cftr*^{tm1Eur} mice. Our findings demonstrate that the F508del mutation in CFTR impacts trabecular bone mass by reducing bone formation (Le Henaff C. et al, Am J Pathol, 2012, in press).

Supported by grants from Vaincre la Mucoviscidose and Inserm/Region Champagne Ardenne convention.

F508del-CFTR ER Retention, Hypoxia and Saturated Fatty Acid Accumulation: A Cross Road to Cystic Fibrosis Pathogenesis

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In the airways of cystic fibrosis (CF) patients, mutation in the CF transmembrane conductance regulator (CFTR) gene leads to an increased mucus viscosity and hypersecretion, impairing mucociliary clearance, which could also cause hypoxia.
CFTR dysfunction is also associated with an altered fatty acid metabolism, which results in low levels of Poly-Unsaturated Fatty Acids (PUFA) in cultured airway epithelial cells from CF patients (Anderson et al., 2008). This defect also appears to impact the overall fatty acyl content of phospholipids, since the amounts of five phosphatidylcholine (PC) species containing unsaturated chains are decreased in the plasma of CF patients.
In this study, we first investigated if such differences in the fatty acyl content of membrane phospholipids could be observed in freshly isolated epithelial cells from CF patient lungs bearing the F508del-CFTR mutation. Surprisingly, HPLC-MS (High-Performance Liquid Chromatography-Mass Spectrometry) analyses revealed an accumulation of PC species with two saturated fatty acyl chains (SFA), in the form of palmitic acid (C16:0), in CF samples, as compared to non-CF samples. This SFA accumulation within PC was not directly related to the presence of the F508del-CFTR mutation, since this phenomenon could not be observed in the CFBE cell line, a bronchial epithelial cell line from F508del-CFTR homozygote patients. We therefore concluded that the expression of the F508del-CFTR protein cannot account, by itself, for the observed palmitate accumulation within PC in lungs, and that another physiological side effect might be responsible for this phenomenon.
Among non-CF patients, two different populations with strikingly different PC profiles could be identified: smokers and non-smokers. Smokers presented a CF-like profile with an accumulation of palmitate within PC. This similar lipid signature between CF patients and non-CF smokers led us to postulate that SFA accumulation could be the result of hypoxia, related to alteration/obstruction of the upper airways, a physiological trait shared by both CF-patients and non-CF smokers. To test this hypothesis, we developed a cellular culture model for hypoxia and showed that oxygen scarcity resulted in palmitate accumulation within PC both in CFBE and wild-type (16HBE) cell lines. This observation reinforces the idea that SFA accumulation in CF is not-directly related to the expression of the F508del-CFTR protein, but could rather be a side-consequence of hypoxia.
Even if it is still a matter of debate for the F508del-CFTR protein, it is known that the accumulation of misfolded proteins in the ER induces a cellular stress response, known as the Unfolded Protein Response (UPR). Interestingly, SFA have a very similar impact on cells, likely because they impair protein folding/translocation in the ER and thus promote a misfolded protein overload in this compartment. Ultimately, under both circumstances, sustained UPR can lead to cell death by apoptosis. In this context, we are presently investigating the possible synergistic effects of F508del-CFTR retention in the ER and its side-related SFA accumulation in CF-patients on the induction of the UPR and cell survival. Preliminary data concerning these aspects will also be presented.

Establishment of an F508del Pseudoislet Model for the Study of CF-Related Diabetes

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Objectives: CF-related diabetes (CFRD) is poorly understood but occurs in approximately 50% of CF patients over the age of 30. Several studies have now shown that the pancreatic β-cells express CFTR (see Polychronakos *et al.* 2004) and a recent report has shown that functional CFTR channels are important in insulin secretion (Edlund *et al.* 2010). CF animal models offer new opportunities to study CFTR deficient islets. However, islet isolation is labour intensive, of low yield and inflicts a significant animal burden. Here, we sought to establish an F508del pseudoislet model using the murine MIN6 β-cell line (which expresses wild-type CFTR). 3D pseudoislets are preferable to monolayer cultures because of the enhanced cell-cell communication achieved through upregulation of gap-junction activity (Hauge-Evans *et al.* 1999). Maintenance of cell-cell contact is needed for normal patterns of insulin secretion (Halban *et al.* 1989).

Methods: Wild-type MIN6 cells, grown in ultra-low attachment plates (Corning), self-formed into pseudoislets over one week. Wild-type pseudoislets were examined for insulin secretion in response to stimuli (RIA) and β-cell markers (Western blot). Additionally, expression of E-cadherin and connexion 36 (gap junction markers) was determined by PCR. Central apoptosis has previously been reported in pseudoislet models (Luther *et al.* 2005). We therefore examined cellular proliferation by chemiluminescent BrdU ELISA and cellular viability by MTT and LDH assay. Freshly isolated mouse islets and MIN6 monolayers served as controls for these experiments. To establish an F508del pseudoislet model, MIN6 monolayer cells were treated with CFTR shRNA (Qiagen), subsequently transfected with F508del cDNA (CFTR Expression Core) and pseudoislets configured as described (Kelly *et al.* 2010).

Conclusions: Wild-type MIN6 pseudoislets are similar in size and morphology to primary mouse islets and release comparable levels of insulin following stimulation with glucose, nutrients and drugs. Furthermore, they showed enhanced expression of β-cell markers (GLUT2, glucokinase) and gap-junction markers when compared with MIN6 monolayers. Individual wild-type pseudoislets were of uniform size and remained static in culture for periods of up to 14 days. Analysis of cellular proliferation and viability revealed that wild-type MIN6 pseudoislets appeared able to roughly balance the rate of cell death with proliferation, likely contributing to their static size in culture. These findings reinforce the utility of this model to study basic mechanisms of β-cell biology. The established F508del pseudoislet model will be used for future studies to determine if CFRD results from perturbation of normal β-cell function because of the basic CFTR defect.

P. 52

Cystic Fibrosis Airway Epithelial Regeneration is Abnormal in Absence of Endogenous Infection and Inflammation

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The main cause of morbidity and death in cystic fibrosis (CF) patient is due to lung dysfunction linked airway epithelium damage. The airway epithelium maintains mucus hydration via ion channels that transport ions and water, plays a role of barrier and of defenses, and is responsible for the mucociliary clearance. In the CF patients, the CFTR chloride channel mutations lead to a chloride secretion dysfunction and sodium absorption deregulation, resulting in mucus dehydration and mucociliary clearance impairment, mucus accumulation in lungs, hyper-infections and hyper-inflammation. In CF lungs, airway epithelial lesions and remodeling are frequently observed. Whether these alterations are related to infection and/or inflammation or to a deregulated regeneration process remains to be elucidated. Indeed, to restore its barrier and defense functions, the CF airway epithelium must repair and rapidly reconstitute its functional cellular structure. Previously, we showed in an aseptic xenograft model that CF epithelial regeneration is abnormal, delayed and reconstitute a remodeled epithelium, excluding infection as the main actor in the CF deregulated epithelial regeneration.

The aim of the present study was to determine if CF airway epithelial regeneration is abnormal in absence of endogenous inflammation.

CF and non-CF airway epithelial cells collected from aseptic nasal polyps were cultured in defined media at the air-liquid interface. Histology and cell proliferation were examined on culture sections after histological or immunological staining. Epithelial functionality was assessed in terms of electrical properties - short circuit current and transepithelial resistance - and bactericidal activity of epithelial secretions against *Staphylococcus aureus*.

In absence of endogenous inflammation, the CF epithelial regeneration is delayed. The regenerated epithelium is remodeled, exhibits basal cell hyperplasia and is significantly thicker compared to non-CF. However, a delay in CF airway cell proliferation, rather than hyperproliferation, is observed. Finally, the CF regenerated epithelium is not functional.

We show here that in absence of exogenous infection and inflammation, the CF regenerated epithelium is a remodeled. This strongly suggests the involvement of the basic CFTR default in this phenomenon. To verify this hypothesis, we will examine the regeneration process of cells after treatment with CFTR correctors (VX-809 combined with VX-770) or CFTR-encoding lentiviral vectors to correct CF cells, as well as CFTR siRNAs to silence CFTR in non-CF cells in order to create “CF-like cells”.

We thank the french Association Vaincre la Mucoviscidose, Région Champagne-Ardenne and INSERM.