2008
European Cystic Fibrosis Society

New Frontiers in Basic Science of Cystic Fibrosis

Régua, Douro, Portugal

Chairpersons

Margarida D. Amaral and Phil Thomas

09 – 13 April 2008

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Grateful Thanks to:

- The Embassy of France for supporting the travel expenses of one Speaker
- FLAD (Luso-American Foundation) for supporting the travel expenses of two Speakers.

Welcome from the ECFS President

Dear Friends and Colleagues,

It is a great pleasure to invite you to the fifth European Cystic Fibrosis Conference entirely dedicated to Basic Science which in 2008 takes place in the Douro region of Portugal. I would like to acknowledge the success of this series of conferences to Professor Margarida Amaral and her team, and welcome Professor Phil Thomas from Texas as this year's co-chairperson.

The ECFS believes the activities of the basic scientists are critical to our understanding of CF with the development of new therapies being adopted by clinicians. We are thus proud to provide you with a platform to discuss your ideas with your colleagues during this important conference which will comprise a series of symposia with invited European and International expert guest speakers. Your active participation will contribute to a productive exchange of information. I extend a very warm welcome to an exciting conference.

Stuart Elborn
President
European Cystic Fibrosis Society

Welcome from the Conference Chairpersons

It is a great pleasure to welcome you to the 2008 – ECFS Basic Science Conference: "New Frontiers in the Basic Science of Cystic Fibrosis". Our aim in organising this venture is to help foster new research interactions towards a better understanding of the pathogenesis of CF and of other related disorders. We have encouraged our invited speakers to present their most recent unpublished results and wish to ensure lively discussion of data and ideas at the forefront of research, all in an informal, co-operative atmosphere.

This year we are for the first time in the North of Portugal in the spectacular Douro valley, the region of the world-famous Port wine. Besides participating in a cutting-edge conference, we hope that you will be able to experience some of the flavour of Douro, a region that was named an UNESCO world heritage site in 2001. Indeed, the Douro Region has been producing wine for nearly two thousand years. It is the oldest marked wine region in the world and since the 18th century its main product, Port wine, has been world-famous for its high quality. The Douro landscape is thus representative of the full range of activities associated with winemaking – vineyards in terraces, quintas (wine-producing farm complexes), picturesque villages and breath-taking winding scenic roads looking down into amazing deep valleys. The cultural landscape of the Douro Region is an outstanding example of a traditional European wine-producing region, reflecting the evolution of this human activity over time.

We thus welcome you to Douro and to a wonderful and exciting conference in the spirit of the traditional Portuguese hospitality!

Margarida D. Amaral
University of Lisboa, Portugal

Phil Thomas,
University of Texas, USA

CONFERENCE PROGRAMME

2008 ECFS Conference

Régua-Douro (Portugal), 9 – 13 April 2008

Programme

Chairpersons: Margarida Amaral (Lisboa, Portugal) - Phil Thomas (Dallas, TX, USA)

Wednesday, 9 April 2008 (Day 1)

Time	Event	Location
13:00-17:00	*Registration, Light Meal & Set-up of Posters*	
17:30-18:00	**Official opening of the Conference** Stuart Elborn, President of the European Cystic Fibrosis Society Margarida Amaral (Lisboa, PT) and Phil Thomas (Dallas, TX, USA), Conference Chairpersons	Room: Auditorium
18:00-19:00	**Opening Lecture**: Convicting HCO^{3-} of Mucoviscidosis – Paul Quinton (San Diego, CA, USA)	Room: Auditorium
19:00-20:30	***Reception and Port***	*Main Lobby*
20:30-21:30	*Dinner*	Restaurant – 3rd Floor

Thursday, 10 April 2008 (Day 2)		
07:30-09:00	*Breakfast*	*Restaurant – 3rd Floor*
09:00-10:30	**Symposium 1 – CFTR Interdomain Interactions** **Chairs: Phil Thomas (Dallas, TX, USA) & Voula Kanelis (Mississauga, ON, CDN)** Organisation and functional relevance of domain interactions	Room: Auditorium
09:00-09:10	Use of REFER Analysis to Map the Gating Pathway of the CFTR Cl- Channel – David Sheppard (Bristol, UK)	
09:10-09:30	CFTR structure and domain localisation using electron cryo-microscopy – Robert C. Ford (Manchester, UK)	
09:30-09:50	Differences in Interactions of Wild Type and F508del NBD1 with Other Regions of CFTR – Voula Kanelis (Mississauga, ON, CDN)	
09:50-10:10	A small molecule corrector (VRT-325) and a potentiator (VRT-532) interact directly with purified and reconstituted G551D-CFTR to enhance its ATPase activity - Christine Bear (Toronto, ON, CDN)	
10:10-10:20	Folding, Processing and Function of Human-Murine CFTR Chimeras: Implications for the Structure of CFTR – Ana Carina Da Paula (Lisboa, PT)	
10:20-10:30	CFTR Alternative Translation Results in N-terminus Truncated Proteins: Structural and Functional Consequences – Anabela Ramalho (Lisboa, PT)	
10:30-11:00	*Coffee break & **Poster viewing***	*Room: Green Room*
11:00-12:30	**Symposium 2 – Genetics of CF - Gene Networks** **Chairs: Harry Cuppens (Leuven, BE) & Ann Harris (Chicago, IL, USA)**	Room: Auditorium
11:00-11:20	Modifier Genes in Cystic Fibrosis - Harry Cuppens (Leuven, BE)	
11:20-11:40	Novel regulatory mechanisms for the CFTR gene - Ann Harris (Chicago, IL, USA)	
11:40-12:00	Towards understanding of the CFTR dynamic interactome: the example of inflammation - Aleksander Edelman (Paris, FR)	
12:00-12:15	Towards in vivo correction of the CFTR F508del allele: design and synthesis of two zinc finger nucleases for homology-directed repair – Ciaran Lee (Cork, IE)	
12:15-12:30	Development of Post-Genomics Microscopy Assays to Automatically Screen for Protein Targets Affecting CFTR Traffic – Shehrazade Dahimène (Lisboa, PT)	
12:30-14:30	*Lunch*	*Restaurant – 3rd Floor*
14:30-16:00	**Symposium 3 – CFTR Traffic** **Chairs: Margarida Amaral (Lisboa, PT) & Gergely Lukacs (Montreal, QC, CDN)**	Room: Auditorium
14:30-14:35	Introduction – Margarida Amaral (Lisboa, PT)	
14:35-14:50	Folding and Misfolding of CFTR – Douglas Cyr (Chapel Hill, NC,USA)	
14:50-15:00	Low Temperature Allows Mutant CFTR to Bypass the Endoplasmic Reticulum Quality Control – Carlos Farinha (Lisboa, PT)	
15:00-15:20	The chaperone-independent role of N-glycans in CFTR folding and trafficking - Gergely Lukacs (Montreal, QC, CDN)	
15:20-15:40	Rescue of F508del-CFTR in CFBE410- cells is dependent on actin cytoskeleton interation with Ezrin and NHERF1 – Valeria Casavola (Bari, IT)	
15:40-16:00	Achieving Proteostasis in Cystic Fibrosis – Bill Balch (La Jolla, CA, USA)	
16:00-16:30	*Coffee Break & **Poster Viewing***	*Room: Green Room*
16:30-18:00	**Special group discussion-I: Using Molecular Information for the development of new therapies** **Moderators: Oscar Moran (Genova, IT) & Alan Cuthbert (Cambridge, UK)**	Room: Auditorium
16:30-18:00	**Special group discussion-II: Ca^{2+} Signalling and CF** **Moderators: Carla Ribeiro (Chapel Hill, NC, USA) & Kevin Foskett (Philadelphia, PA, USA)** Dysfunction of mitochondria Ca2+ uptake in cystic fibrosis airway epithelial cells – Fabrice Antigny (Poitiers, FR)	Room: Miradouro
20:30-21:30	*Dinner*	*Restaurant – 3rd Floor*
21:30-23:30	Evening posters	Room: Green Room

Friday, 11 April 2008 (Day 3)		
07:30-09:00	*Breakfast*	*Restaurant – 3rd Floor*
09:00-10:30	**Symposium 4 – Bicarbonate Transport in CF** **Chairs: Paul Quinton (La Jolla, CA, USA) & Ursula Seidler (Hannover, DE)**	Room: Auditorium
09:00-09:20	Regulation of HCO^{3-} secretion in the pancreatic duct – Shmuel Muallem (Dallas, TX, USA)	
09:20-09:40	Bicarbonate Transport in Human Airway Cells – Mike Gray (Newcastle upon Tyne, UK)	
09:40-10:00	**Key role of CFTR for all modes of intestinal HCO_3^- secretion** – Ursula Seidler (Hannover, DE)	
10:00-10:15	CFTR activation regulates both apical and basolateral Cl-/HCO3- exchange activity in Calu-3 cells – James Garnett (Newcastle upon Tyne, UK)	
10:15-10:30	Role of carbonic anhydrase II in the murine duodenal secretory response to CO_2, secretagogues, and mucosal acidification – Anurag K. Singh (Hannover, DE)	
10:30-11:00	*Coffee break &* ***Poster viewing***	*Room: Green Room*
11:00-12:30	**Symposium 5 – Inflammation and other respiratory responses in CF** **Chairs: G. Döring (Tübigen, DE) & Cristelle Coraux (Reims Cedex, FR)** Room: Auditorium	
11:00-11:20	Airway epithelial repair and regeneration – Christelle Coraux (Reims Cedex, FR)	
11:20-11:40	Immunobiology of a novel genetic modifier of F lung disease – Chris Karp (Cincinnati, OH, USA)	
11:40-12:00	New concepts, how mutated CFTR provokes inflammation and infection in CF – Gerd Döring (Tübigen, DE)	
12:00-12:10	CFTR expression suppresses NFkB-driven inflammatory signalling – Mairi Hunter (Dundee, UK)	
12:10-12:20	Proteomics of membrane microdomains from human bronchial cells subjected to proinflammmatory stimulation – Florence Borot (Site Necker, FR)	
12:20-12:30	Identification of anti-inflammatory potential of *Aegle marmelos* by screening medicinal plant extracts – Giulio Cabrini (Verona, IT)	
12:30-14:00	*Lunch*	*Restaurant – 3rd Floor*
14:00-18:00	***Free Afternoon: Boat Trip to Pinhão (reservation required)***	
18:30-20:00	**Special group discussion-III: CFTR Dysfunction in Surface Epithelium vs Glands** **Moderators: Lucy Clunes (Chapel Hill, NC, USA) & Jeffrey Wine (Stanford, CA, USA)** Reduced A2BR-mediated Regulation of Mucus Clearance in Cystic Fibrosis Airway Epithelium – Lucy Clunes (Chapel Hill, NC,USA) CFTR Dysfunction in Airways: Glands and Innate Immunity – Jeffrey Wine (Stanford, CA, USA) Differential expression of PLUNC proteins in CF Lung Disease – Colin Bingle (Sheffield, UK) Anaesthesia and the elemental content of mouse nasal fluid – Inna Kozlova (Husargatan, SE) Mucin gene expression in airway epithelial cells from children with CF – Angela Fonceca (Liverpool, UK) The NCI-H441 cell line as a model for the study of airway epithelial sodium transport - a comparison with cultured human bronchial epithelial cells – Rosemary Sugar (Horsham, UK)	Room: Miradouro
18:30-20:00	**Special group discussion-IV: CFTR interacting proteins: a source for new therapies?** **Moderators: Anil Mehta (Dundee, UK) & Rebecca Hughey (Pittsburgh, PA, USA)** ENaC activation by proteolysis and related development of therapies – Rebecca Hughey (Pittsburgh, PA, USA) Regulation of the Epithelial Sodium Channel (ENaC) by AMP-activated kinase (AMPK) – Joana Almaça (Regensburg, DE) CFTR interacting proteins - a common link between kinases acting on CFTR and ENaC – Anil Mehta (Dundee, UK) A Role for CK2 and SYK in CFTR Turnover and processing? – Simão Luz (Lisboa, PT)	Room: Auditorium
20:00-21:30	*Dinner*	*Restaurant – 3rd Floor*
21:30-23:30	Evening posters	Room: Green Room

Saturday, 12 April 2008 (Day 4)

Time	Programme	Room
07:30-09:00	*Breakfast*	*Restaurant – 3rd Floor*
09:00-10:40	**Symposium 6 – CFTR–Interacting Proteins** **Chairs: Karl Kunzelmann (Regensburg, DE) & Valeria Casavola (Bari, IT)**	Room: Auditorium
09:00-09:20	Phosphorylation of CFTR AMPK and localized regulation of Cl^- secretion –Karl Kunzelmann (Regensburg, DE)	
09:20-09:40	A CFTR Associated Protein, CAL, regulates the surface expression of CFTR. – Bill Guggino (Baltimore, MD, USA)	
09:40-10:00	The role of Hsp27-mediated sumoylation in CFTR biogenesis/degradation – Ray Frizzell (Pittsburgh, PA, US)	
10:00-10:10	Evidence of an essential role of NHERF1 and NHEF2 in G-protein coupled receptor regulation of CFTR-mediated intestinal anion secretion in vivo – Anurag Singh (Hannover, DE)	
10:10-10:20	Impact of F508 Residue and Small Molecule Correctors on the Interaction of Hsc70 with NBD1 of CFTR – Toby Scott-Ward (Lisboa, PT)	
10:20-10:30	The First Steps in CFTR Biogenesis: Co-translational interactions with nascent CFTR as it exits the Ribosome – Andrey Karamyshev (Dallas, TX, USA)	
10:30- 10:40	Annexin V and S100A8 are binding partners of CFTR and inhibits activation of whole cells currents in *Xenopus* oocytes – Diana Faria (Regensburg, DE)	
10:40-11:00	*Coffee break &* ***Poster viewing***	*Room: Green Room*
11:00-12:30	**EuroCareCF session – WP6 – Animal Models** **Chair: Bob J. Scholte (Rotterdam, NL)**	Room: Auditorium
11:00-11:25	Eurocare CF WP6 Animal models of CF - Inflammation and tissue remodelling in CF mutant mice – Bob J. Scholte (Rotterdam, NL)	
11:25-11:45	Defective CFTR Cl^- channel causes increased proteasomal degradation and reduced NF-κB activity in lung epithelial cells exposed to ROS – Jacky Jacquot (Paris, FR)	
11:45-12:30	New Animal Models for Cystic Fibrosis Research – John Engelhardt (Iowa City, IO, US)	
12:30-14:30	*Lunch*	*Restaurant – 3rd Floor*
14:30-16:00	**EuroCareCF-WP7 – Resources for Basic Research** **Session I: Endpoints of CFTR Rescuing Therapies** **Chairs: Margarida Amaral (Lisboa, PT) & David Sheppard (Bristol, UK)**	Room: Auditorium
14:30-14:50	Overview – Margarida Amaral (Lisboa, PT)	
14:50-15:10	Standardized ICM in the US – Sheriff Gabriel (Chapel Hill, NC, USA)	
15:10-15:30	Measurement of trace gases in the exhaled breath – a new diagnostic option in CF lung disease? – Michael Barker (Berlin, DE)	
15:30-16:00	General Discussion	
16:00-16:30	*Coffee Break &* ***Poster viewing***	*Room: Green Room*
16:30-18:00	**EuroCareCF-WP7 – Resources for Basic Research** **Session II: Cellular Systems for CF Research** **Chairs: Margarida Amaral (Lisboa, PT) & David Sheppard (Bristol, UK)**	Room: Auditorium
16:30-16:50	The Clinical and Functional Translation of CFTR (CFTR2) Project – Garry Cutting (Baltimore, MD, USA)	
16:50-17:10	The culture of primary bronchial epithelial cells from the lungs of people with CF removed at the time of transplantation – A model to study cystic fibrosis lung disease – Malcolm Brodlie (Newcastle-upon-Tyne, UK)	
17:10-17:30	Generating Tools to Aid in CFTR Modulator Drug Discovery – Melissa Ashlock (Bethesda, MD, USA)	
17:30-18:00	General Discussion	
20:00	*Dinner / social event: Meet at the Hotel main Lobby at 19:45*	

Sunday, 13 April 2008 (Day 5)		
08:00-09:30	*Breakfast*	*Restaurant – 3rd Floor*
09:30-11:00	**Symposium 7 – Therapeutic Developments aimed at Correcting CFTR Defects** **Chairs: Olga Zegarra-Moran (Genova, IT) & Alan Cuthbert (Cambridge, UK)** **Room: Auditorium**	
09:30-09:50	CFFT's Pipeline approach to CFTR drug discovery and development – Diana Wetmore (Bethesda, MD, USA)	
09:50-10:10	Emerging therapies in CF: preclinical approaches to tackling CF lung disease in Novartis – Chris Poll (West Sussex, UK)	
10:10-10:30	Pharmacologic activation of chloride transport in CF mutants - Olga Zegarra-Moran (Genova, IT)	
10:30-10:50	Correctors of F508del-CFTR Trafficking – David Thomas (Montreal, QC, CDN)	
10:50-11:00	Poly ADP-Ribose Polymerase-1 inhibitors correct F508del-CFTR trafficking – Suzana Anjos (Montreal, CDN)	
11:10-12:00	**Closing lecture** Inflammation in the CF lung – A therapeutic target - Pamela Davis (Cleveland, OH, USA)	Room: Auditorium
12:00-12:10	**Closing remarks** **Phil Thomas (Dallas, TX, USA) & Margarida Amaral (Lisboa, PT)**	Room: Auditorium
12:10	***End of the Meeting – Departure of Shuttles to the Porto airport***	

POSTER TITLES & AUTHORS

P.1 **Assessment of domain-domain interactions in CFTR**
Linda Millen, Patrick Thibodeau, Juan Mendoza, Jon Moody, Chad Brautigam, Sharon Fischman, Hannoch Senderowitz and Philip J. Thomas

P.2 **CFTR Alternative Translation Results in N-terminus Truncated Proteins: Structural and Functional Consequences**
Anabela S Ramalho, Marzena Lewandowska, Carlos M Farinha, Ann Harris, Margarida D Amaral

P.3 **Folding, Processing and Function of Human-Murine CFTR Chimeras: Implications for the Structure of CFTR**
Ana Carina Da Paula, Marisa Sousa, Elizabeth S. Dawson, Christopher A. Boyd, David N. Sheppard, and Margarida D. Amaral

P.4 **Comparative Analysis of Data from Independent Microarray Studies: Towards a Molecular Signature in CF**
Luka Clarke, Joao Canhita, Lisete da Sousa, Timothy Starner, Paul McCray and Margarida Amaral

P.5 **Towards *in vivo* correction of the CFTR F508del allele: design and synthesis of two zinc finger nucleases for homology-directed repair**
Ciaran M Lee, Rowan Flynn, Patrick T Harrison

P.6 **Mutation panel for Cystic Fibrosis carriers of 941 samples in Greece**
S. Rapti, A. Vasiageorgi, I. Saitis, S. Protopsalti, P.Tsoplou

P.7 **Mutations and polymorphisms in the cystic fibrosis gene in men with severe oligozoospermia**
Maris Teder-Laving, M. Punab, E. Oitmaa, A. Belousova, K. Haller, O. Poolamets, E. Raukas, A. Metspalu, A. Salumets

P.8 **Folding and Misfolding of CFTR**
Douglas M. Cyr, Diane Grove, Hong Yu Ren and Meredith F.N. Rosser

P.9 **Low Temperature Allows Mutant CFTR to Bypass the Endoplasmic Reticulum Quality Control**
Carlos M Farinha, Filipa Mendes, Mónica Roxo-Rosa, Margarida D. Amaral

P.10 **Impact of the revertant mutations G550E and 4RK on the PROCESSING and function of G551D-CFTR**
Luísa S. Pissarra, Zhe Xu, Carlos M Farinha, David N. Sheppard, Margarida D. Amaral

P.11 **Morphological analasys of the secretory traffic of CFTR**
Alberto Luini, Seetheraman Parashuraman, Galina Beznoussenko, Alexander Mironov

P.12 **Characterization of CFTR Mutants and Revertants located in NBD1**
Luísa S. Pissarra, Margarida D. Amaral

P.13 **CFTR activation regulates both apical and basolateral Cl-/HCO3- exchange activity in Calu-3 cells**
JamesP Garnett, Alan W Cuthbert, E Hickman and MA Gray.

P.14 **Evidence of an essential role of NHERF1 and NHEF2 in G-protein coupled receptor regulation of CFTR-mediated intestinal anion secretion *in vivo***

Anurag Kumar Singh, Brigitte Riederer, Anja Krabbenhöft, Brigitte Rausch, Boris M. Hogema, Hugo R. de Jonge, Mark Donowitz, Edward J. Weinman, Olivier Kocher, Ursula Seidler

P.15 **Role of carbonic anhydrase II in the murine duodenal secretory response to CO2, secretagogues, and mucosal acidification**
Anurag Kumar Singh, Markus Sjöblom, Anja Krabbenhöft, Brigitte Riederer, Brigitte Rausch, Michael P. Manns, Gerolf Gros, Ursula Seidler

P.16 **CFTR controls proteasomal degradation and NF-kB activity in lung epithelial cells exposed to oxidative stress**
T. Roque, E. Boncoeur, E. Bonvin, V. Saint Criq, M. Bonora, A. Clement, O. Tabary , J. Jacquot

P.17 **Proteomics of membrane microdomains from human bronchial cells subject to proinflammatory stimulation**
Borot F, Guerrera C, Trudel S, Vieu DL, Tondelier D, Colas J, Brouillard F, Fritsch J, Gruenert D, Chanson M, Edelman A and Ollero M

P.18 **Psoralen derivatives as regulators of P.aeruginosa-dependent transcription of Interleukin-8**
Tamanini, Anna; Lampronti, Ilaria; Nicolis, Elena; Dechecchi, Maria Cristina; Borgatti, Monica; Bezzerri, Valentino; Mancini, Irene; Quiri, Federica; Mazzon, Martina; Rizzotti, Paolo; Cabrini, Giulio, Gambari, Roberto.

P.19 **Identification of anti-inflammatory potential of Aegle marmelos by screening medicinal plant extracts**
Nicolis, Elena; Lampronti, Ilaria; Tamanini, Anna; Dechecchi, Maria Cristina; Borgatti, Monica; Bezzerri, Valentino; Mancini, Irene; Quiri, Federica; Mazzon, Martina; Rizzotti, Paolo; Gambari, Roberto; Cabrini, Giulio

P.20 **Differential expression of PLUNC proteins in CF lung disease**
CD Bingle, FA Barnes, MA Campos, D Rassl, L Bingle

P.21 **CFTR expression suppresses NFkB-driven inflammatory signalling**
M.J. Hunter, D.M.Cassidy, K.J. Treharne, S.C. Land, A. Mehta

P.22 **Influence of leucocytes on Interleukin-8 (IL-8) in sputum and whole blood in cystic fibrosis**
S.Schmitt-Grohé, L.v.d.Boom, D.N´Gampolo, O.Eickmeier, R.Schubert, S.Zielen, B.Zur, M.J.Lentze

P.23 **The Effect Of LTB_4 Receptor Antagonist BIIL 284 BS In The Model Of Chronic Respiratory Infection With *Pseudomonas aeruginosa* In The Rat**
Elena.J. Growcott, D.Yu, N. Dekhtyar, M. Steinkrauss, A. Grevot, L. Thomas, C. Poll, G. Neckermann and K.H. Banner

P.24 **Mucin gene expression in airway epithelial cells from children with CF**
Angela M Fonceca, S Gomez-Aspron, B Pearce, PS McNamara

P.25 **The First Steps in CFTR Biogenesis: Cotranslational Interactions with Nascent CFTR as It Exits the Ribosome**
Andrey L. Karamyshev, Anna E. Patrick, Arthur E. Johnson, Philip J. Thomas

P.26 **Impact of F508 Residue and Small Molecule Correctors on the Interaction of Hsc70 with NBD1 of CFTR**
Toby S Scott-Ward, Margarida D Amaral

P.27 **Regulation of the Epithelial Sodium Channel (ENaC) by AMP-activated kinase (AMPK)**
Joana Almaça, Jiraporn Ousingsawat, Bernhard Hieke, Rainer Schreiber, Karl Kunzelmann

P.28 **Annexin V and S100A8 are binding partners of CFTR and inhibits activation of whole cells currents in Xenopus oocytes**
Diana Faria, Toby S Scott-Ward, Luísa Alessio, Rainer Schreiber, Margarida D. Amaral, Karl Kunzelmann

P.29 **A Role for CK2 and SYK in CFTR Turnover and processing?**
Simão Luz, Carlos M Farinha, Luísa S Pissarra and Margarida D. Amaral

P.30 **Intracellular localization of the putative CA2+ activated CL-Channel Bestrophin-1 and search for interacting protein partners**
Joana Raquel Martins, Toby Scott-Ward, Rainer Schreiber, Karl Kunzelmann, Margarida Amaral

P.31 **The NCI-H441 cell line as a model for the study of airway epithelial sodium transport - a comparison with cultured human bronchial epithelial cells**
Rosemary Sugar, Hazel Atherton, Rachel Burrows, Zaynab Neeto & Henry Danahay

P.32 **Development of Post-Genomics Microscopy Assays to Automatically Screen for Pro Targets Affecting CFTR Traffic**
Simão Luz, Shehrazade Dahimène, Filipa Mendes, Joana Almaça, René Barro-Soria, Karl Kunzelmann and Margarida D Amaral

P.33 **Discerning community interactions using a Drosophila model of polymicrobial infections**
Christopher D. Sibley, Kangmin Duan, Carrie Fischer, Michael D. Parkins, Douglas G. Storey, Harvey Rabin and Michael G. Surette.

P.34 **Human MDR1 and also Wild-type, but not F508del, CFTR/MDR1 chimera Rescue Heavy-Metal resistance Phenotype of MDR-defective c. elegans**
Mário F Neto, Susana M Garcia, M Catarina Silva, Richard I Morimoto, Margarida D Amaral

P.35 **Anaesthesia and the elemental content of mouse nasal fluid**
Inna Kozlova, MH Hühn, M Flodström-Tullberg, M Wilke, BJ Scholte, GM Roomans

P.36 **The F508del mutation reveals a site in NBD1 accessible to drugs that increase F508del-CFTR trafficking**
Malcolm M.C. Pereira, Morgan Pomeroy, Jody Parker, [1]Frédéric Becq, Robert L. Dormer

P.37 **Mechanism of Action and Epithelial Tissue Efficacy of Several F508del-CFTR Correctors**
André Schmidt, Marisa Sousa, Karl Kunzelmann, Margarida D Amaral

P.38 **Guanabenz, an α2-selective adrenergic agonist, activates Ca2+-dependent chloride currents in cystic fibrosis human airway epithelial cell**
Caroline Norez, Clarisse Vandebrouck, Fabrice Antigny, Luc Dannhoffer, Marc Blondel and Frédéric Becq

P.39 **Poly ADP-Ribose Polymerase-1 inhibitors correct F508del-CFTR trafficking**
Suzana M. Anjos, Renaud Robert, Graeme W. Carlile, John W. Hanrahan and David Y Thomas

P.40 **Production of a large genomic CFTR construct inserted into a phage-based**

artificial chromosome (PAC) of potential interest for CF gene therapy
Carla Braz, Anabela Ramalho, Sonja Cattani, Sulith Christan, Andreas Laner, Simone Kraner, Margarida D. Amaral, Dirk Schindelhauer

P.41 **Dysfunction of mitochondria Ca2+ uptake in cystic fibrosis airway epithelial cells**
Fabrice Antigny, Frédéric Becq and Clarisse Vandebrouck

P.42 **CFTR and Tight Junctions in Cultured Normal Bronchial Epithelial Cells**
Nilsson, Harriet E; Johannesson, Marie; Roomans, Godfried M

9 April – 18:00-19:00 **Room: Auditorium**

Opening Lecture

Convicting HCO3- of Mucoviscidosis

Paul M. Quinton (USA)

Division of Respiratory Medicine
Departments of Pediatrics
UCSD School of Medicine and
Biomedical Sciences, UC Riverside, CA., USA

It is widely accepted that abnormal mucus is highly contributory to, if not the basis of, organ failures, morbidity, and mortality in CF. However, for more than 60 years since its recognition, Cystic Fibrosis (CF) has defied formulation of an underlying, unifying hypothesis to explain the formation of abnormal mucus in this genetic disease. Despite the fact that we have known for decades 1.) that mucus appears to be improperly formed in all affected mucin secreting organs and 2.) that electrolyte transport is also seriously deranged in the epithelia of glands and hollow organs, we have entered the 21st century without a proven explanation that consistently connects these two defects in affected organs.

While the very first observations of organ pathology empirically attributed morbidity and mortality to mucoviscidosis (a state of abnormally thick mucus), later observations provided firm evidence to establish that the characteristic defects in salty sweat and then Cl- impermeability were due to site mutations in a gene that coded for a protein found to be an anion channel (CFTR). Thus, we have a firm link from the gene to an electrolyte transport disturbance, but we have no conclusive link from the electrolyte transport defect to the mucus disturbance.

Along the way, several proposals have been put forward to provide a connection between the latter, so that currently, perhaps the most widely accepted hypothesis maintains that mucoviscidosis occurs due to hyperabsorption of NaCl along with fluid, which thereby "desiccates" secreted mucus making it thicker. Other proposals have evoked problems with mucus synthesis, innate immune defenses, fluid secretion, and/or properties of the CFTR molecule *per se*. Possibly because of a widespread and overriding focus on lung pathology, these concepts have not been rigorously applied to explaining the pathogenesis in other target organs, which should probably be a prerequisite for a unifying hypothesis.

In this lecture, first we hope to challenge at least some of these concepts by considering how well they apply to organs other than the lung. Secondly, we will present evidence that the less appreciated defect in HCO_3^- transport is common to affected organs. Third, we will give evidence showing that poor HCO_3^- secretion impedes mucin release, and fourth, we will offer a mechanism by which a defect in HCO_3^- secretion could explain *mucoviscidosis*; that is, without adequate secreted HCO_3^- to immediately sequester and buffer Ca++ and H+ in exocytosed mucin granules, fixed electronegative sites on mucins cannot electrostatically explode these polyanionic macromolecules into the highly expanded volumes required for a normal tangled string network of mucins. Thus, mucus secreted in CF remains pathologically viscid.
This lecture will review the evidence that HCO_3^- transport is defective in affected CF organs and show that the absence of HCO_3^- may be the causative factor in producing mucovisidosis, thereby linking the electrolyte defect to the pathogenically abnormal mucus characteristic of Cystic Fibrosis.

Thursday 10 April – 09:00-10:30 Room: Auditorium

SYMPOSIUM 1 - CFTR Interdomain Interactions
Chairs: Phil Thomas (USA) & Voula Kanelis (CDN)

S1.1 - Organisation and functional relevance of domain interactions
Use of REFER Analysis to Map the Gating Pathway of the CFTR Cl^- Channel

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CFTR is a Cl^- channel gated by ATP-driven nucleotide-binding domain (NBD) dimerisation. To investigate the CFTR gating pathway, we performed rate-equilibrium free energy relationships (REFER) measurements (1, 2). We used site-directed mutations as structural probes, ATP and membrane voltage as environmental probes of channel gating (1) and the $C_1 \leftrightarrow C_2 \leftrightarrow O$ scheme to describe CFTR gating behaviour (3). In this scheme, C_1 is the long duration closed state separating channel openings and $C_2 \leftrightarrow O$ the bursting state. Transitions between $C_1 \leftrightarrow C_2$ and $C_2 \leftrightarrow O$ are described by the forward (β_1 and β_2), and backward α_1 and α_2) rate constants, respectively. As CFTR is gated by intracellular ATP, $C_1 \leftrightarrow C_2$ reflects agonist binding, whereas $C_2 \leftrightarrow O$ reflects the gating step (4). REFER analysis is applied to the gating step. β_2 and α_2 are used to generate a Brønsted plot (log (β_2) plotted vs. log (β_2/α_2)). The slope of the line in a Brønsted plot (Φ) quantifies the relative extent to which the opening (β_2) and closing (α_2) rate constants change during channel gating. Φ ranges between 0 and 1. When Φ is close to 1, the transition-state resembles an open-channel conformation and moves early during gating, whereas when Φ is close to 0, the transition-state resembles a closed-channel conformation and moves late during gating (1, 2).

Brønsted plots of mutations (G550E, G551D, V562I, G551D-G550E, V562I-G550E) in the H5α-helix of NBD1 (n = 3 – 14), different ATP concentrations (0.03 – 5 mM, n = 8 – 33) and a membrane voltage series (-100 – +100 mV, n = 5 – 7) yielded Φ values of 0.64 (r^2 = 0.91), 0.84 (r^2 = 0.55) and 0.18 (r^2 = 0.71), respectively. Good linear fits of these Brønsted plots indicate that CFTR is amenable to REFER analysis. We interpret our data to suggest that at the transition state, i) the structure of the H5α-helix is a hybrid, which is more open than closed; ii) the ATP-binding sites are almost completely in the open state, arguing that the conformations of the ATP-binding sites change early during CFTR gating; iii) the CFTR pore is almost closed at the transition state, suggesting that the conformation of the CFTR pore changes late during CFTR gating. We conclude that there is a spatial gradient of Φ values from ~0.90 at the ATP-binding sites to ~0.20 at the CFTR pore. Thus, as proposed by Grosman *et al* (1), initiation at the effector site and propagation to the active site is likely to be a common theme for the gating of ion channels activated by ligands.

We thank LS Pissarra and MD Amaral for the generous gift of CFTR constructs and Z Xu for excellent assistance. This work was supported by the BBSRC and CF Trust.

References

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S1.2 - CFTR structure and domain localisation using electron cryo-microscopy

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CFTR is a fully paid up member of the ABC family of membrane proteins, although it is somewhat unusual in that it is a channel rather than a transporter and also has an additional cytoplasmic domain (R domain) that activates the channel when it is phosphorylated. Otherwise, CFTR has the usual arrangement of two transmembrane domains (each with six (predicted) membrane-spanning helices), and two cytoplasmic nucleotide-binding domains. Although there is no high resolution structure for the entire CFTR protein, there is a crystal structure for the first nucleotide binding domain, and some data from NMR on portions of the R domain. In addition, structures of entire ABC proteins have been obtained in six cases, including two with an apparently similar topology to CFTR (Sav1866 and MsbA). Hence the stage is set for a concerted effort for the generation of testable molecular homology models for CFTR, perhaps coupled with lower resolution studies of the entire CFTR protein provided by cryo-electron microscopy.

Here I will describe such studies of the entire CFTR protein using electron microscopy, initially beginning with low resolution (20-30Å) data for the protein embedded in a heavy atom stain. These studies have allowed rough localisation of the major domains, as described above. Perhaps surprisingly, the N-terminal portion of the R domain appears to be located relatively close to the transmembrane region, possibly contacting the polar head group region of the lipid bilayer. The C-terminal tail of CFTR is found to be intimately located with NBD2, rather than 'flapping in the breeze' as one might have surmised. Domain movements in the nucleotide binding domains were indicated when comparing low resolution structures of nucleotide-free/non-phosphorylated CFTR with ATP-bound/phosphorylated CFTR. These initial studies have considerably aided the interpretation of the higher resolution (15-12Å) structures that are now emerging by cryo-electron microscopy of unstained preparations of CFTR, which I will also present.

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S1.3 - Differences in Interactions of Wild Type and F508del NBD1 with Other Regions of CFTR

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Cystic fibrosis is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel. CFTR consists of two repeats each comprising a membrane spanning domain (MSD), followed by a cytosolic nucleotide binding domain (NBD). A regulatory (R) region, unique to CFTR, is located between the first NBD and the second MSD. The MSDs are linked to the NBDs via intracellular loops (ICLs). Nucleotide binding and hydrolysis at the NBDs and phosphorylation of the R region are required for channel gating. The most common CF-causing mutation is deletion of F508 (F508del) in the first nucleotide binding domain (NBD1), making studies of NBD1 essential in understanding CFTR function.

We have used NMR spectroscopy to study fragments of wild type and F508del CFTR consisting of NBD1 and the C-terminal regulatory extension (RE), which is comprised of the first 20 residues of the R region. Interactions between multiple sites on the NBD1 core and the regulatory insert (RI), located within the domain, and the RE are disrupted upon phosphorylation. The altered interactions between NBD1 and the RI expose a binding site for the first intracellular loop (ICL1). In contrast, phosphorylation of F508del does not induce binding of ICL1, likely due to conformational differences between wild type and F508del NBD1. These results provide a structural basis by which phosphorylation of CFTR may affect channel gating and expand our understanding of molecular basis of the defect of the F508del mutation.

S1.4 - A small molecule corrector (VRT-325) and a potentiator (VRT-532) interact directly with purified and reconstituted G551D-CFTR to enhance its ATPase activity

Mohabir Ramjeesingh, Francisca Ugwu, Canhui Li and Christine E. Bear

Programme in Molecular Structure and Function. Research Institute, Hospital for Sick Children, Toronto, Canada

Small molecule "correctors", ie.,VRT-325, identified through high-throughput, cell-based screens of chemical libraries, promote the correct trafficking of the major mutant, F508del-CFTR and its functional expression as a phosphorylation and ATP-regulated channel at the cell surface. Once at the surface, its channel activity can be enhanced through the action of other small molecules, called "potentiators", ie., VRT-532. Certain "potentiators" are also effective in enhancing the activity of another disease-causing mutation, G551D-CFTR. This mutant is properly trafficked to the cell surface but exhibits defective channel regulation.

To date, the identity of the molecular target(s) for VRT-325 and VRT-532 is unknown. It has been suggested that these small molecules may bind to mutant CFTR directly, yet this idea remains to be rigorously tested. In this project, we tested this hypothesis for the mutant protein; G551D-CFTR, as we have shown that this protein can be effectively purified and reconstituted, enabling evaluation of the direct effect of the Vertex compounds. Furthermore, the single channel phenotype of this mutant protein has been well described (Bompadre et al, 2008). The G551D mutation falls within the signature motif in NBD1 and the primary catalytic site formed by hetero-dimerization of the two nucleotide binding domains (NBDs): NBD1 and NBD2. As expected on the basis of the position of this mutation, we found that the purified and reconstituted G551D-CFTR is severely defective in intrinsic ATPase activity (Ramjeesingh et al. 2008). The biochemical phenotype reported by our ATPase assay supports the argument that the impaired channel gating behaviour by this mutant is due to defective ATP binding and failure of the CF-NBDs to close in a tight ATP bound heterodimer, the obligatory structure required for subsequent ATPase activity and the putative power stroke required for opening of the gate.

In the present study, we found that both the "corrector" (VRT-325, 2 μM) and the "potentiator" (VRT-532, 5 μM) directly modified the activity of purified and reconstituted G551D-CFTR protein. Both compounds caused a remarkable increase in the specific ATPase activity of reconstituted G551D-CFTR to 640±10% and 610±2 % of the activity of the untreated mutant protein respectively. The increase in ATPase activity of G551D-CFTR by the "potentiator", VRT-532, is consistent with electrophysiological studies showing that this compound can enhance the channel activity of G551D-CFTR (Van Goor et al. 2006). However, the increase in G551D-CFTR ATPase activity by the "corrector" compound (VRT-325) from 0.06±0.05 (nmole/μg/hr) to 0.4±0.05 (nmole/μg/hr), provides the first evidence that this molecule interacts with G551D-CFTR. In our future studies, we will determine the molecular mechanism through which these two molecules modify the function of G551D-CFTR.

Research supported by operating grants awarded to CEB through CCFF, CIHR and the CFF-USA. F. Ugwu is a recipient of a CCFF Fellowship Award.

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S1.5 - Folding, Processing and Function of Human-Murine CFTR Chimeras: Implications for the Structure of CFTR

Ana Carina Da Paula[1,2], Marisa Sousa[1,2], Elizabeth S. Dawson[3], Christopher A. Boyd[3], David N. Sheppard[4], and Margarida D. Amaral[1,2]

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[3]Medical Genetics Section, Molecular Medicine Centre, University of Edinburgh, UK
[4]Department of Physiology & Pharmacology, School of Medical Sciences, University of Bristol, UK

A powerful approach to investigate the structure and function of the CFTR protein is to examine interspecies differences and identify regions of conservation and divergence. Although little is known about the global structure of CFTR, analyses of the crystal structures of the first nucleotide-binding domains (NBD1s) of murine (m) and human (h) CFTR reveal a common fold [1]. However, functional studies demonstrate significant differences in the gating behaviour of hCFTR and mCFTR [2]. To understand the structural basis for the differences between human (h) CFTR and murine (m) CFTR, we generated by homologous recombination hmCFTR chimeras containing mCFTR domains on an hCFTR backbone by replacing all or part of hNBD1, hNBD2 or the hR-domain with the equivalent regions of mCFTR and investigated their biochemical and functional properties following stable expression in BHK cells. Results show that similar to wt-hCFTR, most chimeric proteins are processed to the cell membrane. However, two chimeras fail to mature: clone 12b (mNBD1, aa residues 518-585) and clone 114c (mNBD2, aa 1260-1412). We compared the murine sequence of these two chimeras with that of hCFTR to determine the physico-chemical distances (PCDs) of the respective amino acid (aa) changes. Changes with higher PCD values were selected and *in vitro* mutagenesis performed to introduce these aa alterations into hCFTR cDNA, which, for clone 12b, were: E527Q, E528Q (PCD = 29); S531T (PCD = 58); K536Q (PCD = 53), I539T (PCD = 89) and K584E (PCD = 56). For clone 114c the changes were: P1290T (PCD = 38), K1302Q (PCD = 53), Y1307N (PCD = 143), C1344Y (PCD = 194), D1394G (PCD = 94) and E1409D (PCD = 45). Biochemical and functional analyses of these mutants expressed as above revealed that all of these CFTR mutants were processed except for K584E. Consistently, all these CFTR mutants were found to be functional as cl^- channels albeit displaying some differences relative to wt-CFTR. The absence of processing observed for K584E-CFTR probably derives from its misfolding. In a model of full-length CFTR (R. Ford, personal communication) we found glutamic acid 584 is solvent exposed and interacting with Leu581. Consistent with this model, we establish that changing the human L581 interacting residue into the correspondent murine aa (L581F) rescues the trafficking defect of K584E. Moreover, by combining PCD values with information on disease causing mutations [3] we have identified additional critical residues (S1311K, T1263I, Q1309D, R1325K, V1338T, and L1367I) that may provide information on conformational differences between hCFTR and mCFTR and hence, derive structural implications for this protein. Similarly to our studies for NBD1, these mutants will help to identify critical residues which are responsible for both conformational changes and functional differences between human and murine CFTR.

Work supported by POCTI/MGI/47382/2002 (FCT, Portugal) grant, pluriannual funding of CIGMH (FCT, Portugal) and BBSRC (UK) grant. ACDaPaula is a recipient of PhD fellowship SFRH/BD/17475/2004 (FCT, Portugal).

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P2

S1.6 - CFTR Alternative Translation Results in N-terminus Truncated Proteins: Structural and Functional Consequences

Anabela S Ramalho[1, 2], Marzena Lewandowska[3], Carlos M Farinha[1, 2], Ann Harris[3], Margarida D Amaral[1, 2]

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[3]Childrens Memorial Research Center, Northwestern University Feinberg School of Medicine, Chicago, IL, USA

Most processing and functional disrupting Cystic Fibrosis (CF) mutations lie in the large cytoplasmic domains of CFTR, namely, the two nucleotide binding domains (NBDs) and the R domain (RD). This is not surprising given their role in regulating the channel activity as well as their major impact on the overall protein folding and maturation. The amino (N) terminus of CFTR (N-tail) has been less well studied despite its reported role in regulating the channel by interacting with a number of other proteins [1]. Furthermore, it was reported that a motif between D44-E60 is involved in regulating the channel gating via the RD [2].

We have detected in a Portuguese CF patient a novel mutation, 120del23 (i.e., the deletion of 23 nucleotides, 120 to 142) which thus abolishes the translation initiation codon (AUG pos.133-135). To address the question of whether 120del23 still allows CFTR protein to be produced and to study this putative N-truncated CFTR structurally and functionally, we generated a novel BHK cell line stably transfected with the pNUT-CFTR-120del23 cDNA construct.

Results from protein analysis show that this construct generates CFTR, albeit a shorter form, thus indicating that an internal initiation codon is used. Data from pulse-chase experiments with [^{35}S]methionine followed by CFTR immunoprecipitation, show that the N-terminus truncated CFTR is highly unstable. Moreover, results from iodide efflux (n=6) indicate that this truncated form of CFTR still retains ~25% (with a 2-min delay) of wt-CFTR channel activity, consistently with a previous study [3]. To determine the extent of amino acid loss from the CFTR N-terminus, caused by 120del23, we first analysed the CFTR cDNA *in silico* to search for internal initiation codons, using the AUG evaluator software [4]. Although 81 possible initiation codons were identified in CFTR coding region of these, only 32 are in-frame. By site-direct mutagenesis, we converted each methionine codon M150, M152 and M156 into valines (as well as the triple mutant), using the pNUT-120del23-CFTR cDNA as template. Immunoblot analysis of BHK cells lines stably expressing these mutants revealed the presence of two bands for M150V, M152V and M156V single mutants similar to those detected for 120del23-CFTR. Notwithstanding, for the triple mutant only the lower band was observed. Data from *in vitro* transcription and translation reactions with CFTR constructs lacking exon 1 plus the M82V, M150V single mutations show the same two bands (although additional bands are observed likely resulting from usage of out-of-frame AUG's). Consistently with *in vivo* results, transcription and translation *in vitro* from the M82/M150/M152/M156V construct causes disappearance of the upper of these two bands.

Altogether, our data indicate that Met150, Met152 and/or Met156 are used to produce the major N-truncated CFTR protein. Double mutants and the M82V constructs are being produced to determine which is the preferentially used alternative initiation codon.

Supported by pluriannual funding from CIGMH (FCT, Portugal). ASR is a recipient of postdoctoral fellowship SFRH/BPD/20622/2004 (FCT, Portugal).

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Thursday 10 April – 11:00-12:30 Room: Auditorium

SYMPOSIUM 2 - Genetics of CF - Gene Networks
Chairs: Harry Cuppens (BE) & Ann Harris (USA)

S2.1 - Modifier Genes in Cystic Fibrosis

Cuppens Harry[1], De Boeck Christiane[1], Castellani Carlo[2], Marianne Schwartz[3], Tania Pressler[3], Stuhrmann Manfred[4], Vavrova Vera[5], Macek Milan Jr[6], Schwarz Martin[6], Cassiman Jean-Jacques[1]

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5. Institute of Biology and Medical Genetics, Pneumology Clinic and Pediatrics Clinic, Charles University Prague, School of Medicine and University Hospital Motol Prague, Czech Republic;
6. Molecular Genetics Laboratory, Royal Manchester Children's Hospital Manchester, United Kingdom.

It has been well established that other genetic and environmental factors affect the CF disease phenotype. Although the initial trigger for disease follows a strict mendelian pattern as a result of one mutant gene, the phenotypic presentation follows a multifactorial complex pattern. A few dozen modifier gene studies have been undertaken in the past decennium. Most of these studies follow a variety of approaches and involve small sample sizes, and often did result in contradictory results. The current trend in modifier gene studies and complex genetics studies is to conduct studies in large patient or family cohorts to increase statistical power of association analyses for various clinical traits and specific covariates. High credence is given to the outcomes of these larger studies. In recent years, a large North American and a Canadian study have been undertaken, each of them involving more than 1000 CF patients. In these studies, $TGF\beta_1$ and MBL have been found/confirmed as CF modifier genes. A critical review and view is given on these CF modifier gene studies.
We also have studied the effect of the $TGF\beta_1$ and MBL genes in different populations from Europe (Belgian, Czech, North-Italian, German, Danish, and English CF patients), in order to evaluate the practical use of these findings.
Despite substantial progress, the vast majority of heritability remains unexplained. As in most complex genetic diseases, individual SNPs have very modest effects and account for only a fraction of the overall risk. Very likely there will be few (if any) large effects, a handful of modest effects, and a substantial number of genes generating small or very mall increases in disease risk. Although these modifier genes may be potential targets for (pharmacogenetic) treatment, their relevance for disease severity prediction is not clear and remains challenging.

S2.2 - Novel regulatory mechanisms for the CFTR gene

Neil Blackledge, Christopher Ott, Marzena Lewandowska, Ann Harris

Human Molecular Genetics Program, Children's Memorial Research Center, Northwestern University, Chicago, IL. 60614. USA.

Novel aspects of the tissue-specific regulation of expression of the CFTR gene have been revealed by the application of the new technologies developed for the Encode Project. However, of particular importance in these analyses is the use of primary cell cultures, which give more accurate insight into the normal CFTR regulatory mechanisms than can be provided by long-term cell lines. Cis-acting elements that are responsible for the tissue-specific expression of CFTR are known to include regions that lie outside the gene promoter, within introns and intergenic regions. Our current understanding of the CFTR locus predicts a domain that is flanked by insulator elements and within which a complex chromatin structure is maintained, to enable cell-type-specific expression of the gene. Specific transcription factors binding to cis-acting elements within the gene may recruit histone acetylases and bring distal elements in close proximity to the CFTR promoter to activate gene expression, through protein-protein and protein-DNA interactions. A better understanding of the regulation of expression of CFTR could have direct therapeutic application.

S2.3 - Towards understanding of the CFTR dynamic interactome: the example of inflammation

F. Borot[1], L. Jeanson[1], C. Guerrera[3], J. Colas, P. Pawlowski[4], F. Zehraoui[5], F. d'Alché-Buc, P. Zielenkiewicz[4], E. Escudier[5], F. Brouillard[1], M.Ollero[1], A. Edelman[1,3]

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To better characterize the molecular mechanisms underlying the inflammatory disturbances in CF, and in particular the eicosanoid pathway, we investigated the total protein expression pattern in (i) normal vs CF cells and (ii) in membrane microdomains from cells treated or not by TNFα, a pro-inflammatory cytokine. As an index of activation of the eicosandoid pathway we analysed the synthesis of leukotriene LTB4 and prostaglandin E2. The study was performed on Calu-3 and CFBE cell lines, and primary cultured cells derived from nasal polyps of controls and CF patients. We have used the classical cell and membrane biology methods followed by mass spectrometry identification, including protein quantification (emPAI and ITRAQ). The results indicate that the eicosanoid pathway is activated by TNFα within 10 minutes, showing the importance of this mechanism during acute inflammation. The response to TNFα is correlated with the appearance of a CFTR-annexin-1-cPLA2 macrocomplex in detergent resistant microdomains (DRM), which can be prevented by the inhibition of CFTR function. In addition, double SDS-PAGE analysis suggests that an important number of proteins are recruited in DRM by TNFα, suggesting that the macrocoplex may be much larger. Furthermore, the identification and quantification of differentially expressed proteins in human primary cultured cells suggests that the expression of major known CFTR-interacting proteins is altered. This may result in the disturbance of potential macrocomplexes involved in ion transport, inflammation, and/or protein trafficking. The results will be discussed in the context of all direct protein interactions described in the BioGrid database (i.e. 110 proteins found to interact physically with CFTR on the first or second degree will be analyzed by statistical learning and topological methods). Altogether, the data from experimental and in silico analyses indicate that the formation of dynamic macrocomplexes with or without CFTR is fundamental for an appropriate cell response to inflammation. This further indicates the necessity for studies on CFTR-related spatio-temporary protein-protein interactions at a large scale.

S2.4 - Towards *in vivo* correction of the *cftr* F508del allele: design and synthesis of two zinc finger nucleases for homology-directed repair

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The potential to repair the *cftr* F508del allele *in vivo* would constitute a significant step forward in the treatment of CF sufferers. However, gene repair by homologous recombination (HR) has an efficiency of less than 1:10^5 cells rendering it of little therapeutic value. In 2005, Urnov and colleagues[1] described a technique to increase efficiency of HR in cells by >10,000-fold. They designed a pair of zinc finger nucleases (ZFNs) to induce a double stranded break (DSB) at a unique genomic site. Co-transfection of the ZFNs with a donor repair sequence resulted in homology-directed repair (HDR) of a target allele in up to 20% of transfected cells. This level of HDR has been independently confirmed[2], and can be delivered by virus vectors[3].

Towards the application of this approach to repair mutations in the *cftr* gene, we report the design of two zinc finger proteins which each bind a 9 bp sequence in the cftr gene, separated by a 4 bp spacer, which corresponds to a 22 bp sequence in intron 9 of the *cftr* gene. The DNA sequence of each ZF protein was fused inframe with that from the nuclease domain[4] of *Fok*I in a vector containing both T7 and CMV promoters. The recombinant ZFN proteins (ZFN1 and ZFN2) were produced in a coupled transcription-translation reaction. Incubation of partially purified ZFN1 and ZFN2 proteins with a plasmid (pTarget-1/2) containing the 22 bp target site resulted in >95% digestion at this site. Plasmids expressing ZFN1 and ZFN2 will be co-transfected with a donor repair sequence into a *cftr* F508del$^{-/-}$ cell line. The degree of *cftr* F508del allele repair by ZFN-mediated HDR will be evaluated by RT-PCR. The degree of restoration of CFTR activity will be monitored by iodide efflux activity.

Although a pair of ZFNs can induce efficient HDR, ZFNs can homodimerise in cells and induce high levels of cytotoxicity[5]. Indeed, incubation of partially purified ZFN1 with a plasmid (pTarget-1/1) containing two copies of the 9 bp ZFN1 target site separated by the 4 bp spacer in a palindromic arrangement resulted in >95% digestion of the plasmid at this site. To prevent homodimerisation, two groups recently described similar remodelling strategies of the *Fok*I nuclease domain[2,5]. Introduction of corresponding mutations into ZFN1 and ZFN2 had no deleterious effect on the enzymes' ability to cut the plasmid corresponding genomic target (pTarget-1/2). Plasmids expressing the modified ZFN1 and ZFN2 will also be co-transfected with a donor repair sequence into a *cftr* F508del-homozygous cell line and evaluated for HDR activity. A comparison in the levels of cytotoxicity between the modified and unmodified ZFNs will be performed.

Acknowledgements
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S2.5 - Development of Post-Genomics Microscopy Assays to Automatically Screen for Protein Targets Affecting CFTR Traffic

Simão Luz[1,2], Shehrazade Dahimène[1,2], Filipa Mendes[1,2], Joana Almaça[1,3], René Barro-Soria[3], Karl Kunzelmann[3] and Margarida D Amaral[1,2]

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Systematic approaches like transcriptomics and proteomics have held promise of the next post-genomic challenge, *i.e.*, elucidation of protein function towards the understanding of biological processes in health and disease and as a fast track to therapy. However, these approaches fail to meet their expectations, as commonly they just deliver long lists of "candidate genes" or pathways. Functional microscopy-based assays in intact living cells with the potential for large-scale analyses have been recently developed and applied to membrane trafficking studies [1]. In combination with genome-wide RNA interference or over-expression strategies, these assays promise to help reveal comprehensively the regulatory networks underlying membrane traffic in intact cells.

Our aim here is to develop robust microscopy-based assays monitoring the secretory pathway of wt- and F508del-CFTR to be used in automatic microscopy-based screens so as to identify the relevant intervenients in CFTR membrane traffic. Thus, for the optimal assessment of plasma membrane CFTR, a Flag-tag was inserted at the fourth extracellular loop (EL) of both wt- and F508del-CFTR cloned into pcDNA3, as described [2]. Also, to assess total amount of CFTR expressed (so as to automatically obtain the ratio of 'total membrane CFTR' over the 'total expressed CFTR'), the mCherry fluorescent protein (~30kDa) [2] was fused to CFTR. To this end, we have cloned both wt- and F508del-Flag-CFTR into the pEGFP-C2 vector (Stratagene) and replaced GFP by the mCherry-tag. The expression, traffic and function of the fusion proteins resulting from these wt- and F508del-mCherry-Flag-CFTR-pEGFP-C2 constructs were assessed following their transfection into BHK-L cells. Results from Western blotting and immunocytochemistry show that the double-tagged CFTR proteins are expressed and that the wt-protein is membrane localized. Moreover, using the iodide efflux technique for BHK cells we observed that the wt-mCherry-Flag-CFTR displays Cl^- channel activity function which is ~20% of non-tagged wt-CFTR. Patch-clamp recordings are underway to further characterize the function of these constructs.

To follow the intracellular expression of CFTR *ab initio* and to monitor its traffic through the secretory pathway, we are cloning the mCherry-FLAG CFTR construct wt and F508del into lentiviral vectors with (and without) a tetracycline-inducible (Tet-On) promoter using the Gateway system (Stratagene). After validation, these systems will be adapted for automatic large-scale high-throughput microscopy screens on cDNA or siRNA arrays [4], to overexpress or down-regulate large sets of genes. Image processing routines will quantify the presence of CFTR at the plasma membrane in individual cells as to evaluate the impact of each individual gene on CFTR trafficking.

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Thursday 10 April – 14:30-16:00 Room: Auditorium

SYMPOSIUM 3 – CFTR Traffic
Chairs: Margarida Amaral (PT) & Gergely Lukacs (CDN)

P8

S3.1 - Folding and Misfolding of CFTR

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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 we are identifying steps in CFTR folding that are facilitated by cytosolic and ER localized chaperones. In addition, we have 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. Recently, we investigated the role which ER luminal calnexin plays in CFTR folding. In parallel, we probed the mechanism by which deletion of F508 arrests CFTR folding. Calnexin was found to be required for proper assembly of CFTR's membrane spanning domains (MSDs), which is also required for completion of down stream folding events that involve NBDII. Interestingly, F508del-CFTR exhibited biogenic defects that occurred both before and after the calnexin dependent step in CFTR folding. The RMA1 E3 ubiquitin ligase appeared to detect defects in MSD assembly, where as recognition of misfolded NBDII is mediated by the Hsc70/CHIP E3. Chemical correctors were observed to alter the conformation of specific sub-domains of CFTR and enable it to selectively pass through either the CHIP or Rma1 quality control checkpoints. Models that describe the mechanism by which chemical correctors enable F508del-CFTR to escape ER quality control will be discussed. This work is supported by the NIH and NACFF.

P9

S3.2 - Low Temperature Allows Mutant CFTR to Bypass the Endoplasmic Reticulum Quality Control

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Low temperature rescues numerous disease-associated mutant proteins from endoplasmic reticulum (ER) retention, possibly through higher folding efficiency and/or increased protein stabilization.
Here, we investigated the mechanism of low-temperature rescuing of F508del-CFTR, a temperature-sensitive mutant causing cystic fibrosis. Data show that at 26ºC the immature forms of both F508del- and wt-CFTR accumulate in their ER forms. However, after shifting cells back to 37ºC, most F508del-CFTR is rapidly degraded, whereas wt-CFTR efficiently matures, suggesting that these two proteins accumulate in different conformations. Moreover, data assessing the synergistic effects of low temperature and two genetic revertants (G550E and 4RK) which we recently proposed to rescue the trafficking defect of F508del-CFTR by distinct mechanisms [1] suggest that low-temperature rescuing of F508del-CFTR results from bypassing the ER quality control and not so much from promoting the mutant to acquire a stable conformation.

Work supported by grant POCTI/SAU/MMO/58425/2004 and pluriannual funding of CIGMH (FCT, Portugal).

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S3.3 - The chaperone-independent role of N-glycans in CFTR folding and trafficking

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The permissive effect of N-glycosylation, one of the most common posttranslational modifications, on membrane protein biogenesis and cell surface trafficking is primarily attributed to client protein targeting to the calnexin/calreticulin folding cycle and preventing their premature degradation by the ER quality control. ER resident lectin like-chaperones (calnexin and calreticulin) play a pivotal role in this process. The chaperone-independent role of N-glycans, however, in CFTR energetics (folding and stability) remains elusive. We used genetic and pharmacological techniques to compare the chaperone-independent function of N-linked glycans in the channel biogenesis, as well as secretory and endocytic membrane trafficking. The results suggest that calnexin recruitment modestly enhances the channel folding efficiency without discernable influence on the native state stability, measured by metabolic-pulse chase technique. In contrast, the N-glycan-deficient CFTR has a global conformational defect conceivable due to the destabilization of transmembrane segments and cytosolic domains. This leads to impaired folding yield at the ER, increased ubiquitination in post-Golgi compartments and rapid degradation of the glycosylation-deficient CFTR from the cell surface. Impaired recycling and preferential MVB/lysosomal delivery by the endosomal sorting machinery with the involvement of ESCRT complexes accounts for the short plasma membrane residence time of the glycosylation-deficient CFTR. These results suggest that N-glycans influence both the folding pathway and native state stability independently of lectin-like chaperones and outline a possible paradigm for the role of N-glycans as structural and trafficking determinant of CFTR.

S3.4 - Rescue of F508del-CFTR in CFBE410- cells is dependent on actin cytoskeleton interation with Ezrin and NHERF1

[1]Favia M., [1]Guerra L., [1]Fanelli T., [1]Monterisi S., [1]Cardone R., [2]Castellani S., [2]Conese M., [1]Reshkin SJ., [1]Casavola V.

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We have previously demonstrated that NHERF1 expression has an important role in up-regulating the activity and apical expression of CFTR in the human bronchial 16HBE14o- (HBE) cell monolayers expressing wildtype CFTR. Further, NHERF1 over-expression in CFBE41o- (CFBE) cells homozygous for the F508del mutation induces both a significant redistribution of F508del CFTR from the cytoplasm to the apical membrane and a rescue of CFTR-dependent chloride secretion. This re-distribution of F508del-CFTR could be a consequence of various interacting factors as NHERF1 has been proposed to mediate associations between CFTR and the cytoskeleton via the actin binding protein, ezrin. Confocal microscopy and actin polymerization assay of polarized monolayers showed that normal HBE airway monolayers have a well organized actin cytoskeleton, while CFBE monolayers displayed substantial disassembly of actin filaments and, importantly, over-expressing NHERF1 in CFBE produced a reorganization of the actin cytoskeleton. Transfection with either NHERF1 truncated of the C-terminal ERM domain (NHERF1-ΔERM) or with the dominant negative of ezrin caused reduction of both CFTR-dependent chloride efflux and F-actin content in HBE cells. Moreover, ezrin co-immunoprecipitated with NHERF1 only in HBE and in CFBE over-expressing NHERF1. These results confirmed that an organized cytoskeleton is critical for apical membrane CFTR functional expression and that NHERF1 has an essential role in regulating these mechanisms through its interaction with ezrin.

As only active ezrin is able to bind to NHERF1, we analyzed the distribution of active P-T567-ezrin. Confocal images demonstrated that while phosphorylated ezrin is scarsely and diffusely expressed in non-transfected CFBE cells, it is highly expressed at the apical membrane in monolayers of HBE and CFBE cells over-expressing NHERF1. Interestingly, this apical distribution was dependent on the capability of NHERF1 to interact with the N-terminus of ezrin because transfection of CFBE cells with the NHERF1-ΔERM construct no longer re-distributed phospho-ezrin to the apical membrane, suggesting that NHERF1 over-expression in CFBE cells may regulate ezrin activation and its recruitment on the apical region.

As the small GTPase, RhoA, has been shown to activate ezrin, we then investigated the role of RhoA in the NHERF1-ezrin mediated association of CFTR with actin. Transfection of CFBE cells with constitutively active RhoA (RhoA-V14) increased F-actin content and rescued CFTR-dependent chloride efflux. That this RhoA-dependent rescue of CFTR-dependent chloride efflux and the F-actin content in CFBE cells occurs through a common pathway with NHERF1 over-expression was confirmed by the finding that simultaneous transfection with RhoA-V14 and NHERF1 did not cause further increases of either F-actin level or CFTR-dependent chloride secretion.

This work was supported by the Italian Cystic Fibrosis Research Foundation (grant FFC#2/2007) with the contribution of the Christopher Ricardo Cystic Fibrosis Foundation, Loifur s.r.l. and Associazione Sergio Valente "100 alberi d'Autore".

S3.5 - Achieving Proteostasis in Cystic Fibrosis

W.E. Balch[1,2,5], D. Hutt[1], P. LaPointe[1], A. Koulov[1], Judith Coppinger[1], S. Pandrow[1], S. Stagg[1], B. Carragher[1,6], C. Potter[1,6], E. Powers[3], J.R. Yates 3rd[1,4], J.W. Kelly[1,7]

Departments of Molecular[1] and Cell Biology[2], Chemistry[3], and Chemical Physiology[4], The Institute for Childhood and Neglected Disease[5], The Center for Integrative Molecular Biosciences[6], The Skaggs Institute for Chemical Biology[7], The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037

Cystic fibrosis is a disease in which CFTR protein misfolding leads to an imbalance in membrane trafficking leading to dysregulation of conductance at the cell surface. As a consequence of destabilization of the energetics of the protein fold through mutation, the normal protein homeostasis or proteostasis (Science (2008) 319:916) environment fails to achieve a fold that escapes endoplasmic reticulum-associated degradation (ERAD) pathways. By readjusting the proteostasis environment to more robustly buffer and stabilize the CFTR fold, it may be possible to augment the cell with a repertoire of components that normalize function to provide benefit in disease. We will discuss approaches we have been taking to address cystic fibrosis in the context of specific components and 'systems' level adjustments that promote folding energetics favoring ER stability, export and improved conductance at the cell surface under physiological conditions.

Thursday 10 April – 16:30-18 **Room: Auditorium**

SPECIAL GROUP DISCUSSION I - Using Molecular Information for the development of new therapies

Moderators: Oscar Moran (IT) & Alan Cuthbert (UK)

CFTR has the architecture of an ABC-protein, with two membrane-spanning domains (MSDs) providing the pore like properties, an R domain involved with channel activity, and ATP binding and hydrolysis on two nucleotide binding domains (NBDs) responsible for gating the channel. The only domain whose structure is known is the NBD1. According to X-ray diffraction data from bacterial NBD crystals, the functional conformation of the NBDs would imply a close interaction between the two NBDs, in the 'head-to-tail' conformation. Electrophysiological and biochemical studies of wild-type and single point mutants of NBDs of CFTR support the idea that NBD1 and NBD2 interact closely in a dimer-like conformation, as observed in other ABC proteins.

The human NBD2 structure was predicted from that of the NBD1, and the dimer was modelled by arranging the two monomers in positions equivalent to those of other ABC proteins. More recently, using the structure of six intact ABC transporters, a homology model of the CFTR was constructed. Nonetheless, models are always based on assumptions and may well diverge from the real objects. We ask; 1- *are the models reliable?* 2- *how much experimental data support or contradict these models?* 3- *Do these models provide a good framework for the discovery or development of drugs?*

The most common CF mutation (F508del) occurs in NBD1 causing a traffic/folding problem in the protein. Using recombinant proteins, information on the physical-chemical properties and structural data of the wild type and F508del has been obtained. There is also conspicuous data on the interaction of CFTR (or its domains) with several chaperones. While particular efforts have been directed at understanding the physiopathology of the disease these data have not yet provided a description. Questions are; 1- *what information given by the molecular structure is relevant to understanding F508del misfolding?* 2- *is it possible to have a consensus description of CFTR-chaperone interaction to explain F508del traffic defects?* 3- *are these data sufficient to support the development of CF therapies?*

After several programs of high throughput screening (HTS) carried our by pharmaceutical industries and academia, various CFTR potentiators and correctors belonging to different chemical families have been identified. These data could be used to develop a solid QSAR model, to identify the pharmacophores, and by common chemioinformatics protocols, used for intelligent design of therapeutic drugs. We need to ask 1- *is all the information obtained in HTS projects available to researchers?* 2- *is the available information sufficient to develop a QSAR model?*

Partial support for OM from the Italian Cystic Fibrosis Foundation and for AC from the UK Cystic Fibrosis Trust is gratefully acknowledged.

Thursday 10 April – 16:30-18:00 Room: Miradouro

SPECIAL GROUP DISCUSSION II - Ca^{2+} Signalling and CF
Moderators: Carla Ribeiro (USA) & Kevin Foskett (USA)

Intracellular calcium (Ca^{2+}_i) signals play an important role in many airway epithelial functions, such as ciliary beating and chloride secretion. Previous studies have suggested that Ca^{2+}_i signals resulting from activation of apical G protein-coupled receptors are increased in primary cultures of CF human airway epithelia. The larger Ca^{2+}_i responses in CF airway epithelia are due to endoplasmic reticulum (ER) Ca^{2+} store expansion, which is an acquired response to luminal infection/inflammation. Moreover, the increased Ca^{2+}_i signals in CF epithelia have been linked to ER stress.

This Special Discussion Group will explore the following current issues related to Ca^{2+}_i signaling relevant to CF airway disease: 1) Is F508del-CFTR linked to ER stress in airway epithelia? and 2) The role of ER stress and Ca^{2+}_i-mediated airway inflammatory responses. In addition, the importance of Ca^{2+}_i signaling in airway physiology and CF pathophysiology will be exemplified by the role of Ca^{2+}_i responses in airway epithelial cells regarding regulation of fluid and macromolecule transport and secretion, *P. aeruginosa* exposure, NF-κB activation, production of reactive oxygen species, secretion of inflammatory mediators and mucins, and ATP release.

SGD II - Dysfunction of mitochondria Ca^{2+} uptake in cystic fibrosis airway epithelial cells.

Fabrice Antigny, Frédéric Becq and Clarisse Vandebrouck

Institut de Physiologie et de Biologie Cellulaires, CNRS, Université de Poitiers, Poitiers, France

In the genetic disease cystic fibrosis (CF) mutations of cystic fibrosis transmembrane conductance regulator gene (CFTR), among them the deletion of phenylalanine at position 508 (F508del-CFTR), lead to chloride impermeability in many exocrine glands (salivary, airways, pancreatic) associated with dehydration and reduced volume of the final secretory fluid (**Riordan et al., 1989**). In homozygous F508del-CFTR airway epithelial cells, the histamine Ca^{2+} mobilization is abnormally increased. The ER Ca^{2+} release and plasma membrane Ca^{2+} influx seem to be responsible of this abnormal Ca^{2+} signalling (**Antigny et al., 2007**). Mitochondria are known to play key roles in the Ca^{2+} homeostasis in several cells types **(Carafoli, 1987; Gunter and Pfeiffer, 1990).** Previous and pioneer works revealed that mitochondria are dysfunctional in CF fibroblast cells and that the major site of increased intracellular Ca^{2+} in CF is mitochondrial (**Shapiro and Lam, 1987; Shapiro, 1989**).

In the present work, we studied the mitochondria morphology, the mitochondria membrane potential ($\Delta\Psi_{mit}$) (with TMRE probes) and the rate of caspase 3 activity in human CF tracheal gland cells compared to non CF human tracheal gland cells. Additionally, we examined the CF and non CF mitochondria Ca^{2+}-power-buffering using Rhod2-AM probes to load mitochondria. Mitochondria Ca^{2+} uptake was stimulated by histamine-ER Ca^{2+} release. Our findings show:

- **(i)** -using transmission electron microscopic analysis, we show that the mitochondria network is dispersed and fragmented in CF cells compared to non CF cells where mitochondria are extended and continuous.
- **(ii)** -a mitochondria membrane depolarization in CF cells, independently of specific CFTR activity (no effect of $CFTR_{\text{-inh-172}}$).
- **(iii)** -mitochondria Ca^{2+} buffering is decreased in CF mitochondria compared to non CF mitochondria, due to a dysfunction of mitochondrial Ca^{2+} uniporter.
- **(iv)** -apoptosis inducers in non CF mitochondria provoke a $\Delta\Psi_{mit}$ collapse and a decrease of mitochondria Ca^{2+} uptake to the same level as for CF mitochondria.

These results demonstrate existence of a mitochondria dysfunction in human CF epithelial cells. Our data suggest a pre-apoptotic state of the human CF epithelial cells compared to non-CF cells. This mitochondria Ca^{2+} uptake defect in CF cells participates certainly to the abnormal Ca^{2+} mobilization in CF cells. These observations show that F508del-CFTR mutation have many impact in cell physiology.

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Supported by VLM and CNRS

Friday 11 April – 09:00-10:30 – Room: Auditorium

SYMPOSIUM 4 – Bicarbonate Transport in CF
Chairs: Paul Quinton (USA) & Ursula Seidler (DE)

S4.1 - IRBIT Coordinates fluid and HCO_3^- secretion in the pancreatic duct

Dongki Yang[1], Nikolay Shcheynikov[1], Weizong Zeng[1], Insuk So[2], Hideaki Ando[3], Katsuhiko Mikoshiba[3] and Shmuel Muallem[1]

[1]Department of Physiology, University of Texas Southwestern Medical Center, Dallas, Texas 75390, [2]Department of Physiology and Biophysics, Seoul National University College of Medicine, Seoul 110-799, Korea, and [3]Laboratory for Developmental Neurobiology, Brain Science Institute, Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako, Saitama 351-0198, Japan.

The cardinal function of the secretory glands ductal system is the secretion of a HCO_3^--rich fluid. Aberrant fluid and HCO_3^- secretion by secretory glands leads to several diseases, including cystic fibrosis. The pancreatic duct secretes fluid containing the highest known HCO_3^- concentration of about 140 mM. Pancreatic fluid and HCO_3^- secretion initiates with pNBC1-mediated Na^+-HCO_3^- influx across the basolateral membrane. HCO_3^- exit across the luminal membrane is mediated by the coordinated function of the SLC26 transporters and CFTR. The exact roles of the SLC26 transporters and CFTR in mediating Cl^- absorption and HCO_3^- secretion and how events at the basolateral membrane are coordinated with events at the luminal membrane are not well understood. Recently, it was shown that pNBC1 is regulated by the IRBIT (IP_3Rs binding protein released with inositol 1,4,5-trisphosphate). Regulation of pNBC1 by IRBIT raises the possibility that IRBIT regulates pancreatic duct fluid and HCO_3^- secretion by regulating transporters involved in this process. We will show that IRBIT regulates all forms of pancreatic duct fluid and HCO_3^- secretion by the concomitant regulation of pNBC1 and CFTR. The role of CFTR in pancreatic duct fluid and HCO_3^- secretion and how IRBIT regulates CFTR will be discussed.

S4.2 - Bicarbonate Transport in Human Airway Cells

Mike Gray

Epithelial Research Group, Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne, NE2 4HH, UK.

Despite the growing awareness of the importance of HCO_3^- in CF pathophysiology, our understanding of the mechanism of HCO_3^- secretion in CF-affected epithelial tissues is still unclear, particularly in the lung. HCO_3^- is an important component of epithelial secretions and, via its buffering role, controls the pH at the luminal cell surface. Alterations in surface pH are likely to contribute to CF pathogenesis as efficient solubilisation of secreted proteins such as mucus, is a pH-dependent process. In the airways, any increase in mucus viscosity caused by abnormal airways surface liquid pH, would decrease mucociliary transport, and increase the likelihood of submucosal gland blockage, both effects predisposing the lungs to bacterial infection, and disease.

Work primarily for Shmuel Muallem's group in Dallas has provided strong evidence that CFTR regulates HCO_3^- secretion in the pancreas and salivary glands through the activation of members of the SLC26A family of apically-located anion exchangers (AEs). Loss of CFTR would therefore lead to aberrant HCO_3^- secretion via reduced Cl^-/HCO_3^- exchange activity. We have been investigating HCO_3^- transport in the Calu-3 cell line, which are used as a model of serous cells of the human submucosal glands. These cells have been shown by many researchers to secrete HCO_3^- through a CFTR-dependent mechanism. However, it is not clear what role, if any, the SLC26A transporters play in this process. This talk will describe our recent molecular and functional experiments to determine whether polarised cultures of Calu-3 cells express functional Cl^-/HCO_3^- exchangers and whether activators of anion secretion modulate anion exchange activity.

Work supported by the BBSRC and Novartis

S4.3 - Key role of CFTR for all modes of intestinal HCO_3^- secretion

Anurag Singh[1] Wen Zheng[1], Markus Sjöblom[2], and Ursula Seidler[1]

[1]Dept. of Gastroenterology, Hannover Medical School and [2]Dept. of Neuroscience, Uppsala University

Backround: CF patients suffer from a variety of gastrointestinal problems, which may all be directly or indirectly linked to the inability of the intestinal mucosa to secrete HCO_3^- and to inhibit Na^+/H^+ mediated fluid absorption and proton secretion. This has led to an intense search for alternative modes of intestinal anion transport, and the expression of a variety of potential alternative intestinal anion transporters have been reported.
Aim: To delineate the dependency of different modes of intestinal HCO_3^- secretion on CFTR expression.
Methods and Results: We studied acid-, agonist-, and HCO_3 stimulated, as well as Cl^--dependent HCO_3^- secretion in the $CFTR^{tm1cam}$ and WT murine duodenum *in vivo*. NHE3 and Slc26a6-deficient mice were used for selected questions. In WT mice, luminal acid, forskolin, heat-stable E. coli enterotoxin (STa), PG E_2, carbachol, and an increase of blood HCO_3^- all stimulated duodenal HCO_3^- secretion in anesthetized WT but not in $CFTR^{tm1cam}$ mice. Pharmacological inhibition or genetic ablation of NHE3 resulted in a significantly higher basal HCO_3^- secretory rate, which was electroneutral and therefore due to an unmasking of apical Cl^-/HCO_3^- exchange activity. Accordingly, Slc26a6 ablation attenuated S1611-induced J_{HCO3-}. Removal of luminal Cl^- reverted basal HCO_3^- secretion to H^+ secretion, but surprisingly, forskolin was able to elicit a full HCO_3^- secretory response. In the absence of CFTR, electroneutral NaCl absorptive rates were similar to wt rates, but S1611 induced virtually no increase in HCO_3^- secretion.
Conclusion: This indicates that the apical anion exchangers Slc26a6 and Slc26a3 need proton recycling via NHE3 to operate in the Cl^- absorptive mode, and Cl^- exit via CFTR to operate in the HCO_3^- secretory mode. In addition, Cl^- independent HCO_3^- secretion can be stimulated by cAMP increase, likely from the crypt reagion, which expresses high levels of CFTR but none of the anion exchangers.

S4.4 - CFTR activation regulates both apical and basolateral Cl^-/HCO_3^- exchange activity in Calu-3 cells

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[3]Novarits Insititutes for Biomedical Research (NIBR), Novartis Horsham Research Centre, Wimblehurst Road, Horsham, West Sussex, RH12 5AB, UK.

Calu-3 cells are used as a model of the serous cells of human airway submucosal glands which are involved in CFTR-dependent anion secretion. The aim of this work was to characterise Cl^-/HCO_3^- anion exchange (AE) activity in Calu-3 cells and the dependence of AE activity on CFTR. Control and CFTR knock down (KD) Calu-3 cells (1) were grown as monolayers on Transwell supports. Anion exchange activity was measured in cells loaded with the pH-sensitive dye BCECF-AM. Removal of basolateral Cl^- (substituted with gluconate) under non stimulated conditions produced an alkalinisation in pH_i of 0.45±0.02 (mean ± s.e.m.; P<0.001 paired t-test, n=4), which was abolished by 0.5mM H_2-DIDs, consistent with the presence of a basolateral DIDs-sensitive AE (SLC4A2), as previously reported (2). Stimulation of cells with forskolin completely inhibited this basolateral AE activity. Conversely, apical Cl^- removal had no effect on pH_i under resting conditions, but caused an alkalinisation of 0.51±0.02 (P<0.001, n=4) after forskolin stimulation. This apical alkalinisation was insensitive to H_2-DIDs but prevented by the CFTR inhibitor GlyH-101 (10μM). When cells were stimulated with forskolin in the presence of apical GlyH101, cells now displayed a significant basolateral AE activity (~60 % alkalinisation compared to unstimulated cells) in response to basolateral zero Cl^-. Assuming that GlyH101 did not affect cAMP/PKA activation, these results suggest that anion transport by CFTR is linked to inhibition of basolateral AE activity. The cAMP-dependant switch in basolateral and apical AE activity was also observed with bilateral adenosine (10μM). However, when adenosine was applied to the basolateral membrane only, basolateral AE activity was reduced, but not eliminated, and apical AE was not fully activated (~34% of the bilateral adenosine response). In contrast, apical adenosine fully activated apical AE, but did not cause complete inhibition of the basolateral AE. These results suggest that activation of apical AE alone is not sufficient to inhibit basolateral AE activity.

To further investigate the role of CFTR in regulating AE activity, experiments were also conducted on CFTR-KD Calu-3 cells (1). Forskolin produced qualitatively similar effects on apical and basolateral exchangers observed in wild-type cells. However, under resting conditions basolateral AE was ~32 % less active (calculated from the rate of re-acidification following the re-introduction of Cl^-) compared to WT Calu-3 cells, and CFTR-KD cells also showed some residual basolateral AE activity following forskolin stimulation. Significantly, forskolin-stimulated apical AE activity was markedly decreased (~48%) in CFTR-KD cells.

These data show that increases in intracellular cAMP in Calu-3 cells cause the activation of an apical Cl^-/HCO_3^- exchanger as well as the inhibition of a basolateral Cl^-/HCO_3^- exchanger, through a CFTR-dependent mechanism. Such a switch in AE activity would favour bicarbonate secretion, consistent with the finding that cAMP enhances bicarbonate-dependant fluid secretion from Calu-3 cells. Dysregulation of Cl^-/HCO_3^- exchange would be predicted to reduce HCO_3^- and fluid secretion and contribute to the pathological changes observed in CF submucosal glands

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Friday 11 April – 11:00-12:30 - Room: Auditorium

SYMPOSIUM 5 – Inflammation and other respiratory responses in CF
Chairs: Gerd Döring (DE) & Cristelle Coraux (FR)

S5.1 - Airway epithelial repair and regeneration

CORAUX Christelle, ROUX Jacqueline, JOLLY Thomas, BIREMBAUT Philippe

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In permanent contact with the external milieu, and in numerous respiratory diseases such as Cystic Fibrosis (CF), the airway epithelium is frequently injured by bacteria, viruses as well as by inflammatory mediators. The alterations of the airway epithelial structure may vary from epithelial remodelling, barrier integrity loss due to intercellular junction disruptions, to more or less denudation of the basement membrane, leading to the impairment of the epithelial defense functions. In response to chronic injury, the airway epithelium may be shedded or can transdifferentiate with dynamic changes in cell phenotype, and gene and protein expression. Such a remodelling illustrates the marked plasticity of the epithelium. These transient or permanent alterations in the histological structure and functions of the airway epithelium play an important role in the pathogenesis of CF. In order to restore its defence functions, the respiratory epithelium has to rapidly regenerate. The complete regeneration is a complex phenomenon including, not only the epithelial wound repair through wound-edge basal cell spreading, migration and proliferation, but also the epithelial differentiation to reconstitute a well differentiated and functional epithelium. Each step of this process can be actively modulated by mediators released from epithelial, mesenchymal or inflammatory cell sources. Using *in vitro* and *in vivo* models of human airway epithelial repair and regeneration, we have observed that during the initial epithelial cell migration and proliferation, the pro-inflammatory IL-8 cytokine is markedly expressed whereas pseudostratification and terminal differentiation are associated with an increased expression of epithelial matrix metalloproteinases (MMP)-7 and-9 and a progressive IL-8 decrease demonstrating a spatio-temporal regulation of MMPs and IL-8 during the regeneration process. Interestingly, the incubation of the regenerating epithelial cells with MMP inhibitors leads to a default of mucociliary differentiation and to the generation of a remodeled airway epithelium exhibiting squamous metaplasia with areas of basal cell hyperplasia, demonstrating the involvement of these molecules during the airway epithelial regeneration process, and particularly during the final step of epithelial differentiation. Secretory mucins (MUC5AC and MUC5B) are only expressed at the final step of complete differentiation together with the trefoil factor family peptide TFF3. Whereas exogenous recombinant human TFF3 does not affect MUC5AC-positive mucous cell differentiation and number, it induces an increase in the number of ciliated cells, this promotion of airway epithelial ciliary differentiation occurring in part through an EGF-R dependent pathway. In CF, in absence of infection, the airway epithelial regeneration is altered and delayed, leading to the generation of a remodeled airway epithelium with a pronounced height increase and basal cell hyperplasia in comparison with non-CF airway epithelium, this regeneration process being associated with a deregulated pattern of expression of MMP-7, MMP-9, tissue inhibitor of metalloproteinases (TIMP)-1 and IL-8. Altogether, these results suggest that a dysregulation in the pro-inflammatory cytokines and MMPs may induce an epithelial remodelling and an abnormal regeneration process. Understanding how the biological active molecules are up- or down-regulated during the course of regeneration may lead to the development of novel pro-regenerative therapeutic approaches in respiratory diseases such as CF.

S5.2 - Immunobiology of a novel genetic modifier of F lung disease

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In the CF lung, chronic infection and dysregulated neutrophilic inflammation lead to progressive airway destruction. Despite the molecular insights afforded by identification of the responsible gene, *CFTR*, a clear understanding of the pathogenesis of lung disease in CF remains elusive. The poor correlation between genotype and phenotype in the CF lung indicates that disease expression is influenced by environmental exposures and/or modifier, genes. Published studies indicate significant heritability of lung disease severity in CF, independent of *CFTR* genotype. To search for genes modifying CF lung disease, we performed a genome-wide single nucleotide polymorphism scan in one cohort of patients with CF (the Gene Modifier Study Group) with replication of top candidate modifier genes in a complementary, independent cohort of patients (the U.S. Twin Sib Study). Using this approach, genetic variation in a transcriptional co-regulator was identified and replicated as a modifier of lung disease severity in CF. Data outlining the relevant immunobiology of this novel genetic modifier of CF lung disease will be presented.

S5.3 - Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis

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S5.4 - CFTR expression suppresses NFκB-driven inflammatory signalling

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Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) gene was identified nearly 20 years ago but it remains a puzzle how defects in the gene can cause persistent chronic pulmonary infection and inflammation. Numerous studies have shown that inflammatory signalling through the NFκB pathway is increased in CF lungs and that this is linked to the production of pro-inflammatory cytokines such as interleukin 8 (IL-8), e.g. (Verhaeghe, Delbecque et al. 2007); (Carrabino, Carpani et al. 2006). However, there is little consensus on the mechanism(s) which link CFTR and its inheritable mutant forms to chronic lung inflammation. In this study, we tested the hypothesis that wild-type CFTR participates at a fundamental level in the control of endogenous (i.e. unstimulated) NFκB activity and cytokine secretion. Our results show that the level of CFTR expression correlates inversely with endogenous NFκB activity and IL-8 release.

Methods

We used H441 airway epithelial cells which do not express CFTR under basic culture conditions (RPMI + 10% FCS). Cells were transfected with increasing amounts (0-400ng) of wild type CFTR together with a luciferase reporter vector driven by four NFκB response elements. This allowed us to measure NFκB activity, the transcription factor which drives expression of the majority of inflammatory cytokines. The secretion of one of these cytokines, IL-8 was also measured by ELISA assay.

Results

Cells transfected with a control (empty vector) displayed high endogenous NFκB activity. This high endogenous level of NFκB activity was significantly suppressed by transfection with wild type CFTR in a dose dependent manner and this was paralleled by a decrease in IL-8 secretion, e.g. transfection of 200 ng of CFTR wt vector resulted in a 47% reduction in NFκB activity and a 30% decrease in IL-8 secretion.

Conclusions

This data indicates that CFTR has anti inflammatory properties and that the hyper-inflammation found in CF may be due to a disruption of the signalling link between CFTR and NFκB. This is contrary to the belief that inflammation in the CF lung is due to loss of sodium/ chloride transport causing poor clearing of the lung. Further work is required to identify how CFTR interacts with these inflammatory pathways and how this is regulated. Understanding of this could lead to new treatments for inflammation in CF resulting in prolonged better quality of life for these patients.

This work was supported by the CF trust

Carrabino, S., D. Carpani, et al. (2006). "Dysregulated interleukin-8 secretion and NF-kappaB activity in human cystic fibrosis nasal epithelial cells." J Cyst Fibros **5**(2): 113-9.

Verhaeghe, C., K. Delbecque, et al. (2007). "Early inflammation in the airways of a cystic fibrosis foetus." J Cyst Fibros **6**(4): 304-8.

S5.5 - Proteomics of membrane microdomains from human bronchial cells subjected to proinflammmatory stimulation

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Introduction and aims: In a previous study, we have observed that CFTR, Annexin 1 (A1) and cytosolic phospholipase A2 (cPLA2) are partially recruited in detergent insoluble microdomains (DIM) upon proinflammatory stimulation and that they are involved in the same macrocomplex. A1 and cPLA2 participate in the inflammatory response notably via the eicosanoid pathway. Recruitment is prevented by specific inhibition of CFTR and is concomitant with an increase of eicosanoid (prostaglandin E2 and leukotriene B4) production. It is postulated that CFTR, A1 and cPLA2 participate in the regulation of inflammation by their dynamic interaction within DIM. In this study we aimed to identify other potential partners of this complex by proteomic analysis of DIM.
Methods: We adapted and compared two proteomic approaches: in-gel trypsin digestion and double SDS-Page (dSDS-PAGE), which are compatible with membrane protein analysis. Human epithelial respiratory cells CFBE and IB3/C38 (expressing either WT or F508del-CFTR) were subjected or not to proinflammatory conditions (TNFα, 100U/mL, 3h). DIM were isolated by OptiPrep density gradient, analyzed by either of the two methods, and proteins identified by LC/MSMS. Semi-quantification of identified proteins was performed by the emPAI method, which is function of the ratio between the number of identified peptides for a given protein and the total number of theoretical trypsin-induced peptides.
Results: dSDS-Page permitted the identification of 100 proteins, including 30 membrane proteins, and 40 membrane-associated. The differential analysis showed significant differential expression of cytoskeletal proteins (cytokeratin 18, actinin), inflammation-related proteins (annexins 1 and 2), folding-related proteins and chaperons (PDI, Hsp) and ion channels in DIM between control and TNFα-treated WT vs F508del CFBE cells. In-gel analysis allowed the identification of more than 150 proteins in DIM. By means of emPAI, we etablished a list of proteins differentially expressed in IB3 vs C38 subjected or not to TNFα treatment. This included CFTR-interacting proteins, cytoskeletal proteins, and proteins involved in TNF signalization. As a proof of concept, some characteristic DIM-associated proteins, such as caveolin-1 and 2, flotillin-1 and 2, were detected in all samples subjected to in-gel analysis. In addition, a number of membrane proteins were identified (ion transport proteins, transmembrane proteins and receptors).
Conclusion: dSDS-PAGE and in-gel digestion reveal as useful methods for membrane microdomain proteomic analysis. Proinflammatory treatment induces differential expression of several proteins in DIM from normal versus mutated CFTR-expressing cells. These results will provide valuable information about potential components of a DIM-associated CFTR-containing dynamic macrocomplex, as well as new hypotheses on the participation of CFTR in the regulation of the inflammatory response.

This work has been supported by Vaincre la Mucoviscidose and by the European Commission

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S5.6 - Identification of anti-inflammatory potential of *Aegle marmelos* by screening medicinal plant extracts.

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Attenuation of the chronic inflammatory process in the lung of CF patients is considered a complementary therapeutic strategy to intervene on the progressive tissue damage. Among the different approaches to interrupt this deleterious process, specific reduction of neutrophil chemotaxis, e.g. by controlling IL-8 expression, is under investigation. We recently initiated a screening of plant extracts already utilized in Bangladeshi traditional medicine to select molecular components able to reduce chemokine expression from bronchial epithelial cells challenged with *P.aeruginosa*. To prioritize the screening procedure, we selected firstly those plant extracts showing the capacity of inhibiting the binding of the transcription factor NF-kB with the DNA consensus sequences identified in the promoter of IL-8, since NF-kB has been confirmed playing a critical role in regulating IL-8 expression in respiratory epithelial cells (1, 2). Therefore, extracts have been added to cells 24 hr before pro-inflammatory challenge with *P.aeruginosa* and quantification of IL-8 transcription in the IB3-1 CF bronchial epithelial cells *in vitro*. *P.aeruginosa*-dependent IL-8 mRNA induction was increased by *Argemone mexicana, Vernonia antihelmintica*, no significant modification of transcription was observed with *Aphanamixis polystachya, Lagerstroemia speciosa* and *Hemidesmus indicus* and, finally, inhibition was observed with *Polyalthia longifolia* (IC_{50} = 200 µg/ml) and *Aegle marmelos* (IC_{50} = 20 µg/ml). A proliferation assay performed by pre-incubating the plant extracts of *Polyalthia longifolia* and *Aegle marmelos* with IB3-1 cells for 24 and 48 hr excluded that the inhibitory effect observed on IL-8 transcription was dependent on an unspecific interference with cell viability. Five compounds isolated from the most effective extract *Aegle marmelos* were therefore tested. No significant effect on *P.aeruginosa*-dependent transcription of IL-8 mRNA was observed with butyl-p-tolyl-sulfate, whereas the inhibition obtained with 6-methyl-4-chromanone at 1 mM concentration was due to a possible anti-proliferative effect. On the contrary, 5,6 dimethoxy-1-indanone, 2-hydroxy-cinnamic acid and 5-methoxypsoralen resulted in significant IL-8 inhibition at 1-10 µM concentration without side effects on proliferation. In synthesis, initial screening of plant extracts is starting selecting new molecules with potential therapeutic effects on neutrophil chemotactic signalling.

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2) Bezzerri V, Borgatti M, Nicolis E, Lampronti I, Dechecchi MC, Mancini I, Rizzotti P, Gambari R, Cabrini G.
Am J Respir Cell Mol Biol. In press 2008

Friday 11 April – 18:30-20:00 **Room: Miradouro**

SPECIAL GROUP DISCUSSION - III: CFTR Dysfunction in Surface Epithelium vs Glands
Moderators: Lucy Clunes (USA) & Jeffrey Wine (USA)

Airway glands produce most airway mucus which is then modified and transported out of the airways by the surface epithelium. In small airways that lack glands, mucin and protein secretion are achieved by surface cells, indicating the intimate and complementary relations between glands and surface epithelium in airway defense. Although glands and surface jointly participate in mucociliary clearance, a fair question is how each of them is impacted by loss of CFTR.

Normal superficial epithelia regulate airway surface liquid (ASL) volume by sensing volume changes and altering chloride and sodium transport accordingly. The loss of CFTR results in ASL dysregulation caused by chloride hyposecretion and sodium hyperabsorption. This leads to a failure to adequately hydrate mucus on the airway surface, resulting in mucus plugs, which are first observed in the distal airways (where there are no glands). It has been shown that mucus accumulation in glandless mice produces neutrophilic inflammation and goblet cell hyperplasia, both of which are characteristic of CF lung disease.

For glands, loss of CFTR results in the loss of fluid secretion to VIP and SP and loss of secretion produced by combinations of these agonists with low levels of ACh. However, mucins are still secreted; resulting in viscous secretions. High levels of ACh stimulation recruit CFTR-independent pathways and bulk mucus secretion is near normal, although such mucus is more acidic and more viscous.

It seems likely that both surface and gland dysfunctions contribute to the pathophysiology of CF airways: gland secretions are abnormally concentrated in CF and liquid hyposecretion plus hyperabsorption across the surface epithelium will exacerbate the problem. An unanswered question of practical significance is whether fixing either one alone would be sufficient to restore normal airway function.

The purpose of this Special Group Discussion is to clarify what information is required to complete a comprehensive model of CFTR function in the airways, and what experiments could yield such information.

SGD III - Reduced A2BR-mediated Regulation of Mucus Clearance in Cystic Fibrosis Airway Epithelium

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CFTR is an apical membrane chloride channel whose activity is required to maintain airway surface liquid (ASL) volume and efficient mucociliary clearance; it is known to exist in macromolecular complexes with a variety of signaling and transporter molecules. Altered adenosine signaling has been associated with both asthma and chronic obstructive pulmonary disease (COPD). More recently the A2B subclass of adenosine receptor (A2BR) has been shown to directly drive pulmonary inflammation and lung damage in mice. Accordingly, it has been proposed that specific inhibition of the A2BR be used to treat inflammatory lung disease in humans. However, stimulation of CFTR via adenosine is thought to be a key aspect of the lung's innate defense system and to be crucial in permitting the superficial epithelium to maintain a 7 μm periciliary liquid layer (PCL), which is required to produce hydrated and flowing mucus. We hypothesized that A2BR not only functionally and physically associates with CFTR but helps to stabilize it in the plasma membrane by forming an apical membrane complex. We found that the A2BR was the only adenosine receptor expressed in human bronchial epithelia both molecularly and functionally. The expression levels of this and the other adenosine receptors were unaffected by inflammatory stimuli. However, we found that the A2BR was required to report ASL volume status to the epithelia via changes in airway surface liquid (ASL) adenosine levels. Addition of the specific A2B antagonist (ATL801) attenuated the ability of bronchial epithelia to autoregulate ASL height. Similarly, varying A2BR stimulation by removing adenosine with adenosine deaminase and replacing it with different concentrations of NECA resulted in dose-dependent changes in ASL height. To investigate the physical relationship between the A2BR and CFTR, we designed a fluorescent resonance energy transfer (FRET) pair of cfp-CFTR (labeled at the n-terminus) and yfp-A2BR (labeled at the c-terminus). Significant increases in FRET between A2BR and CFTR were observed upon simulation with adenosine (ADO) that were inhibited by the specific A2BR antagonist 8-SPT, demonstrating that stimulation induces the two proteins to interact more closely with each other. In contrast, no such increase in FRET was observed between CFTR and the 2A-R. Evidence from surface biotinylation studies explained the FRET response by demonstrating that apical stimulation of A2BR induces recruitment of both the receptor and CFTR to the apical membrane.
We conclude that ADO and A2BR are absolutely required for ASL volume homeostasis in human airways. We also speculate that the association of A2BR and CFTR may not only anchor the proteins in the plasma membrane but may also function to stabilize them in a signaling complex. Due to there being no evidence of such a complex between CFTR and the 2A-R we suggest that there are two separate pools of CFTR in the apical membrane that are independently regulated by either adenosine or isoproterenol.

SGD III - Differential expression of PLUNC proteins in CF lung disease.

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We have previously described the characterisation of the PLUNC family of secreted proteins in both man and mice. PLUNCs are expressed in the upper airways, nose and mouth and the family subdivides into short (S) and long (L) proteins, which contain domains structurally similar to one or both of the domains of bactericidal/permeability-increasing protein (BPI). Due to this structural similarity, the sites at which the genes are located and the observation that they are rapidly evolving and have diversified in the mammalian lineage, we hypothesised that PLUNCs may play a role in innate immune defence. Despite the diversification of the PLUNC family between human and mice, SPLUNC1 and LPLUNC1 are the two genes that are most highly expressed in the respiratory tract in both species. A number of studies have suggested that these genes are differentially expressed in human lung diseases and in mouse models.
There are limited studies of the localisation of PLUNCs at the protein level. Our previous studies have however shown that in the respiratory tract SPLUNC1 is predominantly a product of the submucosal glands with less present in the non-ciliated epithelial cells of the large airways and none present in small airways or lung parenchyma. In CF we have shown that staining of SPLUNC1 is significantly increased in diseased airways and is also present in some of the inflammatory infiltrates. We have also shown that it is epithelial cells rather than the inflammatory cells that are the source of this protein. Using novel affinity purified antibodies we have now been able to demonstrate that LPLUNC1 strongly stains a population of airway goblet cells in non-diseased lung. Importantly however, co staining with MUC5B shows that LPLUNC1 is not a surrogate marker for all goblet cells. Submucosal glands stain less intensely with LPLUNC1 than they do for SPLUNC1. Elevated epithelial staining of LPLUNC1 is also seen in diseased small airways in CF and also submucosal glands. It is unclear why expression of these two proteins is increased in CF. We have not been able to show that either gene is transcriptionally regulated by pro-inflammatory mediators in ALI cells in culture. Furthermore, using mouse specific antibodies, we could not identify differential expression of either protein in lungs from CFTR mutant mice. The direct effect of pulmonary infection on PLUNC expression may help to resolve this question. Thus our results show unique expression domains for SPLUNC1 and LPLUNC1 within the airways and suggest that alterations in expression of these putative innate immune molecules, is associated with CF lung disease in humans.

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SGD III - Anaesthesia and the elemental content of mouse nasal fluid

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Currently, only the symptoms of CF can be treated, but there is no treatment for the underlying defect, the defective chloride channel CFTR. During recent years, a number of compounds has been suggested that potentially could correct the defect in CFTR, based mainly on experiments with cultured cells. The next step would be to test these compounds on transgenic animals. Although gene-targeted mouse models for CF have not always developed lung pathology that mimics the human disease, we have shown that both CF-patients and CF null mice have significantly elevated concentrations of Na, Cl, and K in their airway surface liquid (ASL) and their nasal fluid, which probably could be normalized by an effective pharmacological treatment.

C57BL/6J mice (F12 backcross) heterozygous for the F508del mutation ($Cftr^{tm1\ Eur}$) were bred as heterozygotes and offspring was genotyped by PCR analysis. Homozygous F508del mice ($Cftr^{tm1\ Eur}$) and wild-type control mice were temporally anesthetized with a solution of 2,2,2 tribromoethanol in 2-methylbutan-2-ol (Avertin). Nasal fluid was collected in Sephadex G-25 beads by applying the beads to double-side tape attached to a filter paper, that was cut in triangular strips (base about 0.2-0.3 mm, length about 0.8 mm) with a narrow tip and inserted into the nostril of a mouse, and kept there for 10 min. The strip of filter paper was then removed, prepared for analysis, and analyzed by X-ray microanalysis in the scanning electron microscope, as described by Kozlova et al. (2005).

The nasal fluid of F508del-CFTR mice had a higher content of Na, Cl, and K, than the nasal fluid of control mice. Both in the F508del-CFTR mice and in the control mice, there was no significant difference in the composition of the nasal fluid collected from the living mice during the first or second anaesthesia, or at the post-mortem sampling. Hence, repeated anaesthesia did not influence the elemental content of the nasal fluid. During experiments all animals survived and no negative symptoms of the anaesthesia were observed.

CF mice are a convenient model for testing novel therapies such as gene therapy and new pharmaceuticals and their survival in long term experiments will be beneficial for many researchers. CF mouse models will be instrumental not only for understanding the disease better, but also for the discovery of modifier genes. The method presented here will allow the use of CF mice in studies, where repeated measurements of parameters on a single animal have to be carried out.

Kozlova I et al. (2005) Am J Physiol Lung Cell Mol Physiol 288: L874-8.

SGD III - Mucin gene expression in airway epithelial cells from children with CF.

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Mucus hypersecretion is a feature of cystic fibrosis (CF). MUC5AC and MUC5B form the major macromolecular components of the mucus gel and are secreted by airway epithelial cells (AEC). In adults, AEC mucin production has been shown to be low in stable CF but increases in pulmonary exacerbations. AIM: To investigate the influence of Pseudomonas (PsA) infection and clinical status on MUC mRNA expression in AECs from paediatric patients with CF compared to healthy controls. Secondly, to investigate whether MUC gene expression is intrinsic to these cells by measuring mRNA in cultured AECs from these same patients. METHODS: AECs were collected by non-bronchoscopic bronchial brushing of children with CF (n=15; PsA+/-ve; stable/pulmonary exacerbation) and without CF (n=15) undergoing elective surgery. AECs in collected cell suspensions were purified by incubation on anti-CD68 coated plates. Cells were then separated for immediate mRNA analysis or incubated with BEGM growth media at 5% CO_2 until confluence. MUC mRNA expression was quantified using real-time PCR with SYBR Green. RESULTS: Preliminary results indicate that MUC5B mRNA was lower in freshly isolated AEC's from CF compared to healthy patients and also lower in cultured AEC's taken from CF compared to healthy patients. MUC5B expression was not affected by clinical nor PsA status. CONCLUSIONS: MUC5B mRNA expression is decreased in freshly isolated and cultured AECs from CF patients and does not appear to be influenced by PsA or clinical status.

SGD III - The NCI-H441 cell line as a model for the study of airway epithelial sodium transport - a comparison with cultured human bronchial epithelial cells

Rosemary Sugar[1], Hazel Atherton[1], Rachel Burrows[1], Zaynab Neeto[1] & Henry Danahay[1]

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Defective mucus clearance, associated with airway obstruction and infection, is the hallmark of cystic fibrosis (CF). The epithelial sodium channel (ENaC) is considered to be a key regulator in influencing lung fluid reabsorption. It is widely accepted that Na^+ hyperabsorption in the CF airway reduces mucosal hydration thereby attenuating mucus clearance. Consistent with this, enhanced mucus clearance has been demonstrated in CF patients following inhaled amiloride, a blocker of ENaC activity.

The aim of this study was to examine the pharmacological regulation of ENaC in immortalised NCI-H441 cells, a cell line derived from the human distal airway epithelium. When cultured at air liquid interface, NCI-H441 cells form a Na^+ absorptive epithelial monolayer (Ramminger et al, 2004) and may therefore represent an alternative model to the more expensive cultures of bronchial epithelial cells. To this end, we have directly compared the profile of pharmacological regulation of ENaC in NCI-H441 cells with cultures of primary human bronchial epithelial cells (HBECs).

Over the course of these studies, NCI-H441 cells retained an air liquid interface generating a spontaneous short circuit current (I_{SC}) of 24.0±0.72μA/cm^2 (n = 53; inserts) with an epithelial resistance of 214±14Ω·cm^2 (n = 53). This compares well with HBECs which displayed an I_{SC} of 21±0.97μA/cm^2 (n = 43) and had an increased epithelial resistance of 1770±126Ω·cm^2 (n = 43). The amiloride-sensitive short circuit current (I_{amil}; 21.1±0.54μA/cm^2, n = 43) was similar to HBECs (I_{amil} 17.1±0.94μA/cm^2, n = 43). Concentration response data using ENaC blockers, revealed a potency order of CF552 (IC_{50} = 4nM; n = 9) >>> benzamil (IC_{50} = 70nM; n = 9) > phenamil (IC_{50} = 251nM; n = 4) > amiloride (IC_{50} = 863nM; n = 9) in NCI-H441 cells. This was similar to the potency order of CF552 (IC_{50} = 3nM; n = 8) >> benzamil (IC_{50} = 24nM; n = 11) > phenamil (IC_{50} = 94nM; n = 11) ≥ amiloride (IC_{50} = 205nM; n = 43) in HBECs.
ENaC activity in the human airway epithelium is sensitive to the broad spectrum serine protease inhibitors, aprotinin and placental bikunin (Bridges et al, 2001). These inhibitors decrease ENaC mediated current over a much slower time frame compared to the direct blockers of this channel. Aprotinin (IC_{50} = 220nM) induced a time-dependent attenuation of the basal and amiloride-sensitive I_{SC} in NCI-H441 cells that was reversed upon addition of excess trypsin to the apical surface, consistent with HBECs (IC_{50} = 810nM). The basal I_{SC} was insensitive to inhibition by soybean trypsin inhibitor or α-1 antitrypsin.

The elements of ENaC regulation are partially conserved between the glucocorticoid-dependent, small airway epithelial cell line NCI-H441 and primary HBECs derived from the upper airways. The ENaC pharmacology of both models looks largely similar using both the direct ENaC blockers and the channel activating protease inhibitors. Therefore, NCI-H441 cells may provide a useful and more cost effective model for the study of airway ENaC function.

Friday 11 April – 18:30-20:00 Room: Auditorium

SPECIAL GROUP DISCUSSION - IV: CFTR interacting proteins: a source for new therapies? Moderators: Anil Mehta (UK) & Rebecca Hughey (USA)

This Special Group Discussion will include two short presentations by Drs. Rebecca Hughey and Anil Mehta that focus on two CFTR interacting proteins, the epithelial sodium channel (ENaC) and the protein kinase CK2, respectively (see abstracts below). The session will include audience participation in discussions of how this information might be used for development of new therapies for treatment of Cystic Fibrosis.

ENaC activity is also modulated by proteolytic cleavage

Rebecca P. Hughey, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

It is well established that CFTR regulates the epithelial sodium channel (ENaC), and that aberrant ENaC activity in the absence of this regulation contributes to the dehydration of airways in CF patients. Recent publications indicate that CFTR and ENaC are physically associated and that CFTR stabilizes ENaC on the cell surface. Additionally, data from the Hughey and Kleyman labs, as well as other research groups, clearly show that ENaC is independently activated by proteolysis to varying degrees.

ENaCs are composed of three highly homologous subunits (α, β and γ) that likely form a heterotrimer. Each subunit has two transmembrane domains, a large extracellular loop (ECL) and both the amino- and carboxyl-termini in the cytoplasm. Hughey and Kleyman found that maturation of ENaC in the biosynthetic pathway involves proteolysis with furin-dependent cleavage of α at two sites and γ at one site within their extracellular loops (ECLs), releasing a 26-mer peptide from α and producing a channel with “normal” activity. Interestingly, non-cleaved channels have very low activity, *while deletion of the 26-mer tract from α by mutagenesis activates the channel in the absence of proteolysis, and a synthetic version of the 26-mer inhibits wild-type ENaC.* Thus, proteolysis *per se* does not activate ENaC, but rather release of an inhibitory tract (see Hughey *et al.* 2007, Curr Opin Nephrol Hypertens 16, 444-50 for review, and Carattino *et al.* 2008, Am J Physiol Renal Physiol 294, F47-52).

Furthermore, cleavage of the γ subunit by the aprotinin-sensitive serine protease prostasin in a post-Golgi compartment releases a 43-mer inhibitory peptide from γ and fully activates ENaC by further increasing the open probability (Po> 0.9). As neutrophil elastase cleaves near the prostasin cleavage site in γ, it is likely that this exogenous protease fully activates ENaC in the CF lung. Tarran *et al.* (2007, J Gen Physiol 127, 591-604) and Myerburg *et al.* (2007, J Biol Chem 281, 27942-9) also independently observed aprotinin-sensitive and time-dependent increases in ENaC activity in cultured human airway cells after expansion of the luminal volume, consistent with dilution of protease inhibitors and increased ENaC proteolysis. A better understanding of the proteases and protease inhibitors involved in regulating ENaC activation by release of inhibitory peptides could lead to the development of novel therapies for treatment of cystic fibrosis.

Is CFTR a singular or plural noun?

Anil Mehta, University of Dundee Ninewells Hospital and Medical School, Dundee, UK

So many proteins are reported to bind to CFTR that they cannot possibly all do so at the same time. So temporally, at least, CFTR changes partners more often than Hollywood actors. Borthwick *et al.* in Mol Biol Cell 18, 3388-3397 2007 offer an example. Li *et al.* in Cell 131, 940-51 2007 offer another. ENaC is yet a third. Worse still, CFTR may be 'different' when ion concentrations change internally or externally. This is the chromo-dynamics of CFTR bathed in colours of bicarbonate chloride thiocyanate etc, which may relate to its role as a signal transducer, but other kinases (such as the with no lysine kinases (WNK) and their ion regulated kinases acting downstream) are likely candidates as novel signalers (see work of Bliss Forbush III, Kevin Strange and David Cook).

Worse still CFTR may bind different partners for different CFTR molecules. Borthwick *et al.* in Cell Signalling (in press) provide yet another example where F508del has different partners compared to wild type. These partners act as transducers of the calcium cell signal to CFTR *via* a calcium sensitive phosphatase regulated by PKA *via* an unknown step. This notion is not as odd as it seems because a CFTR with a phosphate on it (say serine 660) may have a different shape from a dephospho-S660-CFTR (hence different partners with different exposed internal loops). Treharne *et al.* (JBC 2007), although only reproducible in part by others, show how this may be different for CK2 and CFTR. The data in Treharne *et al.* in 2007 are now superceded and should not be taken to read that CFTR is controlled by serine 511 phosphorylation. More recently Kunzelmann's work shows how CK2 and ENac interact complexly (Proceedings Seventh NDPK Meeting, Dundee 2007 www.dundee.ac.uk/mchs/ndpk).

SGD IV - Regulation of the Epithelial Sodium Channel (ENaC) by AMP-activated kinase (AMPK)

Joana Almaça[1,2], Jiraporn Ousingsawat[1], Bernhard Hieke[1], Rainer Schreiber[1], Karl Kunzelmann[1]

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AMP-activated kinase (AMPK) is a ubiquitous Ser/Thr kinase and metabolic sensor that shows increased activity during metabolic stress. AMPK has emerged as a potential mediator for transport-metabolism coupling, as this kinase has been shown to regulate nutrient transporters as well as Cl^-- and Na^+-channels. It was recently shown that AMPK inhibits the epithelial sodium channel (ENaC) by forcing interaction between ENaC and the ubiquitin ligase Nedd4-2 and enhancing ENaC retrieval from the plasma membrane. Here we report that the AMPK inhibitor compound C increased the amiloride-sensitive short circuit current (Isc-amil) in tracheas of wt animals, but not of AMPKα1-/- mice. Rectal PD measurements on AMPKα1-/- mice showed enhanced amiloride-sensitive transport. Because phenformin inhibits ENaC and the basolateral Na^+/K^+-ATPase, Isc-amil was reduced by phenformin in tracheas of both AMPKα1-/- and AMPK+/+ mice. We expressed αβγrENaC in *Xenopus* oocytes to generate amiloride sensitive whole cell conductance (G_{amil}). The AMPK activator phenformin (5 mM) induced a significant decrease in G_{amil}. The effect of phenformin was no longer significant when αβγrENaC was coinjected with RNAi that knocked down xNedd4-2. Our data suggest that AMPK inhibition of ENaC is physiologically relevant. Further studies aim in understanding the underlying mechanisms.

This study is supported by DFG SFB 699 A6 and by FCT (Fundacao para a Ciencia e Tecnologia).

SGD IV - A ROLE FOR CK2 AND SYK IN CFTR TURNOVER AND PROCESSING?

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The most frequent mutation in the cystic fibrosis (CF) gene, F508del, causes retention of its protein product, F508del-CF transmembrane conductance regulator (CFTR) in the endoplasmic reticulum (ER) as a core-glycosylated intermediate that is rapidly degraded. F508del-CFTR therefore fails to traffic to the plasma membrane, where wild-type (wt) CFTR normally functions as a chloride (Cl^-) channel. Retention, however, is not due to lack of function, since the mutant is still partially functional if it reaches the membrane. ER retention results rather from acquisition of a misfolded conformation which is recognized by the ER quality control (ERQC). Morevoer, when F508del-CFTR is rescued to the membrane by low temperature, it is highly unstable, due to both increased endocytosis and reduced recycling [1]. Many CFTR-interacting proteins (CIPs) have been found to regulate these processes, namley: (i) chaperones that affect the biogenesis and ERQC of CFTR; (ii) Rab GTPases and adaptor proteins that control CFTR traffic; and (iii) kinases and phosphatases that have been mostly implicated in regulating CFTR channel activity.
Casein kinase 2 (CK2) has been previously described to affect CFTR traffic/function through phosphorylation of the S511 residue (P-site) of CFTR [2]. However, CFTR possesses another CK2 consensus P-site - T1471- at the C-terminus. Additionally, it has a putative P-site for spleen tyrosine kinase (SYK) - Y512 - which could directly influence CK2 activity by hierarchical phosphorylation [3]. Our aim here was to determine how mutation of these putative CK2 and SYK P-sites affects the CFTR biogenesis, turnover and processing.
We have produced CFTR mutants in which the consensus residues S511, Y512 and T1471 were substituted by either a neutral (alanine, A) or an acidic residue (aspartic acid, D) in both wt and F508del-CFTR backgrounds by site-directed mutagenesis. Generation of BHK cell lines with these constructs was completed for the S511 A/D mutants and is in progress for the other variants. Metabolic labelling with [^{35}S]methionine and pulse-chase experiments followed by CFTR immunoprecipitation were performed in the produced lines. After quantification of bands B (immature form) and C (mature form) of CFTR, these results show that substitution of S511 does not affect the turnover or processing of either wt- or F508del-CFTR. However, treatment of cells with 20 μM TBB (tetrabromobenzotriazole, a specific inhibitor of CK2) show a significant decrease in processing efficiency of wt-CFTR.
Altogether, our data suggest a putative stabilizing role for CK2 upon wt-CFTR in these cells, but this effect does not appear to be dependent on residue 511 (nor on the putative charge added by aspartic acid replacement at on this residue). Experiments are underway to determine whether this effect is reliant on the T1471 putative CK2 consensus P-site or indirectly dependent on prior phosphorylation by SYK.

Work supported by POCTI/SAU/MMO/58425/2004 and POCTI/PTDC/BIA-BCM/67058/2006 grants and pluriannual funding of CIGMH (FCT, Portugal). LS Pissarra received fellowship SFRH/BD/9095/2002 (FCT, Portugal).

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Saturday 12 April – 09:00-10:30 Room: Auditorium

SYMPOSIUM 6 – CFTR–Interacting Proteins
Chairs: Karl Kunzelmann (DE) & Valeria Casavola (IT)

S6.1 - Phosphorylation of CFTR AMPK and localized regulation of C1⁻ secretion

[1]Patthara Kongsuphol, [1]Bernhard Hieke, [2]Anil Mehta, [2]Kate J. Treharne, [3]Benoit Viollet,[1]Jiraporn Ousingsawat, [1]Rainer Schreiber, [1]Karl Kunzelmann

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The cystic fibrosis transmembrane conductance regulator (CFTR; ABCC7) is a cAMP and PKA regulated Cl^- channel in the luminal membrane of epithelia that secret or absorb NaCl. CFTR therefore is a central control switch that directs transepithelial movement of ions. Regulation of CFTR by PKA – phosphorylation and binding of ATP is well recognized, however, other kinases such as cyclic GMP dependent PKG type II, protein kinase C and the Ca^{2+}/calmoduline dependent kinase also phosphorylate CFTR. We demonstrated recently that wild type CFTR, but not F508del-CFTR binds and translocates casein kinase 2 (CK2) to the cell membrane. The pleiotropic and constitutively active protein kinase CK2 that controls protein trafficking, cell proliferation and development binds wild-type CFTR near F508 and phosphorylates NBD1. Mutation of S511 disrupts CFTR channel gating. Constitutive phosphorylation by CK2 appears to be a prerequisite for CFTR function. Another ubiquitous kinase, the adenosine monophosphate (AMP) activated kinase (AMPK) is a Ser/Thr kinase and functions as a metabolic sensor that shows increased activity during metabolic stress. AMPK has emerged as a potential mediator for transport-metabolism coupling, as this kinase has been shown to regulate nutrient transporters, primary and secondary transport as well as ion channels. AMPK binds to the C-terminal end of CFTR between AA 1420-1457 and phosphorylates serins within two Walker motifs located in the NBDs (S573 in NBD1 and S1248 in NBD2) of CFTR. Two further putative AMPK phosphorylation sites are located in the R domain (S737 and S768). AMPK is translocated to the luminal membrane of native airway epithelial cells, suggesting a physiological relevance of CFTR phosphorylation by AMPK *in vivo*. *In vitro* experiments in *Xenopus* oocytes demonstrate that CFTR is tonically inhibited by AMPK since inhibition of AMPK further activates CFTR. Sequential elimination of AMPK-sites in CFTR strongly augments CFTR currents and renders CFTR insensitive towards AMPK. AMPKα1 is expressed in epithelial tissues and may transmit the inhibitory effects on CFTR. Experiments are on their way on AMPKα1 -/- mice to demonstrate the physiological significans of AMPK-regulation of CFTR.

Supported by DFG SFB699A6

S6.2 - A CFTR Associated Protein, CAL, regulates the surface expression of CFTR.

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Department of Physiology, Johns Hopkins University School of Medicine

The PDZ domain protein, CAL, is a Golgi-associated protein that binds to the c-terminal tail of CFTR. Overexpression of CAL down-regulates total and cell-surface CFTR by targeting CFTR for degradation in the lysosome. The sodium-proton exchanger regulatory factor, NHERF, also binds to the c-terminal tail of CFTR but with a higher affinity than CAL. Whereas, CAL, is primarily localized at the Golgi, NHERF is located at the apical membrane and stabilizes CFTR at the plasma membrane. We investigated the role of a CAL interacting, Nethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein syntaxin 6 (STX6) and the Rho family small molecular weight GTPase, TC10, on the surface expression of CFTR. STX6 colocalizes with CAL at the TGN and co-immunoprecipitates with CAL and CFTR. STX6 overexpression reduces mature CFTR, whereas a dominant-negative mutant (STX6ΔC) has the opposite effect. Silencing of endogenous STX6 with siRNA increases CFTR expression without affecting CAL expression or Golgi morphology. Although STX6 can bind to a CFTR mutant lacking the PDZ motif (CFTRΔTRL), STX6 silencing has no effect on CFTRΔTRL expression. These data together with the finding that CAL silencing eliminates the inhibitory effect of STX6 suggest that the function of STX6 rather than binding directly to CFTR dependents on the presence of CAL. TC10 interacts with CAL and specifically up-regulates CFTR protein expression. Co-expression of the constitutively active form of TC10, TC10Q75L, increases total and cell surface CFTR in a dose-dependent fashion. Thus, both STX6 and TC10, through an interaction with CAL, work oppositely to regulate the surface expression of CFTR. It is known that certain bacterial toxins function by affecting the activity of Rho GTPases. For example, *Clostridium difficile,* toxin B, is a Rho-inactivating toxin and *Pseudomonas aeruginosa* exoenzyme S (ExoS) is a GAP-like 'injected' toxin. Both toxins increase the surface expression of CFTR. The effect of *Clostridium difficile,* toxin B, to increase surface CFTR, requires an intact PDZ binding domain of CFTR suggesting that these bacterial toxins operate via PDZ domain proteins such as CAL. In conclusion, several PDZ domain proteins bind to CFTR at different locations in different cell types. These proteins regulate surface expression of CFTR. They are likely to be involved in how some bacterial toxins impact upon CFTR to enhance the fluid production by mucosal membranes of the gastrointestinal track and in the airways.

S6.3 - The role of Hsp27-mediated sumoylation in CFTR biogenesis/degradation

Annette Ahner and Raymond A. Frizzell

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The cystic fibrosis transmembrane conductance regulator (CFTR) was the first mammalian membrane protein implicated as a substrate for endoplasmic reticulum associated degradation (ERAD), and it has served as a model for the folding or disposal of polytopic membrane proteins. Steps in ERAD usually include the recognition and removal of misfolded proteins from the ER, followed by their ubiquitylation and proteasome-dependent degradation (1). Due to its complex folding and domain assembly requirements, the majority of WT CFTR and ~100% of the common folding mutant, F508del-CFTR, are degraded in most systems.

We initially identified a new interaction between CFTR and small heat shock proteins (sHsps) using yeast genomic analysis (2). In mammalian cells, we have shown that two sHsps, αA-crystalline and Hsp27, target F508del-CFTR for proteasome-dependent degradation, and relative to the WT protein, both sHsps show selective physical interactions with the mutant. The predominant sHsp in airway cells, Hsp27, binds six times more F508del than WT CFTR at steady-state. This pro-degradative action of Hsp27 was linked to its ability to interact with Ubc9, the single E2 enzyme that adds the small ubiquitin-like modifier, SUMO, to substrate proteins. Indeed, the expression of Ubc9 decreased, while expression of the SUMO protease, SENP1, increased the expression of F508del-CFTR, and pulse-chase studies linked these changes in expression to F508del-CFTR ERAD.

To determine the physiological role of the Hsp27/SUMO system in CFTR degradation, we co-expressed Hsp27 shRNA with WT and F508del-CFTR R. A 70-80% reduction in Hsp27 expression led to 2-3 fold increases in the expression of both WT and mutant proteins in the steady-state. Another approach to this issue is provided by expression of the avian adenoviral protein, Gam1, which targets the SUMO E1 component, SAE1, for degradation. Gam1 markedly reduced the expression of SAE1, and similar to Hsp27 knockdown, increased WT and F508del-CFTR expression 2-3 fold. CFTR has three consensus sumoylation sites (ψKxD/E), in which lysine is SUMO modified with an isopeptide bond. One site lies in each nucleotide binding domain (NBD1 and 2) and one is positioned at the C-terminus. Mutation of these sites (K/A) reduced WT and F508del-CFTR expression ~80%, and also eliminated the ability of Hsp27 and Ubc9 (SUMO E2) to promote F508del-CFTR degradation. Hsp27 expression augmented the sumoylation of full-length F508del-CFTR *in vivo*, and of NBD1 *in vitro*, consistent with the concept that Hsp27 serves as an E3 protein that targets Ubc9 to sumoylate specific CFTR sites. Consistent with this, the SUMO modification of full-length CFTR by Hsp27 was eliminated in the sumoylation site mutant. Our findings are consistent with the hypothesis that Hsp27-mediated sumoylation of WT CFTR maintains domain solubility during assembly of the native CFTR structure, but that failure to efficiently remove Hsp27 and SUMO from F508del-CFTR targets its degradation. [Supported by grants from the NIH and CF Foundation]

1. Amaral, M. D. Ped. Pulmonol. 39(6), 479-491, 2005
4. Ahner, A. et al., Mol. Biol. Cell. 18(3): 806-14, 2007

P14

S6.4 - Evidence of an essential role of NHERF1 and NHEF2 in G-protein coupled receptor regulation of CFTR-mediated intestinal anion secretion *in vivo*

Anurag Kumar Singh[1], Brigitte Riederer[1], Anja Krabbenhöft[1], Brigitte Rausch[1], Boris M. Hogema[2], Hugo R. de Jonge[2], Mark Donowitz[3], Edward J. Weinman[4], Olivier Kocher[5], Ursula Seidler[1]

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Background & Aim: The results of heterologous expression studies suggest that the PDZ-proteins NHERF1 and NHERF2 play essential roles in the transduction of receptor-specific signals to the CFTR anion channel. To study their biological roles *in vivo* in an epithelium that highly expresses both CFTR and both NHERF proteins in the same cells, we investigated the effect of NHERF1 and NHERF2 ablation on ß2-adrenergic stimulation and lysophosphatidic acid (LPA) - inhibition of CFTR-regulated duodenal HCO_3^- secretion *in vivo*. **Methods:** The proximal duodenum of anesthetized mice was perfused *in situ,* and HCO_3^- secretion was determined by back-titration. **Results:** NHERF1 ablation strongly reduced basal HCO_3^- secretory rates. The ß2-adrenerigc antagonist ICI-118551 significantly inhibited basal HCO_3^- secretion in wt, but not in NHERF1-deficient mice. Likewise, NHERF1 ablation completely prevented the stimulatory effect of the β_2-adrenergic ligand clenbuterol. NHERF2 ablation resulted in a stronger stimulation by forskolin compared to wt littermates, completely prevented the inhibitory effect of LPA, and partially rescued the suppressed basal HCO_3^- secretion resulting from NHERF1 ablation. The deletion of CFTR dramatically reduced basal HCO_3^- secretion and abolished the effect of NHERF ablation. **Conclusions**: The PDZ-adapters NHERF1 and NHERF2 proteins differentially modulate basal and agonist-mediated duodenal HCO_3^- secretion *in vivo* in a CFTR-dependent fashion. NHERF1 is an obligatory linker for ß2-adrenergic stimulation of CFTR, and strongly augments cAMP-mediated stimulation. NHERF2 confers inhibitory signals, i.e. as a coupling factor between inhibitory LPA receptors and CFTR.

S6.5 - Impact of F508 Residue and Small Molecule Correctors on the Interaction of Hsc70 with NBD1 of CFTR

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The deletion of a phenylalanine residue at position 508 (F508del) in the first nucleotide-binding domain (NBD1) of CFTR is the principal cause of cystic fibrosis (CF). The altered interaction of F508del-CFTR with endoplasmic reticulum (ER) quality control proteins, primarily chaperones, mediates both its folding and proteasomal degradation. Recent high-throughput screens (HTS) have identified several small molecule correctors of CFTR biogenesis with therapeutic potential to CF [1]. However, little real-time information is currently available on factors governing the strength of chaperone-CFTR interactions. Our goals here are to quantify the effect on these interactions of: i) the F508del mutation and ii) small molecule compounds.

We used surface plasmon resonance (SPR; Biacore™) to quantify the real-time binding of molecular chaperone Hsc70 to bacterially expressed wt- and F508del-murine NBD1. Hsc70 was covalently immobilised onto the surface of carboxymethyl dextran (CM5) sensor chips (500 µM). In control studies, denatured lactalbumin (3 µM), a known substrate of Hsc70, bound specifically to immobilised Hsc70 (28.4 ± 0.92 pmol nmol-1; n = 4). In separate experiments, purified NBD1 was found to bind immobilised Hsc70 (43.7 ± 0.32 pmol nmol-1; n = 3) but not BSA (n = 10). Moreover, the Hsc70-binding affinity of NBD1 was significantly strengthened when F508 was deleted (wt, K_D^{app}, 1.20 ± 0.16 µM; F508del, K_D^{app}, 0.41 ± 0.10 µM; n = 3; $p < 0.05$). Interestingly, increasing MgATP concentration dramatically weakened wt-NBD1 (0.5 µM) binding to Hsc70 (IC_{50}, 19.9 ± 2.2 µM ATP; n = 3). Furthermore, deletion of F508 increased 5-fold the concentration of MgATP required to destabilise the NBD1-Hsc70 interaction (n = 3; $p < 0.01$). To better understand the mechanism of action of small molecule correctors with therapeutic potential for CF, we also quantified their effect on the NBD1-Hsc70 interaction. In control studies, pre-incubation of F508del-NBD1 (0.5 µM; 3 µM ATP) with inhibitor $CFTR_{inh-172}$ (50 µM; 30 min, 16 °C) did not destabilise its Hsc70-interaction (n = 3; $p > 0.3$). Surprisingly, corrector VRT-325 [2] (5 - 50 µM) was also without significant affect on the interaction (n = 3; $p > 0.1$). Only a relatively high concentration of corrector 4a [3] (50 µM) significantly reduced F508del-NBD1 binding to Hsc70 (control, 31.5 ± 2.1 pmol nmol-1; Corr-4a, 24.6 ± 3.1 pmol nmol-1; n = 3).

Overall, these data demonstrate that: (i) F508del strengthens the association of NBD1 with Hsc70 in the absence or presence of ATP; and (ii) corrector 4a, but not VRT-325, has a small, albeit measurable, impact on this interaction, suggesting it may help NBD1 to fold and thus be faster released from its Hsc70 interaction.

We are grateful to Prof. John Findlay (Leeds, UK) for SPR training, the CF Folding Consortium (www.cftrfolding.org) for purified NBD1 and CFF (USA) for correctors. Work supported by grant POCTI/SAU/MMO/58425/2004, pluriannual funding of CIGMH (FCT, Portugal) and TSS-W post-doctoral fellowship SFRH/BPD/18989/2004 (FCT, Portugal).

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[3] Pedemonte N *et al* (2005) *JCI* **115**, 2564-71

S6.6 - The First Steps in CFTR Biogenesis: Cotranslational Interactions with Nascent CFTR as It Exits the Ribosome

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During its biogenesis, CFTR is synthesized in the cytosol, integrated into the endoplasmic reticulum (ER) membrane, and finally transported through the Golgi to the plasma membrane. In the current work we have studied cotranslational CFTR interactions during the earliest steps of its biogenesis. Site-specific photocrosslinking was used as a method to detect protein-protein interactions. It is based on tRNA-mediated incorporation of a single photo-reactive probe into CFTR nascent chains at various positions during *in vitro* synthesis. Amber suppressor N^{ε}-(5-azido-2-nitrobenzoyl)-Lys-$tRNA^{amb}$ (εANB-Lys-$tRNA^{amb}$) was used to incorporate the probe into the nascent chain wherever an amber stop codon was introduced into the CFTR mRNA. Ribosome-bound CFTR nascent chains of a defined length were prepared by translation of truncated CFTR mRNAs. Using nascent chains with different lengths and different positions of the photo-reactive probe, we examined CFTR interactions at the different stages of its synthesis. In the process of elongation the CFTR nascent chain was found adjacent to 65, 80, 90, 110, 50, and 70 kDa proteins. The ~50 kDa protein was identified as SRP54, a subunit of the signal recognition particle (SRP) that targets ribosome•nascent chain complexes to the translocon in the ER membrane. SRP did not bind to CFTR until its first transmembrane segment (TM1) had completely emerged from the ribosomal tunnel. The SRP-nascent chain interaction was disrupted when the integrity of TM1 was compromised by a deletion mutation. The involvement of SRP in CFTR biogenesis was also shown *in vivo* by siRNA-mediated depletion of SRP in human cell culture: in cells with reduced SRP levels, nonglycosylated forms of CFTR accumulated and total CFTR levels decreased. Notably, some CF-causing mutations in TM1 also reduced photocrosslinking to SRP. These data reveal that mutational inhibition of nascent CFTR association with SRP may contribute to some types of CF. One of these mutations, W79R, promotes a new interaction with an unidentified ~60-70 kDa protein that was not observed for wild-type protein or other mutants. The experimental system provides an avenue to identify these new interacting protein partners. In summary, our data show that CFTR is proximal to a significant number of proteins during the earliest steps of biogenesis and that disease-associated mutations alter many of these interactions.
(*Supported by NIH NIDDK and the CFF*)

Saturday 12 April – 11:00-12:30 Room: Auditorium

EuroCareCF session – WP6 – Animal Models
Chair: Bob Scholte (NL)

WP6.1 - Eurocare CF WP6 Animal models of CF - Inflammation and tissue remodelling in CF mutant mice.

Bob J Scholte[1], J. Aarbiou[1], R Buijs-Offerman[1], M Wilke[2]

Erasmus Medical Center, department of [1]Cell biology, [2]Biochemistry

Animal models have been instrumental in advancing our understanding of CF pathology, and present a useful platform for testing experimental therapeutic interventions. At least thirteen different strains of genetically modified mice are available, carrying either a CFTR null allele or one of the most relevant specific CFTR mutations (F508del, G551D). EUROCARECF/Animal models supports distribution of breeding pairs from the F508del $Cftr^{tm1eur}$ strain, and the $Cftr^{tm1cam}$ null strain. In addition to mouse strains carrying CFTR mutations, the North Carolina group has presented an alternative model of CF lung pathology that features over expression of the apical sodium channel alpha subunit (tgCC10/ENACa). While the role of ENAC in CF lung pathology and the therapeutic potential of ENAC inhibitors were highlighted in the previous basic science meeting, this year we will focus on chronic activation of inflammatory pathways in CF mutant mice, and the possible relationship with excessive tissue remodelling upon injury in CF lung disease.
Chronic lung disease in Cystic Fibrosis, and likewise in Asthma, COPD and IPF, is characterized by irreversible, and eventually fatal tissue remodeling. Treatment or effective preventive intervention is not available. The complex molecular network of cell signaling that drives airway stenosis, epithelial metaplasia en vascular bed adaptation, and its relationship with inflammation is still the subject of intense investigation. Quantitative PCR array analysis supported by immunohistology and *in situ* hybridization in our lab identified for the first time several genes involved in inflammation and tissue remodeling that are upregulated in F508del mutant mice, both before and after injury. The pattern of gene expression observed in unchallenged F508del mutant mice is consistent with chronic inflammation, most likely mediated by the inflammasome/IL-1B system. This expression pattern includes upregulation of several morphogens known to be involved in tissue injury/repair and morphogenesis, but not previously implicated in CF lung disease. This study supports the view that the CF condition results in chronic lung inflammation, also in the absence of pathogens, which subsequently promotes tissue remodeling upon injury in the CF lung though the hyper activation of specific growth factor related pathways. In a contribution by Dr Jaquot (Paris, Fr) the effect of CFTR dysfunction on proteasome activity and NF-kB activation will be described, a highly relevant link since many genes hyper expressed in the inflammation/injury pathway in mutant mice are at least partly NF-kB dependent.

While mouse models are very useful indeed, they clearly have limitations as a model for human lung disease. It is of great importance therefore that this workshop can present the progress towards Pig and Ferret CF models in a presentation by Dr. John Engelhard of Iowa. On the other side of the evolutionary and size scale, models are presented in the poster section involving genetically engineered nematodes that can be used to study the behaviour of mutant CFTR (Neto et al), and Drosophila that can be used to study bacterial pathogen communities interacting with the inate immune system (Sibley et al).

WP6.2 - Defective CFTR Cl^- channel causes increased proteasomal degradation and reduced NF-κB activity in lung epithelial cells exposed to ROS

T. Roque, E. Boncoeur, E. Bonvin, V. Saint Criq, M. Bonora, A. Clement, O. Tabary and J. Jacquot

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Oxidative stress, by the production of reactive oxydative species (ROS) in the airway epithelium has been identified as an early complication of young children with CF. In CF patients, the loss of functional cystic fibrosis (CF) transmembrane conductance regulator (CFTR) protein in lung epithelial cells leads to reduced volume and increased oxidation of the airway surface liquid. Following exposure to ROS, we have recently reported that reduced caspase-3 activity and no increase of NF-κB activity were observed in two CF lung epithelial cell lines, IB3-1 and CFBE41o- due to an absence of reduction of IκB-α inhibitor in contrast to two CFTRwt-corrected-S9 and normal 16HBE14o- cell lines (*Boncoeur E. et al, IJBCB, 2008*). These results prompted us to investigate whether ROS production in lungs resulted in any CF-specific regulation of caspase-3 and proteasome-proteolytic (UPS) activities and NF-κB/IκB-α signaling through the use of *in vivo* and *in vitro* complementary models of CFTR deficient mice compared to wild-type mice, and a human CFTR-deficient lung cell line compared to the same but corrected CFTR-sufficient lung cell line.
We exposed CFTR deficient ($cftr^{-/-}$) and wild type mice for 64-h to hyperoxia-mediated oxidative stress. CFTR deficient mice exhibited significantly higher lung proteasomal activity than $CFTR^{+/+}$ animals after oxidative stress. This was accompagnied by a reduced lung caspase-3 immunoreactivity and an absence of degradation of NF-κB inhibitor IκB-$\tilde{\alpha}$*In vitro*, human CFTR-deficient lung cells exhibited higher proteasomal activity and a lack of increased NF-κB-dependent transcriptional activity compared to CFTR-sufficient lung cells after oxidative stress. CFTR Cl^- channel inhibition by $CFTR_{inh-172}$ in normal bronchial immortalized cell line 16HBE14o- exposed to oxidative stress caused an increase of proteasomal degradation. Inhibition of caspase-3 by Z-DQMD in CFTR-sufficient lung cells mimicked the response profile of increased proteasomal degradation and lowered NF-κB activity of CFTR-deficient lung cells exposed to oxidative stress. These new data reveal a crucial role of functional CFTR Cl^- channel activity in regulating lung proteasomal degradation, caspase-3 activity and NF-κB-dependent transcriptional activity under oxidative stress conditions (*Boncoeur E., Roque T. et al, Am J Pathol, 172, May 2008*). Further studies are now required to determine which of the components of UPS and caspases complexes might be optimal targets to promote an increase in NF-κB-dependent transcriptional activity in the CF lung epithelium but with low toxicity. Such a strategy might provide more tailor-made therapies for patients with CF who are subject to notable deleterious effects of oxidants.

Supported by the French Cystic Fibrosis Foundation (Vaincre La Mucoviscidose).

WP6.3 - "New Animal Models for Cystic Fibrosis Research"

John F. Engelhardt

Carver College of Medicine, University of Iowa, Department of Anatomy and Cell Biology

The development of effective therapies for cystic fibrosis (CF) lung disease requires animal models that can appropriately reproduce the human disease phenotype. The lack of such animal models has hindered progress toward both the understanding CF lung pathophysiology and the development of gene and pharmacologic therapies. Although much has been learned from current in vitro (polarized human airway epithelial cultures), ex vivo (human airway xenografts), and CFTR knockout mouse model systems, there is an urgent need to develop more authentic models of CF by which to dissect pathophysiology and test therapies. CF mice have been extremely useful in understanding CFTR biology and function in the nasal and intestinal epithelia, but they appear not to accurately model the spontaneous lung infections seen in human CF patients. This is most likely due to the many differences in lung biology between humans and mice including: 1) differences in the types of secretory cells present in human and mouse airways (goblet vs. Clara cells), as well as in their patterns of distribution, 2) differences in the abundance and distribution of submucosal glands in the airways, which are a major site of CFTR-expression, and 3) differences in the abundance and/or activity of Ca^{++} activated Cl^{-} channels in mouse vs. humans airways. Together, these species-specific differences between mouse and human airways impose severe limitations on the use of CF mice as a model to study CF pathophysiology and explore therapeutic approaches for CF lung disease. Due to the limitations in the current CF mouse model, the Welsh and Engelhardt laboratories have collaboratively generated CFTR-deficient models in the pig and ferret, respectively (1,2). These animals were generated using recombinant adeno-associated virus targeting of the CFTR gene in primary fibroblasts, followed by somatic cell nuclear transfer cloning of the animals heterozygous for a CFTR-disrupted allele. This presentation will focus on potential advantages of these species for the study of CF pathophysiology and testing of potential gene therapies. Additionally, recent data evaluating pig and ferret F508del-CFTR has also suggested that these two species may have unique mutation-specific phenotypes. In this context, pig F508del-CFTR appears to be processed normally in CF human airway epithelia and gives rise to functional chloride channels at the apical membrane (3), whereas ferret F508del-CFTR appears to have impaired processing similar to that of human F508del-CFTR. Hence, the ferret may be particularly useful in modeling CF disease caused by the F508del-CFTR mutation.

1. Rogers CS, Hao Y, Rokhlina T, Samuel M, Stoltz DA, Li Y et al. Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. *J Clin Invest* 2008.
2. Sun X, Yan Z, Yi Y, Li Z, Lei D, Rogers CS et al. Adeno-associated virus-targeted disruption of the CFTR gene in cloned ferrets. *J Clin Invest* 2008.
3. Ostedgaard LS, Rogers CS, Dong Q, Randak CO, Vermeer DW, Rokhlina T et al. Processing and function of CFTR-DeltaF508 are species-dependent. *Proc Natl Acad Sci U S A* 2007; 104: 15370-15375.

Saturday 12 April – 14:30-16:00 Room: Auditorium

EuroCareCF session – WP7 – Resources for Basic Research

Moderators: **Margarida Amaral (PT) & Martin Hug (DE)**

SESSION 1 – Endpoints of CFTR Rescuing Therapies

WP7.S1.2 -.Development of Intestinal Current Measurements as a Biomarker of F508del-CFTR Correction in Multi-Center Clinical Trials.

JP Clancy, MD[1]; Sherif Gabriel, PhD[2]; Phillip Karp[3]; David Stoltz, MD, PhD[3]; Michael Welsh, MD, PhD[3]; Marcus Mall, MD[4]; Judy Williams, RN[5]; Melissa Ashlock, MD[6].

[1]University of Alabama at Birmingham; [2]University of North Carolina; [3]University of Iowa; [4]University of Heidelberg; [5]Children's Hospital and Regional Medical Center (Seattle, WA); and [6]Cystic Fibrosis Foundation Therapeutics.

Compounds that 'correct' abnormal folding and/or localization of F508del-CFTR have been identified through high throughput screening and/or other efforts. Lead compounds have been optimized and developed for investigation in human subjects with CF caused by the F508del-CFTR mutation. As these agents near clinical investigation, biomarkers capable of detecting normal maturation, localization and function are of high priority to confirm biologic activity and provide proof of mechanism of investigative agents. Currently, at least two biomarkers specific for CFTR activity have been developed, including the nasal potential difference (NPD) and the sweat Cl- test. Both of these tests have been successfully used in multi-center clinical trials, but concerns over sensitivity, and limitations in the assays due to their in vivo nature have lead investigators to consider additional methods to confirm restoration of F508del-CFTR misprocessing and function in human subjects. One assay of interest is Intestinal Current Measurements (ICM) from rectal biopsy samples. This method is attractive since CFTR expression is high in the rectum, is not altered by disease manifestations, and the tissue can be studied ex vivo, providing flexibility in the techniques to detect/quantify CFTR activity. The CF-TDN is developing a multi-center protocol to examine ICM as a biomarker of F508del-CFTR correction. Rectal ICM is used to diagnose CF, and it has been demonstrated to be safe and well tolerated. The rectal ICM is sensitive and specific for CFTR function, and the assay is highly discriminatory between severe CF (no detectable CFTR activity), milder CF (residual, low level CFTR activity), and non-CF (normal CFTR activity). Our approach involves gaining experience from experts in the assay, development of expertise in animal tissues, and extension to human studies. Currently we are examining a variety of technical issues related to the assay, including tissue acquisition (suction vs forceps), mounting in prototype micro-Ussing chambers, monitoring ion transport function by short circuit current and transepithelial voltage, and testing cAMP agonists, CFTR potentiators, and Cl- transport blockers to isolate CFTR activity and provide interpretable data appropriate for multi-center trials. As conditions are optimized, an ICM training workshop utilizing animal models will be conducted to standardize techniques across centers. Five institutions have been identified for assay implementation, with oversight of conduct provided by the CF-TDN Coordinating Center. Blinded central interpretation of data will be performed within the network, and common SOPs will be used to minimize intrasite variability. This project is intended to yield a sensitive, specific, and flexible biomarker to detect and quantify F508del-CFTR correction that is appropriate for use in multi-center trials of F508del-CFTR corrective and potentiating therapeutics in development. Sponsored by CFFT.

WP7.S1.3 -. Measurement of trace gases in the exhaled breath – a new diagnostic option in CF lung disease?

M. Barker [1,2], G. Brasse [2,3], L. Hülsmeier [4], M. Hengst [4], D. Klemp [3]

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[2] Dept. of Pediatric Pulmonology and Immunology, Charité University Medical Center, Berlin, Germany
[3] Institute of Chemistry and Dynamics of the Geosphere, Div. II: Troposphere, Jülich Research Center, Germany
[4]Dept. of Pediatrics, Aachen University Medical Center, Aachen, Germany

Background: Early, sustained and severe airway inflammation and bacterial infection are characteristic features of pulmonary involvement in cystic fibrosis (CF). The immune response is dominated by neutrophils, which have been shown to generate oxygen- and halogen-derived radicals as part of their host defense armamentarium. Local release of reactive substances by microorganisms and host cells contributes to epithelial damage and bronchiectasis formation. While pulmonary function and imaging studies only reveal the consequences of these processes, a new and non-invasive diagnostic window could be opened by biochemical breath analysis. However, current knowledge on methodological standards and clinical data are very limited.
Aims: To gather pilot data on the pulmonary output or uptake of selected organic trace gases in CF patients and healthy controls; and to explore the impact of pulmonary exacerbations and antibiotic treatment.
Methods: A customized system for sampling and storing human breath in evacuated stainless steel canisters and trace gas determination by gas chromatography with flame ionization, electron capture and mass spectrometry detectors was developed and thoroughly evaluated for performance and error estimation. Subsequently, children and adults with CF as well as healthy controls were included in several study series. From corresponding samples of ambient air and exhaled breath, we determined the mixing ratios of a variety of volatile organic compounds and calculated alveolar gradients.
Key results: Compared to healthy control subjects, CF patients showed a significantly higher net release of pentane, benzene, methylchloride, trichloromethane and trichloroethene but a lower respiratory output of methanol, ethanol, acetaldehyde and dimethylsulfide. The pulmonary uptake of carbonyl sulfide was decreased in CF patients versus controls and correlated with FEV1. These differences were most pronounced in patients with acute exacerbations. A relevant scatter and overlap between groups was noted for ethane, propane, butane, acetone, isoprene, toluene, and tetrachloromethane. After antibiotic treatment, the output of benzene, methylchloride and trichloromethane decreased by 40-250%.
Conclusion: With off-line technology adapted from atmospheric science, a broad spectrum of organic trace gases can be accurately quantified in the human breath. The increased respiratory output of halocarbons and lipid peroxidation products in CF patients may be a non-invasive indicator of airway inflammation. Although important pathways remain incompletely understood, the local biochemical milieu in CF lungs thus appears to be informative and accessible through biochemical breath analysis. Future studies should focus (1) on parameter selection according to their sensitivity and specificity, (2) description of their pathophysiological role and reference range, (3) data acquisition in larger clinical studies, and (4) development of real-time analytical methods such as laser spectroscopy. While exhaled breath measurements must be considered as insufficiently evaluated to serve as treatment endpoints to date, they do promise a broad diagnostic potential.

Saturday 12 April – 16:30-18:00 Room: Auditorium

EuroCareCF session – WP7 – Resources for Basic Research

SESSION 2 – Cellular Systems for CF Research

Chairs: Margarida Amaral (PT) & David Sheppard (UK)

WP7.S2.1 - The Clinical and Functional TRanslation of CFTR (CFTR2) Project

Garry Cutting on behalf of the CFTR2 Project

Johns Hopkins University School of Medicine

Over 1500 putative disease-associated mutations have been identified in the CFTR gene. Approximately two dozen of these mutations have a world-wide frequency in CF patients that exceed one percent. Clinical and functional studies document the deleterious effect of these “common” CFTR mutations. However, the disease-causing potential of most of the remaining “rare” mutations are largely unknown. The increasing role of mutation detection in the diagnosis and prognosis of CF and the widespread implementation of CF newborn screening has prompted the Cystic Fibrosis Foundation in the U.S. to initiate a project to clinically and functionally annotate the “rare” mutations in CFTR. Approximately half of the rare mutations can be predicted to have deleterious effects based on the nature of the change (i.e. frameshift, nonsense, or canonical splice-site) and can be predicted to cause CF with moderate confidence. However, the remaining “rare” mutations primarily consist of missense and intronic mutations of unknown effect. The Clinical and Functional TRanslation of CFTR (CFTR2) project proposes to annotate each reported CFTR mutation using rigorously vetted clinical data and, when necessary, functional assays of CFTR bearing the mutation. The first phase of this project is to create the CFTR2 database that will be linked to the Toronto CF Mutation database, providing a simple means to access clinical and functional data. As opposed to the genotype-driven CF Mutation Database, CFTR2 will be a phenotype-driven database. Clinical data associated with each CFTR mutation will be derived from CF patients attending CF Centers in the North America, Europe, and Australia. The CFTR2 database will consist of three levels; 1) a public domain that provides an estimate of the disease-causing liability of each mutation vetted by an expert panel. 2) a qualified user domain (e.g. investigators and clinicians), that contains the primary data used to generate the expert opinion. 3) a developer domain for the CFTR2 team and expert consultants. Mutations with unclear clinical consequences will be prioritized for functional studies in later phases of the project. Mutations that are predicted to affect RNA splicing will be analyzed by RT-PCR studies of nasal epithelial cells from patients carrying the respective mutations. Mutations that cause an amino acid substitution in CFTR will be analyzed in cell lines that have stable expression of mutant CFTR. These cell lines will be stored for potential future studies of mutation-specific therapies. CFTR2 is proposed as a world-wide collaborative effort to annotate each CFTR mutation with uniform clinical data and functional analyses.

WP7.S2.2 - The culture of primary bronchial epithelial cells from the lungs of people with cystic fibrosis removed at the time of transplantation – A model to study cystic fibrosis lung disease

Brodlie M[1, 2], Johnson GE[1], Perry J[3], Hinchcliffe M[4], Fisher A[1], Corris PA[1], Pearson JP[4], McKean MC[2], Lordan JL[1] and Ward C[1]

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Background
Studies involving animal models and immortalised cell lines have contributed significantly to our current understanding of cystic fibrosis (CF) lung disease. There are inherent limitations to this indirect approach however. The opportunity to work on primary tissue from patients with CF is rare and scientifically very important but involves significant technical difficulties, most notably due to infection.
Aim
To culture primary bronchial epithelial cells (PBECs) from lungs removed at the time of transplantation from people with CF.
Methods
Pieces of segmental bronchus were removed from lungs immediately after explantation. The tissue was treated with antimicrobials and mucolytics to achieve washing and disinfection prior to harvesting of PBECs. Submerged PBEC cultures were established initially before transfer to an air-liquid interface (ALI). Culture supernatants and fixed cells were assessed for mucus production by enzyme-linked immunosorbent assay (ELISA) and amylase-periodic acid schiff staining.
Results
To date we have successfully cultured PBECs from two out of three patients attempted. Mucociliary differentiation at ALI was denoted by mucus staining and ELISA for MUC5B. Cultures from one patient initially became established but then overgrew with pan-resistant *Burkholderia* species that were known to be colonising the lungs pre-transplant.
Conclusions
PBEC culture is possible from lungs removed at the time of transplantation from people with CF. This technique represents a valuable resource that provides a model to elucidate the pathogenic mechanisms in CF lung disease and to investigate potential therapeutic targets.

WP7.S2.3. - Generating Tools to Aid in CFTR Modulator Drug Discovery

Chris Penland, Ph.D., Elizabeth Joseloff, Ph.D., Diana Wetmore, Ph.D., Melissa Ashlock, MD.

Cystic Fibrosis Foundation Therapeutics, Department of Drug Discovery, 20814 Bethesda, MD, United States

The US Cystic Fibrosis Foundation (CFF) has numerous collaborations to discover and develop potent and effective compounds that improve the chloride channel function of the defective CFTR protein (i.e., CFTR modulators). To expedite CFTR modulator drug discovery by CF scientists in academia and industry worldwide, CFF facilitates the development and distribution of various research tools including: 1) antibodies, 2) protein, 3) control modulators (inhibitors, potentiators and correctors), 4) assays of chloride channel function (both high-throughput and secondary electrophysiological assays), 5) assays to assess CFTR modulators' mode of action and 6) epithelial cells for use in these assays.

A primary source of new tool development is the "CFTR Consortium", an international group of academic CF scientists with extensive experience in CFTR research (for information see http://www.cftrfolding.org). In addition to the Consortium, CFF supports several other programs for the development and distribution of research tools (for information contact DrugDiscoveryResources@cff.org). An important new project is the generation of cell lines containing various uncommon CFTR mutations. These cell lines should aid in the characterization of the physiological consequences of the particular mutant CFTR. Through all of these efforts, CFF hopes to deliver promising therapies more quickly to CF patients.

Sunday 13 April – 09:30-11:00 Room: Auditorium

SYMPOSIUM 7 – Therapeutic Development aimed at Correcting CFTR Defects
Chairs: Olga Zegarra-Moran (IT) & Alan Cuthbert (UK)

S7.1 - CFFT'S PIPELINE APPROACH TO CFTR DRUG DISCOVERY AND DEVELOPMENT

Diana R. Wetmore, Ph.D., Melissa Ashlock, M.D., Robert Beall, Ph.D., Preston Campbell, III, M.D., Christopher Penland, Ph.D.

Cystic Fibrosis Foundation Therapeutics

The Cystic Fibrosis Foundation (CFF) was started over fifty years ago by a group of parents with children that had cystic fibrosis (CF), to help advance knowledge about the disease and improve clinical care. At that time, the average lifespan of a child with CF was just a few years, whereas today the average life expectancy has increased to 37 years. These gains have been achieved through a combination of improved care and the availability of new disease management drugs such as TOBI ™ and Pulmozyme ™. However, many CF researchers believe that in order to increase survival to a normal lifespan it will be necessary to address the basic functional defects of CFTR, the protein that, when mutated, causes CF. In the late 1990's, CFF embarked on a new business model that is now known as "Venture Philanthropy". On the assumption that new and emerging drug discovery technology would only be applied to the CFTR target if the Foundation helped with both funding and access to expertise in the clinical and research fields, it founded two new organizations: Cystic Fibrosis Foundation Therapeutics, and the CFFT-Therapeutics Development Network (CF-TDN). Since 1998, CFFT has invested close to $300M US in a diverse array of discovery platforms with Industry and Academic collaborators. The CF-TDN, a network of CF clinical care centers with expertise in designing and conducting clinical trials with CF patients, has enabled an efficient path for new candidate CFTR drugs to be evaluated in CF populations for clinical safety and efficacy. This Venture Philanthropy model has been validated by recent clinical successes with several new candidate drugs that target CFTR, including PTC-124 from PTC Therapeutics and VX-770 from Vertex Pharmaceuticals. This presentation will provide an overview of the diverse discovery programs currently in place as well as recent progress in clinical trials.

S7.2 - Emerging therapies in CF: preclinical approaches to tackling CF lung disease in Novartis

Kathy Banner, Henry Danahay, Martin Gosling, Chris Poll

Novartis Institutes for Biomedical Research, Wimblehurst Road,Horsham,West Sussex, RH12 4AB, UK

Cystic Fibrosis (CF) is the most common lethal genetic disease of Caucasians affecting approximately 1 in 2500- 3500 newborns with an estimated CF patient population of >50,000 individuals worldwide . Despite the success of improved therapeutic management in extending the median age of survival for CF patients to >35 years of age there remains a pressing unmet medical need for new therapies particularly those with disease modifying and curative properties.

The current model of CF lung disease pathogenesis hypothesises a pathphysiological cascade in which mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, results in the defective trafficking, expression or activation of the CFTR protein leading to decreased epithelial cell chloride secretion and sodium hyperabsorption. This altered profile of epithelial ion transport results, in turn, in depletion of the airway surface liquid layer and impaired mucociliary transport. Bronchial obstruction by thickened retained mucus most likely provides a favourable environment for the initiation of bacterial colonisation and a vicious cycle of infection and excessive lung inflammation and damage ensues. Chronic bacterial infection and inflammation leading to lung destruction are the major cause of morbidity and mortality in CF.

Emerging therapeutic approaches aimed at intervening in the above pathophysiological pathway are described including enhancing mucociliary clearance by increasing the airway surface liquid available for mucus hydration. Potential anti-inflammatory approaches for tackling CF lung disease are also discussed.

S7.3 – Pharmacologic activation of chloride transport in CF Mutants

Olga Zegarra-Moran[1], Nicoletta Pedemonte[1], Cristina Barsanti[1], Emanuela Caci[1], Martino Monteverde[1], Loretta Ferrera[1], Oscar Moran[2], Luis J.V. Galietta[1]

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[2]Istituto di Biofisica, Consiglio Nazionale delle Ricerche, via de Marini, 6. Genova-16149. ITALY

The most common CF mutation, F508del, causes CFTR dysfunction principally by disrupting its processing and delivery to the apical membrane of epithelia. In addition, a faulty channel gating in the few molecules that do reach the plasma membrane accentuates this abnormality. Several other less common mutations like G551D and G1349D display also an impaired opening. Most of our efforts in the last years have been devoted to the identification of small chemical compounds able to recover the activity of gating and of processing mutations. These compounds have been called potentiators and correctors, respectively. By using a high throughput approach to screen large chemical libraries of organic compounds and a small library of drugs approved for human use we have identified several families of compounds, most of them active as potentiators (Yang et al, *JBC* 278:35079-85, 2003; Pedemonte et al, *J Clin Invest* 115:2564-71, 2005; Pedemonte et al, *Mol Pharmacol*, 68:1736-46, 2005, Pedemonte et al, *Mol Pharmacol*, 72: 197-207, 2007). Some of these compounds, such as benzothiophenes, phenylglycines, sulfonamides and 1,4-dihydropyridines displayed potencies in the order of tens of nM. Analysis of the effect of several compounds on CF mutants have shown that mutations in conserved residues of the nucleotide binding domains (NBDs), such as G551D and G1349D, exhibit a shift in the affinity for potentiators (Zegarra-Moran et al, *Br J Pharmacol* 137, 504-512, 2002; Moran et al, *Cell Mol Life Sci.* 62, 446-460, 2005). This has allowed us to model the human NBD dimer and identify a site that shows good correlation between theoretical data and experimental dissociation constants. Then, we have produced mutations on some residues predicted to form part of the binding site for CFTR potentiators and have analysed the activating (at low concentrations) and the inhibitory (at higher concentrations) effects of different potentiators, confirming that residues R553 in NBD1 and V1293 in NBD2 form close interactions with the bound compound (Zegarra-Moran et al, *JBC* 282:9098-104, 2007). We have also found an inverse correlation between potentiator dissociation constants for activation and inhibition, suggesting that the binding sites for these two actions may not be independent. Patch-clamp analysis has confirmed for several compounds that activation and inhibition by potentiators are mainly a consequence of the modification of a single channel parameter, the duration of the interburst closed state. While the target for potentiators is the CFTR protein itself, correctors probably may act on different proteins involved in the folding, maturation, trafficking or recycling of CFTR. This makes the identification of correctors more difficult. Nevertheless, screening for CFTR correctors have yielded a few compounds, such as bithiazole Corr-4a, that worked at relative high concentrations on primary bronchial epithelial cells. The reasons for the low yield of correctors from high throughput analysis deserve a critical discussion.

This work was supported by the Cystic Fibrosis Foundation Therapeutics, Italian Cystic Fibrosis Foundation and Telethon-Italy.

S7.4 - Correctors of F508del CFTR trafficking

Graeme W. Carlile, Renaud Robert, Donglei Zhang, Heidi M. Sampson, Suzana Anjos, Peter Thornhill, Katrina A. Teske, Yishan Luo, John W. Hanrahan and David Y. Thomas

Biochemistry Department, Physiology Department, McGill University, Montreal H3G 1Y6, Quebec, Canada

We have developed an epitopically-tagged version of F508del-CFTR and have used this in a cell-based high throughput screen (HTS) to identify corrector genes and compounds that restore normal trafficking. We have used this assay to screen >72000 chemically diverse compounds from combichem, natural product and proprietary libraries and identified a variety of chemically distinct hit compounds. We have followed up these some of these hits with CFTR functional assays and with tests in a mouse model. For one of these compound classes of compounds we have obtained a number of available analogues and found a compound, KM11060, 7-chloro-4-{4-[(4-chlorophenyl)sulfonyl]piperazino}quinoline, that gives in vitro, correction of the F508del-CFTR trafficking defect in less than 2 hours of treatment and at a concentration of 10nM. This correction persisted for up to 24 hours after removal of KM11060 and also corrected in the F508del-CFTR mouse model. We have shown that KM11060 is a potent inhibitor of phosphodiesterase 5 and that siRNA knock downs of PDE5 are similar in their correction of F508del-CFTR trafficking. We have also used the corrector and functional assays with compounds and siRNAs to known genes, to determine the contributions of the ER luminal quality control system to F508del-CFTR.

Supported by the Canadian Cystic Fibrosis Foundation, Cystic Fibrosis Foundation Therapeutic Inc and the Canadian Institutes of Health Research.

S7.5 - Poly ADP-Ribose Polymerase-1 inhibitors correct F508del-CFTR trafficking

Suzana M. Anjos, Renaud Robert, Graeme W. Carlile, John W. Hanrahan and David Y Thomas

Departments of Biochemistry and Physiology, McGill University, Montreal Canada

Our work is based on the hypothesis that increasing the trafficking of the ER-retained F508del-CFTR mutant by small molecule correctors will be sufficient in alleviating the disease phenotype. We carried out a fluorescence-based High–Throughput Screen (HTS) of CCBN Maybridge library compounds in BHK cells expressing F508del-CFTR and identified a series of compounds capable of increasing the cell-surface expression of F508del-CFTR. One such compound, a benzo-isoquinoline dione (BID) derivative was identified as a strong hit in our screen with 70% similarity to known Poly ADP-ribose polymerase inhibitors (PARP-1). PARP-1 plays a role in a variety of genomic processes, including the regulation of chromatin structure and transcription in response to specific cellular signals. PARP-1 also plays important roles in many stress-induced disease states. Potential therapeutic use of drugs that target PARP-1's enzymatic activity for the treatment of human diseases is the focus of intense research. Identification of PARP-1 inhibitors as correctors of F508del-CFTR R trafficking may elucidate a novel target in the treatment of Cystic Fibrosis.

Aim: To determine the effect of BID and a panel of known PARP-1 inhibitors on the trafficking and function of F508del-CFTR.

Methods: We validated the compounds identified in the screen and their structural analogs by monitoring the appearance of complex glycosylated F508del-CFTR (Band C, ~170kda) and functionally, by its ability to transport halides. Appearance of Band C measured by Western blotting suggests increased trafficking and maturation along the secretory pathway. Halide transport measured by NaI Efflux and single chloride channel measurements by Ussing chamber in polarized human lung epithelial cells determine if the channels are functional at the cell surface.

Results: We found a 50% increase of complex glycosylated F508del-CFTR in BHKHis cells upon treatment with BID in the 1-0.1 μM range. The effect was dose-dependent. We confirmed increased trafficking in human airway epithelial cell line CFBe41o-F508del-CFTR, observing a 25% increase in Band C upon treating with BID in the 1-0.1μM concentration range. Treating with BID also resulted in a 3-fold increase in F508del-CFTR mRNA, measured by qRT-PCR at the corrective concentration in polarized CFBe41o- cells. In addition, we detected a 2.5 fold increase in NaI efflux in BHK(His) F508del-CFTR cells treated with BID for 24 hours. We identified a series of PARP-1 inhibitors sharing 70% structural similarity to our parent compound. Subsequent screening by Western blotting identified a potent PARP-1 inhibitor, PARPinh1 as a F508del-CFTR trafficking corrector. We detected a ~20% increase in Band C in airway epithelial cells in the 1-0.1uM range, within the IC50 for PARP-1 inhibition. NaI efflux assays in BHK cells treated with PARPinh1 showed a significant increase in channel activity relative to untreated F508del-CFTR (3 fold). Single channel measurements by Ussing chamber in polarized CFBe41o- F508del-CFTR show a 3 fold increase in current in PARPinh1 treated cells. Other known PARP-1 inhibitors, structurally unrelated to BID, have also been validated as correctors of F508del-CFTR trafficking.

Conclusion: Our study identifies novel small molecule F508del-CFTR trafficking correctors belonging to the PARP-1 inhibitor family. This suggests a novel target pathway for Cystic Fibrosis regulation and modulation.

Closing Lecture

Inflammation in the CF Lung – a Therapeutic Target

David Nichols, Aura Perez, Assem Ziady, Pamela B. Davis

Case Western Reserve University School of Medicine, Cleveland OH

In cystic fibrosis, the inflammatory response to infectious or inflammatory stimuli is excessive compared to non-CF patients, mice, or cells, and prior data indicate that inflammation is an independent therapeutic target in CF. However, whether this excess response relates directly to deficiency of CFTR function, or to other associated defects such as the increase in activity of the epithelial sodium channel, has been unclear. Recently, we demonstrated that inhibiting the function of CFTR with I-172 in airway epithelial cells in primary culture, grown at the air-liquid interface, results in increased IL-8 response to inflammatory stimuli, which reverses when the CFTR inhibition is withdrawn. In this model, CFTR inhibition does not result in increased activity of the epithelial sodium channel, so the pro-inflammatory effect seems to be associated with inhibition of CFTR per se. This would imply that correcting the CFTR defect should improve the inflammatory response. However, no therapies that have been proven to have this effect are now available for general use. Therefore, novel therapies for inflammation are urgently needed. We have considered local therapies and also alternative drugs. Because high dose ibuprofen is effective in patients, but has rare but dramatic adverse effects, we considered the possible mechanisms by which ibuprofen at these doses – but not low doses – might exert its effect. Two possibilities – inhibition of IKK and binding to PPARγ – come to mind. Triterpenoids may have both these effects. Therefore we tested CDDO for its effect on inflammatory responses in CF phenotype airway cells as well as CF mice. CDDO, given prior to inflammatory stimulus, was effective in reducing NF-κB driven reporter gene transcription, as well as IL-8 release from primary cultures of airway epithelial cells in response to inflammatory stimuli. CDDO also reduced lung inflammation induced by either LPS or flagellin in CF mice. Because of their wide therapeutic window and very few adverse effects, this excellent anti-inflammatory effect makes these drugs of interest in further studies.

P. 1

Assessment of domain-domain interactions in CFTR

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The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) is a member of the ABC transporter supergene family. It is composed of two cytosolic nucleotide binding domains (NBDs), two transmembrane domains and a regulatory region. When these domains fold and associate successfully, they form a structure which mediates ATP-gated chloride conductance across membranes. Most of the mutations which cause Cystic Fibrosis (CF), including the most predominant F508del mutation, alter this process and, thus, affect the maturation of the protein to its functional native structure. When CFTR folding is disrupted, cellular quality control systems target the protein for degradation. Recent structural results reveal that F508 is partially exposed on the surface of the isolated NBD1, suggesting that its deletion affects domain-domain interactions in addition to its known effects on NBD1 folding. To study the folding and assembly process of this protein, we have utilized a double mutant approach to determine which areas/sequences are responsible for stability contacts within and between protein domains. For example, high-resolution structures of related ABC transporters suggest that NBD1 interacts with intracellular loops (ICLs) 1 and 4 or ICL2 and 3. To test these models, F508 was either deleted or mutated to a charged residue, and second-site changes in the ICLs were assessed for their ability to restore maturation of CFTR. The results indicate that F508 is in proximity to ICL4 and that changes that stabilize the NBD1 ICL interaction can partially correct the folding of the most common CF-causing mutation.

CFTR Alternative Translation Results in N-terminus Truncated Proteins: Structural and Functional Consequences

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Most processing and functional disrupting Cystic Fibrosis (CF) mutations lie in the large cytoplasmic domains of CFTR, namely, the two nucleotide binding domains (NBDs) and the R domain (RD). This is not surprising given their role in regulating the channel activity as well as their major impact on the overall protein folding and maturation. The amino (N) terminus of CFTR (N-tail) has been less well studied despite its reported role in regulating the channel by interacting with a number of other proteins [1]. Furthermore, it was reported that a motif between D44-E60 is involved in regulating the channel gating via the RD [2].

We have detected in a Portuguese CF patient a novel mutation, 120del23 (i.e., the deletion of 23 nucleotides, 120 to 142) which thus abolishes the translation initiation codon (AUG pos.133-135). To address the question of whether 120del23 still allows CFTR protein to be produced and to study this putative N-truncated CFTR structurally and functionally, we generated a novel BHK cell line stably transfected with the pNUT-CFTR-120del23 cDNA construct.

Results from protein analysis show that this construct generates CFTR, albeit a shorter form, thus indicating that an internal initiation codon is used. Data from pulse-chase experiments with [^{35}S]methionine followed by CFTR immunoprecipitation, show that the N-terminus truncated CFTR is highly unstable. Moreover, results from iodide efflux (n=6) indicate that this truncated form of CFTR still retains ~25% (with a 2-min delay) of wt-CFTR channel activity, consistently with a previous study [3]. To determine the extent of amino acid loss from the CFTR N-terminus, caused by 120del23, we first analysed the CFTR cDNA *in silico* to search for internal initiation codons, using the AUG evaluator software [4]. Although 81 possible initiation codons were identified in CFTR coding region of these, only 32 are in-frame. By site-direct mutagenesis, we converted each methionine codon M150, M152 and M156 into valines (as well as the triple mutant), using the pNUT-120del23-CFTR cDNA as template. Immunoblot analysis of BHK cells lines stably expressing these mutants revealed the presence of two bands for M150V, M152V and M156V single mutants similar to those detected for 120del23-CFTR. Notwithstanding, for the triple mutant only the lower band was observed. Data from *in vitro* transcription and translation reactions with CFTR constructs lacking exon 1 plus the M82V, M150V single mutations show the same two bands (although additional bands are observed likely resulting from usage of out-of-frame AUG's). Consistently with *in vivo* results, transcription and translation *in vitro* from the M82/M150/M152/M156V construct causes disappearance of the upper of these two bands.

Altogether, our data indicate that Met150, Met152 and/or Met156 are used to produce the major N-truncated CFTR protein. Double mutants and the M82V constructs are being produced to determine which is the preferentially used alternative initiation codon.

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Folding, Processing and Function of Human-Murine CFTR Chimeras: Implications for the Structure of CFTR

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A powerful approach to investigate the structure and function of the CFTR protein is to examine interspecies differences and identify regions of conservation and divergence. Although little is known about the global structure of CFTR, analyses of the crystal structures of the first nucleotide-binding domains (NBD1s) of murine (m) and human (h) CFTR reveal a common fold [1]. However, functional studies demonstrate significant differences in the gating behaviour of hCFTR and mCFTR [2]. To understand the structural basis for the differences between human (h) CFTR and murine (m) CFTR, we generated by homologous recombination hmCFTR chimeras containing mCFTR domains on an hCFTR backbone by replacing all or part of hNBD1, hNBD2 or the hR-domain with the equivalent regions of mCFTR and investigated their biochemical and functional properties following stable expression in BHK cells. Results show that similar to wt-hCFTR, most chimeric proteins are processed to the cell membrane. However, two chimeras fail to mature: clone 12b (mNBD1, aa residues 518-585) and clone 114c (mNBD2, aa 1260-1412). We compared the murine sequence of these two chimeras with that of hCFTR to determine the physico-chemical distances (PCDs) of the respective amino acid (aa) changes. Changes with higher PCD values were selected and *in vitro* mutagenesis performed to introduce these aa alterations into hCFTR cDNA, which, for clone 12b, were: E527Q, E528Q (PCD = 29); S531T (PCD = 58); K536Q (PCD = 53), I539T (PCD = 89) and K584E (PCD = 56). For clone 114c the changes were: P1290T (PCD = 38), K1302Q (PCD = 53), Y1307N (PCD = 143), C1344Y (PCD = 194), D1394G (PCD = 94) and E1409D (PCD = 45). Biochemical and functional analyses of these mutants expressed as above revealed that all of these CFTR mutants were processed except for K584E. Consistently, all these CFTR mutants were found to be functional as cl^- channels albeit displaying some differences relative to wt-CFTR. The absence of processing observed for K584E-CFTR probably derives from its misfolding. In a model of full-length CFTR (R. Ford, personal communication) we found glutamic acid 584 is solvent exposed and interacting with Leu581. Consistent with this model, we establish that changing the human L581 interacting residue into the correspondent murine aa (L581F) rescues the trafficking defect of K584E. Moreover, by combining PCD values with information on disease causing mutations [3] we have identified additional critical residues (S1311K, T1263I, Q1309D, R1325K, V1338T, and L1367I) that may provide information on conformational differences between hCFTR and mCFTR and hence, derive structural implications for this protein. Similarly to our studies for NBD1, these mutants will help to identify critical residues which are responsible for both conformational changes and functional differences between human and murine CFTR.

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Room: Green Room - Basement
Poster Session – Genetics of CF – Gene Networks

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Comparative Analysis of Data from Independent Microarray Studies: Towards a Molecular Signature in CF

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Measurement of global changes in mRNA transcript abundance using microarrays can potentially be used to identify consistent alterations within the proteome (or the interactome) downstream of a genetic defect. For complex genetic disorders including cancer, "molecular signatures" – complex biomarkers with diagnostic utility - have already been revealed using microarrays [1].

In Cystic Fibrosis (CF), the changes in gene expression caused by CFTR dysfunction appear to be subtle once secondary effects from chronic infection or inflammation are excluded [2]. Primary disturbances to redox and antioxidant systems have been reported in an F508del-CFTR mouse model [3], while in human nasal epithelium, genes involved in airway defence, protein ubiquitination and mitochondrial oxidoreductase activity have been associated with differences in CF lung phenotype [4]. A coordinated comparative study of individual experimental datasets (*i.e.*, meta-analysis) is required to clarify whether CF has a robust molecular signature.

We have previously used custom Affymetrix airway arrays [5] to determine mRNA expression profiles in nasal epithelial cells, a readily and non-invasively collected CF model tissue, to identify genes or pathways differentially expressed in CF that would be either potentially related to CF pathology *in vivo*, or good biological markers of CF respiratory disease. In two separate experiments, we compared gene expression in both pooled and individual RNA samples [6]. However, the two lists of differentially expressed genes generated, while each containing several genes highly relevant for CF pathophysiology, had limited overlap. We hypothesized that, as in independent studies of molecular signatures in tumour progression [4] individual studies of CF-related gene expression may identify different subgroups of a larger underlying gene signature.

We have therefore used Gene Set Enrichment Analysis (GSEA) [7] to locate the most highly regulated and significant genes from three independent microarray datasets (Lisbon and Iowa) within their respective ranked gene lists. Results from these GSEA comparisons support the hypothesis of a wider gene signature in CF. Using pathway software we have constructed basic and enhanced pathways based on the isolated gene lists and a "meta-list" composed of genes from all studies combined, which includes such potential CF biomarkers as s100a8, cd44, gja1, areg and ereg. The grouping of data from independent studies fills in many gaps seen when the gene datasets are taken alone. Following validation of these data (comparison with proteomics datasets; quantitative PCR), a firm list of potential CF marker genes will be drawn up.

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Towards *in vivo* correction of the *cftr* F508del allele: design and synthesis of two zinc finger nucleases for homology-directed repair

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The potential to repair the *cftr* F508del allele *in vivo* would constitute a significant step forward in the treatment of CF sufferers. However, gene repair by homologous recombination (HR) has an efficiency of less than 1:10^5 cells rendering it of little therapeutic value. In 2005, Urnov and colleagues[1] described a technique to increase efficiency of HR in cells by >10,000-fold. They designed a pair of zinc finger nucleases (ZFNs) to induce a double stranded break (DSB) at a unique genomic site. Co-transfection of the ZFNs with a donor repair sequence resulted in homology-directed repair (HDR) of a target allele in up to 20% of transfected cells. This level of HDR has been independently confirmed[2], and can be delivered by virus vectors[3].

Towards the application of this approach to repair mutations in the *cftr* gene, we report the design of two zinc finger proteins which each bind a 9 bp sequence in the cftr gene, separated by a 4 bp spacer, which corresponds to a 22 bp sequence in intron 9 of the *cftr* gene. The DNA sequence of each ZF protein was fused inframe with that from the nuclease domain[4] of *Fok*I in a vector containing both T7 and CMV promoters. The recombinant ZFN proteins (ZFN1 and ZFN2) were produced in a coupled transcription-translation reaction. Incubation of partially purified ZFN1 and ZFN2 proteins with a plasmid (pTarget-1/2) containing the 22 bp target site resulted in >95% digestion at this site. Plasmids expressing ZFN1 and ZFN2 will be co-transfected with a donor repair sequence into a *cftr* F508del$^{-/-}$ cell line. The degree of *cftr* F508del allele repair by ZFN-mediated HDR will be evaluated by RT-PCR. The degree of restoration of CFTR activity will be monitored by iodide efflux activity.

Although a pair of ZFNs can induce efficient HDR, ZFNs can homodimerise in cells and induce high levels of cytotoxicity[5]. Indeed, incubation of partially purified ZFN1 with a plasmid (pTarget-1/1) containing two copies of the 9 bp ZFN1 target site separated by the 4 bp spacer in a palindromic arrangement resulted in >95% digestion of the plasmid at this site. To prevent homodimerisation, two groups recently described similar remodelling strategies of the *Fok*I nuclease domain[2,5]. Introduction of corresponding mutations into ZFN1 and ZFN2 had no deleterious effect on the enzymes' ability to cut the plasmid corresponding genomic target (pTarget-1/2). Plasmids expressing the modified ZFN1 and ZFN2 will also be co-transfected with a donor repair sequence into a *cftr* F508del-homozygous cell line and evaluated for HDR activity. A comparison in the levels of cytotoxicity between the modified and unmodified ZFNs will be performed.

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Mutation panel for Cystic Fibrosis carriers of 941 samples in Greece

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Genetic screening for CF mutations was carried out to identify carriers among couples currently planning a pregnancy, couples seeking prenatal care, partners of individuals with CF (CF carriers) and a small group of men with azoospermia or oligospermia.
Cystic Fibrosis is the most common autosomal recessive disease in white population and is characterized by wide heterogeneity at the clinical and the molecular level. The carrier frequency is approximately 5 % in Caucasian North Europe. Among the Greek population, cystic fibrosis constitutes the second most common disease after β-thalassemia.
For the last two years, we have analyzed CFTR mutations in genomic DNA from 941 people, using a mutation screening test (INNOLiPA CFTR 17+Tn and INNOLiPA CFTR 19 by Innogenetics)
We have examined 1882 chromosomes and 40 of them were carrying a CFTR mutation. The mutations that we identified were: F508del (28 cases -35%), N1303K (2 cases, 2.5%), I148T (6 cases, 7.5%), 621+1G→T (1 case, 1.25%), 2789+5G→A (1 case, 1.25%), G85E (1 case, 1.25%), G542X (1 case, 1.25%).
The mutation (variant) I148T, had a significant higher ratio, than the given for the Greek population and for this reason an additional confirmation will be performed by denaturing gradient gel electrophoresis (DGGE). We are also proceeding to screen specific exons of the CFTR gene, in order to detect other (less frequent) mutations in our cohort of samples.

Mutations and polymorphisms in the cystic fibrosis gene in men with severe oligozoospermia.

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Background: Majority of male cystic fibrosis (CF) patients are infertile because of congenital bilateral absence of vas deferens (CBAVD). In addition, male infertility as a result of isolated CBAVD is also recognized as a primary genital form of CF. Mutations in cystic fibrosis transmembrane conductance regulator (CFTR) gene have been found in more than 85% of CBAVD cases. Possible involvements of CFTR mutations in other forms of male infertility have been suggested but remain controversial. In sperm, CFTR may be important in transport of various anions and sperm capacitation, hence making it possible that certain CFTR mutations may lead to reduced sperm fertilizing capacity and male infertility in other forms of male infertility rather than CBAVD.
Objective: To compare the frequency of CFTR gene mutations between oligozoospermic and healthy fertile men.
Materials and Methods: The study populations consisted of 124 oligo- and 90 normozoospermic men. We screened simultaneously for 254 different CFTR mutations and variations using arrayed primer extension (APEX) genotyping microarray (Asper Biotech Ltd).
Results: CFTR mutations and variants were demonstrated in 22 (17.7%) of 124 oligozoospermic patients and in 13 (14.4%) of 90 control men. In addition, the total frequency of mutant/variant alleles in infertility group was slightly, but not significantly higher than in controls (9.7 vs 7.2%). Similar trend was also observed for IVS8-5T allele frequencies (3.6 vs 2.2%, respectively). Although we demonstrate comparable CFTR mutation/variation frequencies in both groups, the causal relationships between specific CFTR mutations and male infertility cannot be completely ruled out.

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FOLDING AND MISFOLDING OF CFTR

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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 we are identifying steps in CFTR folding that are facilitated by cytosolic and ER localized chaperones. In addition, we have 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. Recently, we investigated the role which ER luminal calnexin plays in CFTR folding. In parallel, we probed the mechanism by which deletion of F508 arrests CFTR folding. Calnexin was found to be required for proper assembly of CFTR's membrane spanning domains (MSDs), which is also required for completion of down stream folding events that involve NBDII. Interestingly, F508del-CFTR exhibited biogenic defects that occurred both before and after the calnexin dependent step in CFTR folding. The RMA1 E3 ubiquitin ligase appeared to detect defects in MSD assembly, where as recognition of misfolded NBDII is mediated by the Hsc70/CHIP E3. Chemical correctors were observed to alter the conformation of specific sub-domains of CFTR and enable it to selectively pass through either the CHIP or Rma1 quality control checkpoints. Models that describe the mechanism by which chemical correctors enable F508del-CFTR to escape ER quality control will be discussed. This work is supported by the NIH and NACFF.

LOW TEMPERATURE ALLOWS MUTANT CFTR TO BYPASS THE ENDOPLASMIC RETICULUM QUALITY CONTROL

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Low temperature rescues numerous disease-associated mutant proteins from endoplasmic reticulum (ER) retention, possibly through higher folding efficiency and/or increased protein stabilization.
Here, we investigated the mechanism of low-temperature rescuing of F508del-CFTR, a temperature-sensitive mutant causing cystic fibrosis. Data show that at 26ºC the immature forms of both F508del- and wt-CFTR accumulate in their ER forms. However, after shifting cells back to 37ºC, most F508del-CFTR is rapidly degraded, whereas wt-CFTR efficiently matures, suggesting that these two proteins accumulate in different conformations. Moreover, data assessing the synergistic effects of low temperature and two genetic revertants (G550E and 4RK) which we recently proposed to rescue the trafficking defect of F508del-CFTR by distinct mechanisms [1] suggest that low-temperature rescuing of F508del-CFTR results from bypassing the ER quality control and not so much from promoting the mutant to acquire a stable conformation.

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IMPACT OF THE REVERTANT MUTATIONS G550E AND 4RK ON THE PROCESSING AND FUNCTION OF G551D-CFTR

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G550E and 4RK (the simultaneous mutation of four arginine-framed tripeptides (AFTs): R29K, R516K, R555K and R766K) are second-site mutations that rescue the processing and function of F508del-CFTR [1,2]. These revertant mutations appear to rescue F508del-CFTR from retention within the endoplasmic reticulum (ER) by distinct mechanisms: while G550E likely alters the conformation of the first nucleotide-binding domain (NBD1), 4RK plausibly allows F508del-CFTR to escape ER retention/retrieval mediated by AFTs [3]. Both G550E and one of the AFTs (R555K) lie close to the residue G551, where G551D, the second most common CF-causing mutation occurs, generating a correctly localized Cl^- channel with a severe gating defect.

In this study our aim was to assess whether the F508del-revertants G550E and 4RK also influence the folding, processing and/or gating behaviour of the G551D mutant, by employing biochemical and functional approaches. To test this idea, we introduced the G551D mutation into either G550E- or 4RK-CFTR cDNAs cloned in the pNUT vector and stably expressed these constructs in BHK cells. Results from pulse-chase experiments with [^{35}S]methionine followed by CFTR immunoprecipitation indicate that the turnover rate of the core-glycosylated form of G551D-CFTR (band B) was not significantly affected by the presence of either 4RK or G550E and that the maturation of G551D-CFTR was slightly, albeit not significantly, increased by the presence of G550E but unaffected by 4RK.

Analysis of CFTR-mediated iodide efflux from these cells revealed that G550E is unable to rescue the functional defect of G551D. However, 4RK-G551D-CFTR generated an efflux of iodide larger than that elicited by cells expressing G551D-CFTR, albeit 63% smaller than that of cells expressing wild-type (wt) CFTR. Consistent with these data, analysis of single-channel gating revealed that neither 4RK nor G550E rescued the defective channel gating of G551D-CFTR. However, both revertants augmented the activity of G551D-CFTR by attenuating the mutant's greatly prolonged interburst interval (IBI); G550E halved IBI, whereas 4RK reduced IBI 10-fold.

Altogether, our data suggest that at least when in *cis* with the G551D mutation, the AFTs (together or individually) might have a direct effect on CFTR channel gating. This indicates that the revertants affect CFTR in a mutation-specific manner and raises the possibility that 4RK, in addition to its well-described effect on trafficking, may also act on G551D-CFTR structure and/or folding.

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Morphological analasys of the secretory traffick of CFTR

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Cystic fibrosis (CF) is a severe and frequent genetic disease that results from a mutation in a ion channel protein called CFTR. Normal CFTR resides and functions at the cell surface, but the mutated protein is unable to leave the intracellular organelle where it is synthesized, and to reach the surface of the cell.
Therefore, this 'trafficking' defects is the ultimate cause of the disease. In order to develop a rational therapy for this condition, it is therefore important to understand both the trafficking behaviour of the normal protein and the reasons for which the mutated protein is unable to traffic normally.
While some aspects of the intracellular movement of CFTR have been thoroughly investigated, other aspects remains little understood because of the lack of the necessary technologies.
We have set up the required methodologies (expecially traffic synchronization approaches), and we are using them to analyse the progression of CFTR along its secretory route. We are also using high-throughput microscopy to identify the kinases and phosphatases that regulate the trafficking of CFTR and its D508-CFTR mutant. These results will be presented at the meeting.

CHARACTERIZATION OF CFTR MUTANTS AND REVERTANTS LOCATED IN NBD1

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Despite the large number of mutations reported in the *CFTR* gene [1], the large majority of these have not been molecularly and functionally characterized at the protein level, being the effects of missense mutations the most difficult to predict. Most functional studies have been focused on the two most common missense mutations, F508del and G551D, both located in the first nucleotide binding domain (NBD1), which bears ~10% of all reported disease-causing mutations [1]. In order to further understand how other missense mutations in NBD1 affect the processing of CFTR and to identify the structural motifs critical for the folding of this domain, we have studied the processing and function of two NBD1 missense mutations detected in CF patients that had not been molecularly characterized.
These are S549N [2] and Y563N [3], located in the signature and Walker B motifs, respectively. We also assessed the effect of putative revertants on these mutants, including G330S and T1134P, described to rescue an equivalent mutation (S507N) in the yeast ABC transporter STE6 [4], and of the previously characterized F508del-CFTR revertants G550E and 4RK [5].
Biochemical data show that the S549N mutation does not disrupt CFTR processing into its fully-glycosylated form (band C) but, as expected from its signature motif location, does affect CFTR function as a Cl^- channel, as determined by iodide efflux measurements. This functional defect is partially corrected by both T1134P and G550E. In contrast, Y563N, which is buried inside NBD1 close to F508, severely affects both CFTR processing and the channel activity, likely affecting NBD1 folding differently than F508del, as it is not rescued by either G550E or 4K, the two known revertants of F508del-CFTR that also rescue another NBD1 mutant [5].
To further characterize the mechanisms by which second-site mutations located in NBD1 achieve correction of CFTR processing mutants, we analysed the combined effect of revertants previously described to individually rescue F508del-CFTR, namely G550E, R555K [5,6] and R553M/Q [7] on the expression of F508del-CFTR. Our data show a cumulative effect in terms of band C percentage at steady-state (G550E ~ 36 %; R553M ~ 31 %; R553Q ~ 28 %; R555K ~ 36 %; G550E/R553M ~ 59 %; G550E/R553Q ~ 64 %; R553M/R555K ~ 60 %; R553Q/R555K ~ 70 %), suggesting that these mutations promote correction at distinct checkpoints of the ER quality control or at different points of the NBD1 structure. Further studies are required to understand and discriminate between those mechanisms and to clarify the kinetics of processing and the gating properties of the individual and double revertants preliminarily analyzed here.

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CFTR activation regulates both apical and basolateral Cl^-/HCO_3^- exchange activity in Calu-3 cells

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Calu-3 cells are used as a model of the serous cells of human airway submucosal glands which are involved in CFTR-dependent anion secretion. The aim of this work was to characterise Cl^-/HCO_3^- anion exchange (AE) activity in Calu-3 cells and the dependence of AE activity on CFTR. Control and CFTR knock down (KD) Calu-3 cells (1) were grown as monolayers on Transwell supports. Anion exchange activity was measured in cells loaded with the pH-sensitive dye BCECF-AM. Removal of basolateral Cl^- (substituted with gluconate) under non stimulated conditions produced an alkalinisation in pH_i of 0.45±0.02 (mean ± s.e.m.; P<0.001 paired t-test, n=4), which was abolished by 0.5mM H_2-DIDs, consistent with the presence of a basolateral DIDs-sensitive AE (SLC4A2), as previously reported (2). Stimulation of cells with forskolin completely inhibited this basolateral AE activity. Conversely, apical Cl^- removal had no effect on pH_i under resting conditions, but caused an alkalinisation of 0.51±0.02 (P<0.001, n=4) after forskolin stimulation. This apical alkalinisation was insensitive to H_2-DIDs but prevented by the CFTR inhibitor GlyH-101 (10µM). When cells were stimulated with forskolin in the presence of apical GlyH101, cells now displayed a significant basolateral AE activity (~60 % alkalinisation compared to unstimulated cells) in response to basolateral zero Cl^-. Assuming that GlyH101 did not affect cAMP/PKA activation, these results suggest that anion transport by CFTR is linked to inhibition of basolateral AE activity. The cAMP-dependant switch in basolateral and apical AE activity was also observed with bilateral adenosine (10µM). However, when adenosine was applied to the basolateral membrane only, basolateral AE activity was reduced, but not eliminated, and apical AE was not fully activated (~34% of the bilateral adenosine response). In contrast, apical adenosine fully activated apical AE, but did not cause complete inhibition of the basolateral AE. These results suggest that activation of apical AE alone is not sufficient to inhibit basolateral AE activity.

To further investigate the role of CFTR in regulating AE activity, experiments were also conducted on CFTR-KD Calu-3 cells (1). Forskolin produced qualitatively similar effects on apical and basolateral exchangers observed in wild-type cells. However, under resting conditions basolateral AE was ~32 % less active (calculated from the rate of re-acidification following the re-introduction of Cl^-) compared to WT Calu-3 cells, and CFTR-KD cells also showed some residual basolateral AE activity following forskolin stimulation. Significantly, forskolin-stimulated apical AE activity was markedly decreased (~48%) in CFTR-KD cells.

These data show that increases in intracellular cAMP in Calu-3 cells cause the activation of an apical Cl^-/HCO_3^- exchanger as well as the inhibition of a basolateral Cl^-/HCO_3^- exchanger, through a CFTR-dependent mechanism. Such a switch in AE activity would favour bicarbonate secretion, consistent with the finding that cAMP enhances bicarbonate-dependant fluid secretion from Calu-3 cells. Dysregulation of Cl^-/HCO_3^- exchange would be predicted to reduce HCO_3^- and fluid secretion and contribute to the pathological changes observed in CF submucosal glands

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Evidence of an essential role of NHERF1 and NHEF2 in G-protein coupled receptor regulation of CFTR-mediated intestinal anion secretion *in vivo*

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Background & Aim: The results of heterologous expression studies suggest that the PDZ-proteins NHERF1 and NHERF2 play essential roles in the transduction of receptor-specific signals to the CFTR anion channel. To study their biological roles *in vivo* in an epithelium that highly expresses both CFTR and both NHERF proteins in the same cells, we investigated the effect of NHERF1 and NHERF2 ablation on ß2-adrenergic stimulation and lysophosphatidic acid (LPA) - inhibition of CFTR-regulated duodenal HCO_3^- secretion *in vivo*.
Methods: The proximal duodenum of anesthetized mice was perfused *in situ,* and HCO_3^- secretion was determined by back-titration.
Results: NHERF1 ablation strongly reduced basal HCO_3^- secretory rates. The ß2-adrenerigc antagonist ICI-118551 significantly inhibited basal HCO_3^- secretion in wt, but not in NHERF1-deficient mice. Likewise, NHERF1 ablation completely prevented the stimulatory effect of the β_2-adrenergic ligand clenbuterol. NHERF2 ablation resulted in a stronger stimulation by forskolin compared to wt littermates, completely prevented the inhibitory effect of LPA, and partially rescued the suppressed basal HCO_3^- secretion resulting from NHERF1 ablation. The deletion of CFTR dramatically reduced basal HCO_3^- secretion and abolished the effect of NHERF ablation. **Conclusions**: The PDZ-adapters NHERF1 and NHERF2 proteins differentially modulate basal and agonist-mediated duodenal HCO_3^- secretion *in vivo* in a CFTR-dependent fashion. NHERF1 is an obligatory linker for ß2-adrenergic stimulation of CFTR, and strongly augments cAMP-mediated stimulation. NHERF2 confers inhibitory signals, i.e. as a coupling factor between inhibitory LPA receptors and CFTR.

Role of carbonic anhydrase II in the murine duodenal secretory response to CO_2, secretagogues, and mucosal acidification

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Background and aim: Duodenal HCO_3^- secretion (DBS) is considered the main protective mechanism against gastric acid, and is defective in duodenal ulcer disease. Recent investigations have provided evidence for a pivotal role of CO_2 and extra- and intracellular carbonic anhydrases (CA) in enterocyte sensing of luminal acid. We studied acid-, agonist and CO_2-induced DBS in CA II-deficient mice and their WT counterparts *in vivo*.
Method: The proximal duodenum of anesthetized mice was perfused *in situ,* and HCO_3^- secretion was determined by back-titration.
Results: Basal DBS was not different in CAII-deficient mice and wt, but the DBS response to high luminal CO_2 was abolished, and that to acid was strongly decreased. The small acid-induced increase in DBS in CA II-deficient mice was abolished by perfusing the duodenal lumen with a membrane impermeable CA-inhibitor, suggesting that uptake of HCO_3^- from the lumen, possibly via the anion exchanger Slc26a6, may also play a (minor) part in acid-stimulated HCO_3^- secretion.
Conclusion: CAII is not involved in basal, but of major importance for CO_2 and acid- stimulated DBS *in vivo*. The data suggest that in the duodenal mucosa *in vivo*, Na^+/HCO_3^- cotransporter provides HCO_3^- for basal secretion, but the CAII-mediated CO_2 hydration is essential for the acid- induced HCO_3^- secretory response.

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CFTR controls proteasomal degradation and NF-κB activity in lung epithelial cells exposed to oxidative stress

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Oxidative stress, by the production of reactive oxydative species (ROS) in the airway epithelium has been identified as an early complication of young children with CF. In CF patients, the loss of functional cystic fibrosis (CF) transmembrane conductance regulator (CFTR) protein in lung epithelial cells leads to reduced volume and increased oxidation of the airway surface liquid. Following exposure to ROS, we have recently reported that no increase of NF-κB activity and reduced caspase-3 activity were observed in two CF lung epithelial cell lines, IB3-1 and CFBE41o- due to an absence of reduction of IκB-α inhibitor in contrast to two CFTRwt-corrected-S9 and normal 16HBE14o- cell lines (*Boncoeur E. et al, Int J Biochem & Cell Biol, 2007*). These results prompted us to investigate whether ROS production in lungs resulted in any CF-specific regulation of proteasome-proteolytic (UPS) and caspase-3 activities and NF-κB/IκB-α signaling through the use of *in vivo* and *in vitro* complementary models of CFTR deficient mice compared to wild-type mice, and a human CFTR-deficient lung cell line compared to the same but corrected CFTR-sufficient lung cell line.
We exposed CFTR deficient ($cftr^{-/-}$) and wild type mice for 64-h to hyperoxia-mediated oxidative stress. CFTR deficient mice exhibited significantly higher lung proteasomal activity than $CFTR^{+/+}$ animals after oxidative stress. This was accompagnied by a reduced lung caspase-3 immunoreactivity and an absence of degradation of NF-κB inhibitor IκB-α. *In vitro*, human CFTR-deficient lung cells exhibited higher proteasomal activity and a lack of increased NF-κB-dependent transcriptional activity compared to CFTR-sufficient lung cells after oxidative stress. CFTR Cl^- channel inhibition by $CFTR_{inh\text{-}172}$ in normal bronchial immortalized cell line 16HBE14o- exposed to oxidative stress caused an increase of proteasomal degradation. Inhibition of caspase-3 by Z-DQMD in CFTR-sufficient lung cells mimicked the response profile of increased proteasomal degradation and lowered NF-κB activity of CFTR-deficient lung cells exposed to oxidative stress. These new data reveal a crucial role of CFTR Cl^- channel activity in regulating lung proteasomal degradation, caspase-3 activity and NF-κB-dependent transcriptional activity, a regulatory process that is deficient in cells lacking functional CFTR when exposed to oxidative stress (*Boncoeur E., Roque T. et al, 2008, Am J Pathol, in press*). Further studies are now required to determine which of the components of UPS and caspases complexes might be optimal targets to promote an increase in NF-κB-dependent transcriptional activity in the CF lung epithelium but with low toxicity. Such a strategy might provide more tailor-made therapies for patients with CF who are subject to notable deleterious effects of oxidants.
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Proteomics of membrane microdomains from human bronchial cells subjected to proinflammmatory stimulation

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Introduction and aims: In a previous study, we have observed that CFTR, Annexin 1 (A1) and cytosolic phospholipase A2 (cPLA2) are partially recruited in detergent insoluble microdomains (DIM) upon proinflammatory stimulation and that they are involved in the same macrocomplex. A1 and cPLA2 participate in the inflammatory response notably via the eicosanoid pathway. Recruitment is prevented by specific inhibition of CFTR and is concomitant with an increase of eicosanoid (prostaglandin E2 and leukotriene B4) production. It is postulated that CFTR, A1 and cPLA2 participate in the regulation of inflammation by their dynamic interaction within DIM. In this study we aimed to identify other potential partners of this complex by proteomic analysis of DIM.
Methods: We adapted and compared two proteomic approaches: in-gel trypsin digestion and double SDS-Page (dSDS-PAGE), which are compatible with membrane protein analysis. Human epithelial respiratory cells CFBE and IB3/C38 (expressing either WT or F508del CFTR) were subjected or not to proinflammatory conditions (TNFα, 100U/mL, 3h). DIM were isolated by OptiPrep density gradient, analyzed by either of the two methods, and proteins identified by LC/MSMS. Semi-quantification of identified proteins was performed by the emPAI method, which is function of the ratio between the number of identified peptides for a given protein and the total number of theoretical trypsin-induced peptides.
Results: dSDS-Page permitted the identification of 100 proteins, including 30 membrane proteins, and 40 membrane-associated. The differential analysis showed significant differential expression of cytoskeletal proteins (cytokeratin 18, actinin), inflammation-related proteins (annexins 1 and 2), folding-related proteins and chaperons (PDI, Hsp) and ion channels in DIM between control and TNFα-treated WT vs F508del CFBE cells. In-gel analysis allowed the identification of more than 150 proteins in DIM. By means of emPAI, we etablished a list of proteins differentially expressed in IB3 vs C38 subjected or not to TNFα treatment. This included CFTR-interacting proteins, cytoskeletal proteins, and proteins involved in TNF signalization. As a proof of concept, some characteristic DIM-associated proteins, such as caveolin-1 and 2, flotillin-1 and 2, were detected in all samples subjected to in-gel analysis. In addition, a number of membrane proteins were identified (ion transport proteins, transmembrane proteins and receptors).
Conclusion: dSDS-PAGE and in-gel digestion reveal as useful methods for membrane microdomain proteomic analysis. Proinflammatory treatment induces differential expression of several proteins in DIM from normal versus mutated CFTR-expressing cells. These results will provide valuable information about potential components of a DIM-associated CFTR-containing dynamic macrocomplex, as well as new hypotheses on the participation of CFTR in the regulation of the inflammatory response.

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Psoralen derivatives as regulators of *P.aeruginosa*-dependent transcription of Interleukin-8

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Persistent recruitment of inflammatory cells within the bronchial wall and lumen of CF airways strongly contributes to airway tissue damage and progression of lung disease. The massive neutrophil infiltrate, continuously activated by bacterial components produced during *P.aeruginosa* chronic colonization, releases Reactive Oxigen Species and lytic enzymes which degrade bronchial tissue and disable bacterial killing (1). Among the different approaches to interrupt this deleterious process, reduction of neutrophil chemotaxis represents a potential therapeutic target in CF. As specific drugs against neutrophil recruitment are not available, molecular approaches targeted to reduce the major neutrophilic chemokines released by epithelial respiratory cells in the airway surface fluids, e.g. Interleukin-8, are under investigation (2). As broad-spectrum anti-inflammatory drugs, such as corticosteroids and ibuprofen, do not reach this specific molecular target and already presented side effects in CF patients, we started searching novel compounds from a library of plant extracts largely utilized in traditional Bangladeshi medicine. Initial screening indicates that the plant extract from *Aegle marmelos* significantly reduces the *P.aeruginosa*-dependent transcription of IL-8 mRNA and that three of the major compounds identified in the extract by HPLC/GM spectrometry, namely 5,6 dimethoxy-1-indanone, 2-hydroxy-cinnamic acid and 5-methoxypsoralen (5-MOP), reproduce the inhibition of IL-8 transcription. Interestingly, 5-MOP and its 8-MOP furocoumarin analogue are FDA-approved drugs largely utilized in chronic inflammatory skin diseases. Therefore we tested the effect of 5-MOP and 8-MOP in human bronchial IB3-1 cells in dependence of the pro-inflammatory challenge with the *P.aeruginosa* laboratory strain PAO1. Both 5-MOP and 8-MOP inhibited PA01-dependent transcription of IL-8 mRNA in IB3-1 cells (IC_{50} = 10 μM), without interference with the proliferation activity tested with up to 100 M concentration for 24 and 48 hr. These molecules were tested also in Calu-3 cells grown polarized on Transwell insert filters. 5- and 8-MOP inhibited the PAO1-dependent transcription of IL-8 mRNA, but not that of other chemokines expressed at lower levels, such as GRO-α/γ, MIP-1α and IP-10 in Calu-3 cells. To extend the analysis of the effect of the furocoumarin class of compounds, the angular psoralen angelicin was tested. Angelicin inhibited significantly PAO1-dependent IL-8 mRNA in Calu-3 and IB3-1 cells (IC_{50} = 10 μM), without reducing cell proliferation. In conclusion, analysis of linear and angular psoralen analogues, inspired from preliminary screening of medicinal plant extracts, identify promising molecules to be investigated as regulators of the signalling involved in neutrophil chemotaxis.

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Identification of anti-inflammatory potential of *Aegle marmelos* by screening medicinal plant extracts.

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Attenuation of the chronic inflammatory process in the lung of CF patients is considered a complementary therapeutic strategy to intervene on the progressive tissue damage. Among the different approaches to interrupt this deleterious process, specific reduction of neutrophil chemotaxis, e.g. by controlling IL-8 expression, is under investigation. We recently initiated a screening of plant extracts already utilized in Bangladeshi traditional medicine to select molecular components able to reduce chemokine expression from bronchial epithelial cells challenged with *P.aeruginosa*. To prioritize the screening procedure, we selected firstly those plant extracts showing the capacity of inhibiting the binding of the transcription factor NF-kB with the DNA consensus sequences identified in the promoter of IL-8, since NF-kB has been confirmed playing a critical role in regulating IL-8 expression in respiratory epithelial cells (1, 2). Therefore, extracts have been added to cells 24 hr before pro-inflammatory challenge with *P.aeruginosa* and quantification of IL-8 transcription in the IB3-1 CF bronchial epithelial cells *in vitro*. *P.aeruginosa*-dependent IL-8 mRNA induction was increased by *Argemone mexicana, Vernonia antihelmintica*, no significant modification of transcription was observed with *Aphanamixis polystachya, Lagerstroemia speciosa* and *Hemidesmus indicus* and, finally, inhibition was observed with *Polyalthia longifolia* (IC_{50} = 200 µg/ml) and *Aegle marmelos* (IC_{50} = 20 µg/ml). A proliferation assay performed by pre-incubating the plant extracts of *Polyalthia longifolia* and *Aegle marmelos* with IB3-1 cells for 24 and 48 hr excluded that the inhibitory effect observed on IL-8 transcription was dependent on an unspecific interference with cell viability. Five compounds isolated from the most effective extract *Aegle marmelos* were therefore tested. No significant effect on *P.aeruginosa*-dependent transcription of IL-8 mRNA was observed with butyl-p-tolyl-sulfate, whereas the inhibition obtained with 6-methyl-4-chromanone at 1 mM concentration was due to a possible anti-proliferative effect. On the contrary, 5,6 dimethoxy-1-indanone, 2-hydroxy-cinnamic acid and 5-methoxypsoralen resulted in significant IL-8 inhibition at 1-10 µM concentration without side effects on proliferation. In synthesis, initial screening of plant extracts is starting selecting new molecules with potential therapeutic effects on neutrophil chemotactic signalling.

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Differential expression of PLUNC proteins in CF lung disease.

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We have previously described the characterisation of the PLUNC family of secreted proteins in both man and mice. PLUNCs are expressed in the upper airways, nose and mouth and the family subdivides into short (S) and long (L) proteins, which contain domains structurally similar to one or both of the domains of bactericidal/permeability-increasing protein (BPI). Due to this structural similarity, the sites at which the genes are located and the observation that they are rapidly evolving and have diversified in the mammalian lineage, we hypothesised that PLUNCs may play a role in innate immune defence. Despite the diversification of the PLUNC family between human and mice, SPLUNC1 and LPLUNC1 are the two genes that are most highly expressed in the respiratory tract in both species. A number of studies have suggested that these genes are differentially expressed in human lung diseases and in mouse models.
There are limited studies of the localisation of PLUNCs at the protein level. Our previous studies have however shown that in the respiratory tract SPLUNC1 is predominantly a product of the submucosal glands with less present in the non-ciliated epithelial cells of the large airways and none present in small airways or lung parenchyma. In CF we have shown that staining of SPLUNC1 is significantly increased in diseased airways and is also present in some of the inflammatory infiltrates. We have also shown that it is epithelial cells rather than the inflammatory cells that are the source of this protein. Using novel affinity purified antibodies we have now been able to demonstrate that LPLUNC1 strongly stains a population of airway goblet cells in non-diseased lung. Importantly however, co staining with MUC5B shows that LPLUNC1 is not a surrogate marker for all goblet cells. Submucosal glands stain less intensely with LPLUNC1 than they do for SPLUNC1. Elevated epithelial staining of LPLUNC1 is also seen in diseased small airways in CF and also submucosal glands. It is unclear why expression of these two proteins is increased in CF. We have not been able to show that either gene is transcriptionally regulated by pro-inflammatory mediators in ALI cells in culture. Furthermore, using mouse specific antibodies, we could not identify differential expression of either protein in lungs from CFTR mutant mice. The direct effect of pulmonary infection on PLUNC expression may help to resolve this question. Thus our results show unique expression domains for SPLUNC1 and LPLUNC1 within the airways and suggest that alterations in expression of these putative innate immune molecules, is associated with CF lung disease in humans.

CFTR expression suppresses NFκB-driven inflammatory signalling

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Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) gene was identified nearly 20 years ago but it remains a puzzle how defects in the gene can cause persistent chronic pulmonary infection and inflammation. Numerous studies have shown that inflammatory signalling through the NFκB pathway is increased in CF lungs and that this is linked to the production of pro-inflammatory cytokines such as interleukin 8 (IL-8), e.g. (Verhaeghe, Delbecque et al. 2007); (Carrabino, Carpani et al. 2006). However, there is little consensus on the mechanism(s) which link CFTR and its inheritable mutant forms to chronic lung inflammation. In this study, we tested the hypothesis that wild-type CFTR participates at a fundamental level in the control of endogenous (i.e. unstimulated) NFκB activity and cytokine secretion. Our results show that the level of CFTR expression correlates inversely with endogenous NFκB activity and IL-8 release.

Methods

We used H441 airway epithelial cells which do not express CFTR under basic culture conditions (RPMI + 10% FCS). Cells were transfected with increasing amounts (0-400ng) of wild type CFTR together with a luciferase reporter vector driven by four NFκB response elements. This allowed us to measure NFκB activity, the transcription factor which drives expression of the majority of inflammatory cytokines. The secretion of one of these cytokines, IL-8 was also measured by ELISA assay.

Results

Cells transfected with a control (empty vector) displayed high endogenous NFκB activity. This high endogenous level of NFκB activity was significantly suppressed by transfection with wild type CFTR in a dose dependent manner and this was paralleled by a decrease in IL-8 secretion, e.g. transfection of 200 ng of CFTR wt vector resulted in a 47% reduction in NFκB activity and a 30% decrease in IL-8 secretion.

Conclusions

This data indicates that CFTR has anti inflammatory properties and that the hyper-inflammation found in CF may be due to a disruption of the signalling link between CFTR and NFκB. This is contrary to the belief that inflammation in the CF lung is due to loss of sodium/ chloride transport causing poor clearing of the lung. Further work is required to identify how CFTR interacts with these inflammatory pathways and how this is regulated. Understanding of this could lead to new treatments for inflammation in CF resulting in prolonged better quality of life for these patients.

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Influence of leucocytes on Interleukin-8 (IL-8) in sputum and whole blood in cystic fibrosis

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Factors that influence IL-8 in the CF lung are (a) downregulation of IL-8 receptors in the presence of elastase (Hartl et al 2006), (b) ribonuclear protein hnRNP (A2B1) binding to the IL-8 promoter and hyperproduction of IL-8 mRNA in bronchial epithelial cells (Pollard et al 2006) and (c) exposure to LPS (Sadikot et al 2005). The aim of this study was to elucidate the influence of leucocytes on Interleukin-8 (IL-8) in sputum and whole blood. We were interested how sputum IL-8 levels and leucocytes correlate with IL-8 secretion before and after ex vivo stimulation with LPS well as leucocytes in whole blood in patients with the Delta F 508 (homozygous) mutation.
26 patients (median age 20.5 y, m/f 16/10, BMI 19.52 kg/m^2, Shwachman score 75, FEV$_1$(%) 83) and 15 controls (median age 30 y, m/f 5/10, BMI 21.4 kg/m^2) were examined. The following tests were performed: Interleukin-8 in whole blood before (WB BEFORE) and after (WB AFTER) ex vivo stimulation with 5 ng/ml LPS as well as in induced sputum (IS) was measured by a chemiluminescent immunometric assay (DPC Bad Nauheim, Germany).
IL-8 in CF is regulated not only by LPS dependent mechanisms in the lung (CF patients: median WB BEFORE/AFTER/IS 10.4/181.5/5791 pg/ml//controls: 5.0/65.1/62.5). Leucocytes in sputum (CF/controls median 478.75/ 80 /µl, p= 0.008) were significantly different between patients and controls but not in whole blood (CF/controls median 6550/ 7200 /µl, n.s.). There was no correlation between IL-8 in IS and WB BEFORE or AFTER LPS stimulation. Interestingly there was no correlation between IL-8 in IS and sputum leukocytes but there was a correlation between blood leucocytes and IL-8 (r=0.528, p=0.007) in patients but not in controls. This might be explained by an increased apoptosis of airway leucocytes compared to blood leucocytes in CF (Tabary et al 2006).
Conclusion: IL-8 in the CF lung is regulated not only by LPS dependent mechanisms. There is a correlation between sputum IL-8 and leucocytes in whole blood. We assume that blood cells contribute critically to IL-8 secretion in the CF lung.

The Effect Of LTB_4 Receptor Antagonist BIIL 284 BS In The Model Of Chronic Respiratory Infection With *Pseudomonas aeruginosa* In The Rat

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Morbidity and mortality in cystic fibrosis (CF) are primarily caused by progressive lung damage. Excessive inflammatory responses to chronic bacterial infection of the airways, dominated by neutrophils and consequent release of oxidants and proteases, is thought to play a central role in this tissue destruction. Inhibition of LTB_4, a known potent chemoattractant for neutrophils, may represent an effective anti-inflammatory treatment for CF. However, a phase 3 study with an LTB_4 antagonist, BIIL 284 BS, had to be terminated early due to a significant number of people experiencing pulmonary related serious adverse events (Konstan *et al*., 2005). One possible explanation for this might be that the inhibitory effect of BIIL 284 BS on the LTB_4 pathway resulted in impaired anti-microbial defenses.

Here, we evaluated if BIIL 284 BS could attenuate inflammation, in a rat model of chronic respiratory infection with *Pseudomonas aeruginosa*, without compromising host defence.

Male Sprague Dawley rats (160-220g; n=12/group) were dosed with BIIL 284 BS (0.3mg/kg *p.o.*) or vehicle (1:1 PEG200:saline). 3 hours later, under anaesthesia, the left lung was inoculated with agar beads containing 10^5 colony forming units (cfu) *P.aeruginosa* PAO1 strain via tracheotomy. A sham treated group of rats (n=6) was given sterile beads. Rats were dosed again 4 h post surgery and *b.i.d.* for 1 further day prior to being culled. Cfu counts and histopathological scoring were performed on lung tissues; total and differential cell counts were performed on broncheoalveolar lavage fluid (BALF). Data are expressed as mean ± SEM and analysed using a Students paired t-test or a one-way ANOVA with a Tukey's post test.

Infected vehicle and compound treated animals, had left lung infection: 6.7 ± 0.2 and 6.1 ± 0.2 log cfu respectively. Vehicle treated infected rats had a significantly greater number of total cells in BALF versus sham treated rats ($6.4 \pm 1.1 \times 10^6$ and $1.2 \pm 0.1 \times 10^6$ cells respectively, $P<0.001$), with significant increases in macrophages, neutrophils and lymphocytes. BIIL 284 BS significantly reduced the total cell number versus vehicle treated animals ($3.4 \pm 0.4 \times 10^6$ and $6.4 \pm 1.1 \times 10^6$ cells respectively, $P<0.05$) reflected by a reduction in macrophages ($3.4 \pm 0.6 \times 10^6$ versus $1.4 \pm 0.2 \times 10^6$ cells, $P<0.01$). In contrast to BALF, BIIL 284 BS caused a trend towards a minimal increase in inflammation of the left lung reflected by the histopathological score.

In conclusion, whilst BIIL 284 BS reduced macrophages in BALF, histological scoring suggested a minimal augmentation of inflammation in the lung tissue. This study highlights the importance of examining lung tissue in addition to BALF, and suggests BIIL 284 BS may be having some pro-inflammatory effects in lung tissue.

Konstan *et al* on behalf of the Investigators and Coordinators of BI Trial 543.45. Abstract 2005 Cystic Fibrosis Conference.

Mucin gene expression in airway epithelial cells from children with CF.

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Mucus hypersecretion is a feature of cystic fibrosis (CF). MUC5AC and MUC5B form the major macromolecular components of the mucus gel and are secreted by airway epithelial cells (AEC). In adults, AEC mucin production has been shown to be low in stable CF but increases in pulmonary exacerbations. AIM: To investigate the influence of Pseudomonas (PsA) infection and clinical status on MUC mRNA expression in AECs from paediatric patients with CF compared to healthy controls. Secondly, to investigate whether MUC gene expression is intrinsic to these cells by measuring mRNA in cultured AECs from these same patients. METHODS: AECs were collected by non-bronchoscopic bronchial brushing of children with CF (n=15; PsA+/-ve; stable/pulmonary exacerbation) and without CF (n=15) undergoing elective surgery. AECs in collected cell suspensions were purified by incubation on anti-CD68 coated plates. Cells were then separated for immediate mRNA analysis or incubated with BEGM growth media at 5% CO_2 until confluence. MUC mRNA expression was quantified using real-time PCR with SYBR Green. RESULTS: Preliminary results indicate that MUC5B mRNA was lower in freshly isolated AEC's from CF compared to healthy patients and also lower in cultured AEC's taken from CF compared to healthy patients. MUC5B expression was not affected by clinical nor PsA status. CONCLUSIONS: MUC5B mRNA expression is decreased in freshly isolated and cultured AECs from CF patients and does not appear to be influenced by PsA or clinical status.

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The First Steps in CFTR Biogenesis: Cotranslational Interactions with Nascent CFTR as It Exits the Ribosome

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During its biogenesis, CFTR is synthesized in the cytosol, integrated into the endoplasmic reticulum (ER) membrane, and finally transported through the Golgi to the plasma membrane. In the current work we have studied cotranslational CFTR interactions during the earliest steps of its biogenesis. Site-specific photocrosslinking was used as a method to detect protein-protein interactions. It is based on tRNA-mediated incorporation of a single photo-reactive probe into CFTR nascent chains at various positions during *in vitro* synthesis. Amber suppressor N^{ε}-(5-azido-2-nitrobenzoyl)-Lys-$tRNA^{amb}$ (εANB-Lys-$tRNA^{amb}$) was used to incorporate the probe into the nascent chain wherever an amber stop codon was introduced into the CFTR mRNA. Ribosome-bound CFTR nascent chains of a defined length were prepared by translation of truncated CFTR mRNAs. Using nascent chains with different lengths and different positions of the photo-reactive probe, we examined CFTR interactions at the different stages of its synthesis. In the process of elongation the CFTR nascent chain was found adjacent to 65, 80, 90, 110, 50, and 70 kDa proteins. The ~50 kDa protein was identified as SRP54, a subunit of the signal recognition particle (SRP) that targets ribosome•nascent chain complexes to the translocon in the ER membrane. SRP did not bind to CFTR until its first transmembrane segment (TM1) had completely emerged from the ribosomal tunnel. The SRP-nascent chain interaction was disrupted when the integrity of TM1 was compromised by a deletion mutation. The involvement of SRP in CFTR biogenesis was also shown *in vivo* by siRNA-mediated depletion of SRP in human cell culture: in cells with reduced SRP levels, nonglycosylated forms of CFTR accumulated and total CFTR levels decreased. Notably, some CF-causing mutations in TM1 also reduced photocrosslinking to SRP. These data reveal that mutational inhibition of nascent CFTR association with SRP may contribute to some types of CF. One of these mutations, W79R, promotes a new interaction with an unidentified ~60-70 kDa protein that was not observed for wild-type protein or other mutants. The experimental system provides an avenue to identify these new interacting protein partners. In summary, our data show that CFTR is proximal to a significant number of proteins during the earliest steps of biogenesis and that disease-associated mutations alter many of these interactions.

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Impact of F508 Residue and Small Molecule Correctors on the Interaction of Hsc70 with NBD1 of CFTR

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The deletion of a phenylalanine residue at position 508 (F508del) in the first nucleotide-binding domain (NBD1) of CFTR is the principal cause of cystic fibrosis (CF). The altered interaction of F508del-CFTR with endoplasmic reticulum (ER) quality control proteins, primarily chaperones, mediates both its folding and proteasomal degradation. Recent high-throughput screens (HTS) have identified several small molecule correctors of CFTR biogenesis with therapeutic potential to CF [1]. However, little real-time information is currently available on factors governing the strength of chaperone-CFTR interactions. Our goals here are to quantify the effect on these interactions of: i) the F508del mutation and ii) small molecule compounds.

We used surface plasmon resonance (SPR; Biacore™) to quantify the real-time binding of molecular chaperone Hsc70 to bacterially expressed wt- and F508del-murine NBD1. Hsc70 was covalently immobilised onto the surface of carboxymethyl dextran (CM5) sensor chips (500 μM). In control studies, denatured lactalbumin (3 μM), a known substrate of Hsc70, bound specifically to immobilised Hsc70 (28.4 ± 0.92 pmol nmol-1; n = 4). In separate experiments, purified NBD1 was found to bind immobilised Hsc70 (43.7 ± 0.32 pmol nmol-1; n = 3) but not BSA (n = 10). Moreover, the Hsc70-binding affinity of NBD1 was significantly strengthened when F508 was deleted (wt, K_D^{app}, 1.20 ± 0.16 μM; F508del, K_D^{app}, 0.41 ± 0.10 μM; $n = 3$; $p < 0.05$). Interestingly, increasing MgATP concentration dramatically weakened wt-NBD1 (0.5 μM) binding to Hsc70 (IC_{50}, 19.9 ± 2.2 μM ATP; n = 3). Furthermore, deletion of F508 increased 5-fold the concentration of MgATP required to destabilise the NBD1-Hsc70 interaction ($n = 3$; $p < 0.01$). To better understand the mechanism of action of small molecule correctors with therapeutic potential for CF, we also quantified their effect on the NBD1-Hsc70 interaction. In control studies, pre-incubation of F508del-NBD1 (0.5 μM; 3 μM ATP) with inhibitor $CFTR_{inh-172}$ (50 μM; 30 min, 16 °C) did not destabilise its Hsc70-interaction ($n = 3$; $p > 0.3$). Surprisingly, corrector VRT-325 [2] (5 - 50 μM) was also without significant affect on the interaction ($n = 3$; $p > 0.1$). Only a relatively high concentration of corrector 4a [3] (50 μM) significantly reduced F508del-NBD1 binding to Hsc70 (control, 31.5 ± 2.1 pmol nmol-1; Corr-4a, 24.6 ± 3.1 pmol nmol-1; n = 3).

Overall, these data demonstrate that: (i) F508del strengthens the association of NBD1 with Hsc70 in the absence or presence of ATP; and (ii) corrector 4a, but not VRT-325, has a small, albeit measurable, impact on this interaction, suggesting it may help NBD1 to fold and thus be faster released from its Hsc70 interaction.

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Regulation of the Epithelial Sodium Channel (ENaC) by AMP-activated kinase (AMPK)

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AMP-activated kinase (AMPK) is a ubiquitous Ser/Thr kinase and metabolic sensor that shows increased activity during metabolic stress. AMPK has emerged as a potential mediator for transport-metabolism coupling, as this kinase has been shown to regulate nutrient transporters as well as Cl^-- and Na^+-channels. It was recently shown that AMPK inhibits the epithelial sodium channel (ENaC) by forcing interaction between ENaC and the ubiquitin ligase Nedd4-2 and enhancing ENaC retrieval from the plasma membrane. Here we report that the AMPK inhibitor compound C increased the amiloride-sensitive short circuit current (Isc-amil) in tracheas of wt animals, but not of AMPKα1-/- mice. Rectal PD measurements on AMPKα1-/- mice showed enhanced amiloride-sensitive transport. Because phenformin inhibits ENaC and the basolateral Na^+/K^+-ATPase, Isc-amil was reduced by phenformin in tracheas of both AMPKα1-/- and AMPK+/+ mice. We expressed $\alpha\beta\gamma$rENaC in *Xenopus* oocytes to generate amiloride sensitive whole cell conductance (G_{amil}). The AMPK activator phenformin (5 mM) induced a significant decrease in G_{amil}. The effect of phenformin was no longer significant when $\alpha\beta\gamma$rENaC was coinjected with RNAi that knocked down xNedd4-2. Our data suggest that AMPK inhibition of ENaC is physiologically relevant. Further studies aim in understanding the underlying mechanisms.

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Annexin V and S100A8 are binding partners of CFTR and inhibits activation of whole cells currents in Xenopus oocytes

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Deletion of a phenylalanine residue at position 508 (F508del) in the first nucleotide-binding domain (NBD1) of the cystic fibrosis transmembrane conductance regulator (CFTR) is the principal cause of cystic fibrosis (CF). Altered interactions of F508del CFTR with endoplasmic reticulum quality control proteins promote its proteasomal degradation. However, it is believed that crucial CFTR-interacting proteins (CIPs) important for trafficking and function of CFTR remain unknown. In this study, the NBD1 of CFTR was used as 'bait' in affinity chromatography to capture novel interacting proteins. Analysis of the recovered proteins by high resolution two-dimensional electrophoresis followed by mass spectrometry (Maldi-Toff) identified annexin V and S100A8 as binding partners of CFTR-NBD1.
Annexin V belongs to a class of calcium-dependent phospholipids binding proteins, some of which have been implicated in membrane-related events along exocytosis and endocytosis pathways. Annexin V is a protein kinase C (PKC) inhibitor protein, which binds acidic phospholipids. This protein is overexpressed in CF epithelial cells and it was recently shown that annexin V is necessary for CFTR membrane expression and function [1]. However, we report here that annexin V has an inhibitory effect on CFTR-mediated Cl^- currents in *Xenopus* oocytes. Since annexin proteins are well known to control protein trafficking, we examined if compounds that are know to interfere with trafficking are able to abolish the effects of annexin V. The actin filament disrupter cytochalasin D (CD) reduced activation of CFTR by IBMX/Forskolin. However, in the presence of CD, annexin V had no further inhibitory effects on CFTR, sugesting an actin dependent mechanism. We further elucidate whether annexin V facilitates endocytosis of CFTR by using the endocytosis (dynamin) inhibitor dynasore. In the presence of dynasore, CFTR was no longer inhibited by annexin V. This points out to a stimulatory effect of annexin V on the endocytic retrieval pathway of CFTR. This was further confirmed by inhibiting PKC, a known activator of endocytosis, by BIM. Prolonged activation of PKC has been shown to inhibit membrane targeting of CFTR. Here, we observed that inhibition of PKC suppressed the effect of annexin V on CFTR.
S100A8 (calgranulin A, MRP8) belongs to the S100A8 family characterized by two Ca^{2+}-binding sites of the EF-hand type. S100A8 and S100A9 (calgranulin B, MRP14) form a stable Ca^{2+}-dependent complex (called Calprotectin) [2] that modulates the activity of casein kinase II (CKII) by inactivating it [3]. S100 proteins regulate a broad spectrum of cellular proteins involved in phosphorylation, transcription, Ca^{2+} homeostasis and cytoskeletal dynamics. S100A8 (the so-called "CF protein" [4]) was found to be transcriptionally upregulated in CF and during inflammatory processes. By co-expressing CFTR and S100A8 in *Xenopus* oocytes, we found an inhibitory effect of S100A8 on CFTR-mediated Cl^- currents. Current experiments aim at understanding the underlying mechanism.

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A ROLE FOR CK2 AND SYK IN CFTR TURNOVER AND PROCESSING?

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The most frequent mutation in the cystic fibrosis (CF) gene, F508del, causes retention of its protein product, F508del-CF transmembrane conductance regulator (CFTR) in the endoplasmic reticulum (ER) as a core-glycosylated intermediate that is rapidly degraded. F508del-CFTR therefore fails to traffic to the plasma membrane, where wild-type (wt) CFTR normally functions as a chloride (Cl^-) channel. Retention, however, is not due to lack of function, since the mutant is still partially functional if it reaches the membrane. ER retention results rather from acquisition of a misfolded conformation which is recognized by the ER quality control (ERQC). Morevoer, when F508del-CFTR is rescued to the membrane by low temperature, it is highly unstable, due to both increased endocytosis and reduced recycling [1]. Many CFTR-interacting proteins (CIPs) have been found to regulate these processes, namley: (i) chaperones that affect the biogenesis and ERQC of CFTR; (ii) Rab GTPases and adaptor proteins that control CFTR traffic; and (iii) kinases and phosphatases that have been mostly implicated in regulating CFTR channel activity.

Casein kinase 2 (CK2) has been previously described to affect CFTR traffic/function through phosphorylation of the S511 residue (P-site) of CFTR [2]. However, CFTR possesses another CK2 consensus P-site - T1471- at the C-terminus. Additionally, it has a putative P-site for spleen tyrosine kinase (SYK) - Y512 - which could directly influence CK2 activity by hierarchical phosphorylation [3]. Our aim here was to determine how mutation of these putative CK2 and SYK P-sites affects the CFTR biogenesis, turnover and processing.

We have produced CFTR mutants in which the consensus residues S511, Y512 and T1471 were substituted by either a neutral (alanine, A) or an acidic residue (aspartic acid, D) in both wt and F508del-CFTR backgrounds by site-directed mutagenesis. Generation of BHK cell lines with these constructs was completed for the S511 A/D mutants and is in progress for the other variants. Metabolic labelling with [^{35}S]methionine and pulse-chase experiments followed by CFTR immunoprecipitation were performed in the produced lines. After quantification of bands B (immature form) and C (mature form) of CFTR, these results show that substitution of S511 does not affect the turnover or processing of either wt- or F508del-CFTR. However, treatment of cells with 20 μM TBB (tetrabromobenzotriazole, a specific inhibitor of CK2) show a significant decrease in processing efficiency of wt-CFTR.

Altogether, our data suggest a putative stabilizing role for CK2 upon wt-CFTR in these cells, but this effect does not appear to be dependent on residue 511 (nor on the putative charge added by aspartic acid replacement at on this residue). Experiments are underway to determine whether this effect is reliant on the T1471 putative CK2 consensus P-site or indirectly dependent on prior phosphorylation by SYK.

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INTRACELLULAR LOCALIZATION OF THE PUTATIVE CA^{2+} ACTIVATED CL^{-} CHANNEL BESTROPHIN-1 AND SEARCH FOR INTERACTING PROTEIN PARTNERS

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CFTR and Ca^{2+}-activated Cl^{-} channels (CaCCs), stimulated by cAMP and Ca^{2+}, respectively, are main Cl^{-} channels present in respiratory epithelia [1]. Data demonstrating an upregulation of CaCCs when CFTR is absent or defective [2,3] have triggered further studies of these channels and prompted further elucidation of the functional (and possibly also physical) interactions between CaCCs and CFTR. Although searched for long, the molecular identity of CaCCs is still under debate. While the role of bestrophin family proteins as putative candidates for CaCCs is discussed controversially, bestrophin 1 (Best-1) has been proposed to form Ca^{2+} activated Cl^{-} channels in epithelial cells [4]. Moreover, a novel role of Best-1 in cell proliferation has been established [5], which may be of relevance for the regeneration of the epithelia in CF and also for alternative therapies for CF. Furthermore

Our goal here is two-fold: (i) to investigate the intracellular localization of wild-type (wt) and Best disease-causing mutants of Best-1; (ii) furthermore, to identify novel Best-1 interacting proteins (BiPs) to better understand the role and function of bestrophin(s) in epithelial cells.

In order to enable the study of the trafficking properties of human (h) Best-1 and to clarify the membrane topology of this protein, an N-glycosylation site was inserted in the first putative extracellular loop of hBest-1 (N-Gly-hBest-1). Results from immunoblot analysis following electroporation of this construct into Fisher Rat Thyroid (FRT) cells evidence the presence of a higher molecular mass band which is sensitive to N-glycosidase F (N-glyc F). These data show that N-Gly-hBest-1 is glycosylated and thus plausibly membrane inserted. Similar analysis of some of the described disease-causing mutants, generated by mutagenesis of this construct, will clarify whether these mutations affect hBest-1 membrane traffic. Another construct was also generated by insertion of a FLAG epitope into the first putative extracellular loop of the hBest-1 cDNA in order to clearly determine whether both the wt and mutated proteins reach the cell membrane.

To identify BiPs, two putative cytoplasmic domains of Best-1, N-term (aa 1-30, Best-1-N) and C-term (aa 291-584, Best-1-C), were cloned into the pET-SUMO bacterial expression vector fused to a polyhistidine-tagged (pHis). These domains were then immobilized onto metal-affinity resin to capture interacting proteins from human T84 whole cell and sub-cellular lysates. Protein-containing fractions recovered from Best-1-N/C-coated and blank resins were subjected to 2D-gel and protein identification is underway. It is expected that functional characterization of the interaction between the captured proteins and Best-1 will give new insights into the biological role, plausibly of relevance to CF.

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The NCI-H441 cell line as a model for the study of airway epithelial sodium transport - a comparison with cultured human bronchial epithelial cells

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Defective mucus clearance, associated with airway obstruction and infection, is the hallmark of cystic fibrosis (CF). The epithelial sodium channel (ENaC) is considered to be a key regulator in influencing lung fluid reabsorption. It is widely accepted that Na^+ hyperabsorption in the CF airway reduces mucosal hydration thereby attenuating mucus clearance. Consistent with this, enhanced mucus clearance has been demonstrated in CF patients following inhaled amiloride, a blocker of ENaC activity.

The aim of this study was to examine the pharmacological regulation of ENaC in immortalised NCI-H441 cells, a cell line derived from the human distal airway epithelium. When cultured at air liquid interface, NCI-H441 cells form a Na^+ absorptive epithelial monolayer (Ramminger et al, 2004) and may therefore represent an alternative model to the more expensive cultures of bronchial epithelial cells. To this end, we have directly compared the profile of pharmacological regulation of ENaC in NCI-H441 cells with cultures of primary human bronchial epithelial cells (HBECs).

Over the course of these studies, NCI-H441 cells retained an air liquid interface generating a spontaneous short circuit current (I_{SC}) of 24.0±0.72μA/cm^2 (n = 53; inserts) with an epithelial resistance of 214±14Ω·cm^2 (n = 53). This compares well with HBECs which displayed an I_{SC} of 21±0.97μA/cm^2 (n = 43) and had an increased epithelial resistance of 1770±126Ω·cm^2 (n = 43). The amiloride-sensitive short circuit current (I_{amil}; 21.1±0.54μA/cm^2, n = 43) was similar to HBECs (I_{amil} 17.1±0.94μA/cm^2, n = 43). Concentration response data using ENaC blockers, revealed a potency order of CF552 (IC_{50} = 4nM; n = 9) >>> benzamil (IC_{50} = 70nM; n = 9) > phenamil (IC_{50} = 251nM; n = 4) > amiloride (IC_{50} = 863nM; n = 9) in NCI-H441 cells. This was similar to the potency order of CF552 (IC_{50} = 3nM; n = 8) >> benzamil (IC_{50} = 24nM; n = 11) > phenamil (IC_{50} = 94nM; n = 11) ≥ amiloride (IC_{50} = 205nM; n = 43) in HBECs.
ENaC activity in the human airway epithelium is sensitive to the broad spectrum serine protease inhibitors, aprotinin and placental bikunin (Bridges et al, 2001). These inhibitors decrease ENaC mediated current over a much slower time frame compared to the direct blockers of this channel. Aprotinin (IC_{50} = 220nM) induced a time-dependent attenuation of the basal and amiloride-sensitive I_{SC} in NCI-H441 cells that was reversed upon addition of excess trypsin to the apical surface, consistent with HBECs (IC_{50} = 810nM). The basal I_{SC} was insensitive to inhibition by soybean trypsin inhibitor or α-1 antitrypsin.

The elements of ENaC regulation are partially conserved between the glucocorticoid-dependent, small airway epithelial cell line NCI-H441 and primary HBECs derived from the upper airways. The ENaC pharmacology of both models looks largely similar using both the direct ENaC blockers and the channel activating protease inhibitors. Therefore, NCI-H441 cells may provide a useful and more cost effective model for the study of airway ENaC function.

Development of Post-Genomics Microscopy Assays to Automatically Screen for Protein Targets Affecting CFTR Traffic

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Systematic approaches like transcriptomics and proteomics have held promise of the next post-genomic challenge, *i.e.*, elucidation of protein function towards the understanding of biological processes in health and disease and as a fast track to therapy. However, these approaches fail to meet their expectations, as commonly they just deliver long lists of "candidate genes" or pathways. Functional microscopy-based assays in intact living cells with the potential for large-scale analyses have been recently developed and applied to membrane trafficking studies [1]. In combination with genome-wide RNA interference or over-expression strategies, these assays promise to help reveal comprehensively the regulatory networks underlying membrane traffic in intact cells.

Our aim here is to develop robust microscopy-based assays monitoring the secretory pathway of wt- and F508del-CFTR to be used in automatic microscopy-based screens so as to identify the relevant intervenients in CFTR membrane traffic. Thus, for the optimal assessment of plasma membrane CFTR, a Flag-tag was inserted at the fourth extracellular loop (EL) of both wt- and F508del-CFTR cloned into pcDNA3, as described [2]. Also, to assess total amount of CFTR expressed (so as to automatically obtain the ratio of 'total membrane CFTR' over the 'total expressed CFTR'), the mCherry fluorescent protein (~30kDa) [2] was fused to CFTR. To this end, we have cloned both wt- and F508del-Flag-CFTR into the pEGFP-C2 vector (Stratagene) and replaced GFP by the mCherry-tag. The expression, traffic and function of the fusion proteins resulting from these wt- and F508del-mCherry-Flag-CFTR-pEGFP-C2 constructs were assessed following their transfection into BHK-L cells. Results from Western blotting and immunocytochemistry show that the double-tagged CFTR proteins are expressed and that the wt-protein is membrane localized. Moreover, using the iodide efflux technique for BHK cells we observed that the wt-mCherry-Flag-CFTR displays Cl^- channel activity function which is ~20% of non-tagged wt-CFTR. Patch-clamp recordings are underway to further characterize the function of these constructs.

To follow the intracellular expression of CFTR *ab initio* and to monitor its traffic through the secretory pathway, we are cloning the mCherry-FLAG CFTR construct wt and F508del into lentiviral vectors with (and without) a tetracycline-inducible (Tet-On) promoter using the Gateway system (Stratagene). After validation, these systems will be adapted for automatic large-scale high-throughput microscopy screens on cDNA or siRNA arrays [4], to overexpress or down-regulate large sets of genes. Image processing routines will quantify the presence of CFTR at the plasma membrane in individual cells as to evaluate the impact of each individual gene on CFTR trafficking.

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Discerning community interactions using a Drosophila model of polymicrobial infections

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Bacterial infections are often characterized by more then one species. The lung disease of cystic fibrosis (CF) patients provides examples of polymicrobial infections whereby diverse and dynamic microbial communities are a characteristic of CF airways. The significance of microbe-microbe interactions and the interplay of the communities with the host have not been investigated. We describe a novel *Drosophila* model to discern the biological interactions between microbes within communities, as well as between the communities and the innate immune system. *Drosophila* serves as a surrogate environment for polymicrobial communities and fly survival gives readout of relevant interactions. Using this novel infection model, the oropharyngeal microbial population in CF patients has been differentiated, revealing three classes of microorganisms, one of which has the capacity to influence the outcome of polymicrobial disease through the ability to act synergistically with the principal CF pathogen *Pseudomonas aeruginosa.* This synergy involves microbe-microbe interactions that result in the modulation of *P. aeruginosa* virulence factor gene expression within polymicrobe infected *Drosophila.* The innate immune response to microbial communities is complex and the activation of antimicrobial peptide gene expression can also involve a form of synergy. We postulate that the biological interactions exposed using this model may contribute to the transition from chronic stable infections to acute pulmonary exacerbation infections in CF - the cause of the majority of irreversible lung damage.

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human MDR1 and also Wild-type, but not F508del, CFTR/MDR1 chimera Rescue Heavy-Metal resistance Phenotype of MDR-defective *c. elegans*

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Despite Cystic Fibrosis (CF) being a monogenic disease caused by mutations in the gene coding for the CF transmembrane conductance regulator (CFTR or ABCC7), a number of other genetic factors (modifier genes) are believed to play a role in this disorder. Some of these are expected to be involved in the endoplasmic reticulum quality control (ERQC) responsible for the retention and degradation of misfolded F508del-CFTR, the most frequent mutation in CF patients. The nematode *C. elegans* is an excellent multicellular model to study human diseases, as it is easily cultured, its ~20,000-genes genome has been fully sequenced, it has a short life span (2-3 weeks) and life cycle (3.5 days at 20ºC) and both forward and reverse genetics, including gene disruption both by knockout or RNAi, are easy to perform.

To generate a *C. elegans* model for the CFTR folding defect, we constructed two previously described human (h) MDR1 (ABCB1)/CFTR chimeras (hMDR1/wt-CFTR and hMDR1/F508del-CFTR) [1] and, after cloning into a *C. elegans* ubiquitous expression vector (pS235), injected these constructs into MDR-defective *C. elegans* strains [2]. In parallel, intact human wt-hMDR1 was also injected. The phenotypes of the resulting nematodes were evaluated by the sensitivity to arsenite (2.0 mM), as described [2]. Our quantitative results show that the arsenite-sensitivity of the *pgp-1*/*pgp-3 C. elegans* double mutant (NL130) is significantly rescued by injection of the hMDR1/wt-CFTR chimeric construct, as well as by hMDR1, but not by the hMDR1/F508del-CFTR chimera. Preliminary data also indicate that Y490del-hMDR (the hMDR1 mutation correspondent to F508del-CFTR) is unable to rescue the resistance phenotype. These results suggest that it is possible to generate nematodes with distinguishable phenotypes by expressing two proteins differing solely in the same amino acid residue that causes CF (or in its hMDR1 counterpart). They also suggest that this phenotype results from the same folding defect occurring for F508del-CFTR. Nevertheless, analysis of these nematode strains by RT-PCR showed that the transgenes have low mRNA expression levels of each respective transgene. We are thus producing more robust *C. elegans* models, namely by: 1) inserting the above chimeric constructs under either the *C. elegans* endogenous *pgp-1* or an intestinal-specific (more potent) promoters; and 2) producing P-gp chimeras with a full CFTR-NBD1 (wt and F508del). We are also generating transgenic nematode lines to stable express hMDR1 (wt and Y490del) or the hMDR1/ CFTR (wt and F508del) chimeras on a *pgp-1, pgp-3, mrp-1* triple mutant [2], that might improve the distinction of the sensitivity and resistance phenotypes.

These *C. elegans* models will be used in genome-wide RNAi screens to search for genes involved in the folding, ERQC and/or degradation of the P-gp/CFTR chimeras and, possibly, also of F508del-CFTR.

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Anaesthesia and the elemental content of mouse nasal fluid

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Currently, only the symptoms of CF can be treated, but there is no treatment for the underlying defect, the defective chloride channel CFTR. During recent years, a number of compounds has been suggested that potentially could correct the defect in CFTR, based mainly on experiments with cultured cells. The next step would be to test these compounds on transgenic animals. Although gene-targeted mouse models for CF have not always developed lung pathology that mimics the human disease, we have shown that both CF-patients and CF null mice have significantly elevated concentrations of Na, Cl, and K in their airway surface liquid (ASL) and their nasal fluid, which probably could be normalized by an effective pharmacological treatment.

C57BL/6J mice (F12 backcross) heterozygous for the F508del mutation ($Cftr^{tm1\ Eur}$) were bred as heterozygotes and offspring was genotyped by PCR analysis. Homozygous F508del mice ($Cftr^{tm1\ Eur}$) and wild-type control mice were temporally anesthetized with a solution of 2,2,2 tribromoethanol in 2-methylbutan-2-ol (Avertin). Nasal fluid was collected in Sephadex G-25 beads by applying the beads to double-side tape attached to a filter paper, that was cut in triangular strips (base about 0.2-0.3 mm, length about 0.8 mm) with a narrow tip and inserted into the nostril of a mouse, and kept there for 10 min. The strip of filter paper was then removed, prepared for analysis, and analyzed by X-ray microanalysis in the scanning electron microscope, as described by Kozlova et al. (2005).

The nasal fluid of F508del-CFTR mice had a higher content of Na, Cl, and K, than the nasal fluid of control mice. Both in the F508del-CFTR mice and in the control mice, there was no significant difference in the composition of the nasal fluid collected from the living mice during the first or second anaesthesia, or at the post-mortem sampling. Hence, repeated anaesthesia did not influence the elemental content of the nasal fluid. During experiments all animals survived and no negative symptoms of the anaesthesia were observed.

CF mice are a convenient model for testing novel therapies such as gene therapy and new pharmaceuticals and their survival in long term experiments will be beneficial for many researchers. CF mouse models will be instrumental not only for understanding the disease better, but also for the discovery of modifier genes. The method presented here will allow the use of CF mice in studies, where repeated measurements of parameters on a single animal have to be carried out.

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The F508del mutation reveals a site in NBD1 accessible to drugs that increase F508del-CFTR trafficking.

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The CFTR-activating benzo[c]quinolizinium (MPB) compounds were the first small molecules shown to cause rapid and selective correction of F508del-CFTR trafficking in CF cells [1] and are important as model compounds to investigate the mechanism by which correctors can act. We have established a model protein consisting of NBD1 and the R- domain (NBD1-R) of CFTR, shown to bind both ATP and cyclic nucleotides [2] to test our earlier hypothesis that MPB compounds act by binding to NBD1 [3]. In the present study we demonstrate that MPB compounds bind to NBD1 by showing that they displace ATP and cyclic AMP from wild type and F508del NBD1-R proteins. The pattern of nucleotide displacement from wild type protein was not consistent with a mechanism for activation of chloride channel activity. Thus, MPB-07 and MPB-27, which are less effective activators than MPB-91 and MPB-104, were 4- to 8-fold more effective in displacing ATP and cyclic AMP. Also, the inactive analogues, MPB-02 and MPB-04 bound as effectively as MPB-07. The pattern of nucleotide displacement from the F508del NBD1-R domain protein was markedly different from wild type, being more consistent with the action of MPB compounds in increasing F508del-CFTR trafficking. Thus, MPB-91 and MPB-104 displaced both ATP and cyclic AMP to a significantly greater extent, similar to displacement by MPB-07. The data suggest that the additional butyl group at the 5-position of MPB-91 and MPB-104 inhibits binding to the wild type protein but, in the F508del protein, the binding site is more open, allowing accommodation of the bulky side chain.

Our studies showed two-affinity binding of ATP and cyclic AMP to wild type NBD1 [2]. ATP probably exhibits two-affinity binding at a single site since the G551D mutation caused a reduction both in high (from 4μM to 45μM) and low (10^{-3}M to 10^{-2}M) affinity binding. However, the high and low affinity cyclic AMP binding sites may be in different regions of NBD1. Thus, whereas the T421A mutation decreased high but not low affinity cyclic AMP binding [2] the F508del mutation decreased low affinity cyclic AMP binding approximately 10-fold (1.5mM for F508del; 191μM for wild type) with no effect on high affinity binding.

Overall, the data suggest that the low affinity cyclic AMP binding site is important in the mechanism of CFTR trafficking, the defect in trafficking caused by the F508del mutation and the mechanism by which MPB compounds increase F508del-CFTR trafficking in CF cells. The methodology also provides the potential for rapid screening for discovery and development of specific F508del-CFTR trafficking drugs.

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Mechanism of Action and Epithelial Tissue Efficacy of Several F508del-CFTR Correctors.

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Recent high-throughput screens identified several novel small molecules that partially rescue the trafficking defect of F508del-CFTR both in recombinant and human bronchial epithelial (HBE) cells. These include correctors VRT-325, VRT-640 and C4 and potentiator VRT-532 [1,2]. N-butyldeoxynojirimycin (miglustat), an inhibitor of the enzyme glucosylceramide synthase (and of glycosphingolipids synthesis) in clinical usage to treat Gaucher disease, was also reported to cause the same effect [3].
Our aims here are: (i) to investigate the mechanism of action by which these correctors rescue F508del-CFTR maturation in stable BHK cells; and (ii) to determine their efficacy in native human tissues *ex vivo* and in HBE cells.
Pulse-chase experiments with [^{35}S]methionine followed by CFTR immunoprecipitation, show that F508del-CFTR is fully processed, albeit at low levels in comparison to wt-CFTR: 6.7μM, 24h VRT-325 (~6.0%), 20μM, 48h VRT-532 (~6.5%), 6μM, 24h VRT-640 (~4.5%) and 10μM C4 (~2.0%). Similar experiments with F508del-revertants [4] show that VRT-325 (but not VRT-532, VRT-640 nor C4) increases the efficiency of maturation of F508del-4RK-CFTR, whereas F508del-G550E-CFTR remains unaffected, suggesting an effect directly on the folding of F508del-CFTR. Preliminary data show no rescue of F508del-CFTR in BHK cells after 100μM, 16h miglustat. Additionally, in iodide efflux experiments using BHK-F508del-CFTR cells (n=10), no CFTR function was observed after 100μM, 16h miglustat. All compounds tested here showed a variable degree of cytotoxic effect: VRT-325=miglustat<VRT-532<VRT-640<C4. This makes it difficult to carry out rigorous comparative quantitative experiments for effect on processing. Currently, Ussing chamber experiments are underway to assess the function of F508del-CFTR after treatment with these compounds in HBE (UNCN3T and UNCCF3T) cells.
We also adapted a previous micro-Ussing chamber protocol using colonic biopsies for CF diagnosis and prognosis [5], to determine efficacy of small molecules on human tissues *ex vivo.* In colonic tissue from non-CF individuals (n=3) incubation with corrector VRT-325 (6h) increased CFTR-mediated Cl^- currents by ~40% compared with tissues incubated with the vehicle (DMSO) alone. Preliminary data, also in non-CF colonic tissues (n=6), indicate that potentiator VRT-532 applied acutely (i.e., without incubation) stimulates CFTR-mediated Cl^- currents by ~28%. This effect will probably be superior in CF tissues given their greater scope for CFTR function enhancement.
Overall, our data show that, despite the observed rescue of F508del-CFTR (except for miglustat), further chemical engineering is required to find suitable therapeutic small molecules that cause similar effects without cytotoxicity. Our experiments also demonstrate the potential of the micro-Ussing chamber technique to assess the efficacy of compounds directly in native human native tissues *ex vivo*.
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Guanabenz, an α2-selective adrenergic agonist, activates Ca^{2+}-dependent chloride currents in cystic fibrosis human airway epithelial cell

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In Cystic Fibrosis (CF) respiratory epithelial cells, the absence or dysfunction of the chloride channel CF transmembrane conductance regulator (CFTR) results in reduced chloride ion transport. In contrast, Ca^{2+}-stimulated Cl^- secretion is intact in CF airway epithelia. One possible target for drug discovery aiming at treating CF is to correct the ionic imbalance through stimulation of alternative ionic pathways that may compensate the failure of epithelial Cl^- conductance. Here, using a simple high throughput screening assay to search for Cl^- channels modulators in the CF nasal epithelial cell line JME-CF15, the compound guanabenz (Wytensin®), an α2-selective adrenergic agonist was found positive. Using iodide efflux and electrophysiological recordings, we showed that guanabenz activated (EC_{50} = 822 nM) a DIDS-sensitive and Ca^{2+}-dependent Cl^- channel (CaCC). Guanabenz activated a linear Cl^- channel with unitary single-channel conductance of 8 pS. Recording calcium signals in CF15 cells showed that guanabenz increased the intracellular Ca^{2+} concentration stimulating an influx of Ca^{2+}. In the absence of extracellular Ca^{2+}, the guanabenz effects on Ca^{2+} influx and activation of CaCC were both abolished. These data demonstrate that guanabenz activates Ca^{2+}-dependent Cl^- channels via a Ca^{2+} influx in human CF airway epithelial cells.

Poly ADP-Ribose Polymerase-1 inhibitors correct F508del-CFTR trafficking

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Our work is based on the hypothesis that increasing the trafficking of the ER-retained F508del-CFTR mutant by small molecule correctors will be sufficient in alleviating the disease phenotype. We carried out a fluorescence-based High–Throughput Screen (HTS) of CCBN Maybridge library compounds in BHK cells expressing F508del-CFTR and identified a series of compounds capable of increasing the cell-surface expression of F508del-CFTR. One such compound, a benzo-isoquinoline dione (BID) derivative was identified as a strong hit in our screen with 70% similarity to known Poly ADP-ribose polymerase inhibitors (PARP-1). PARP-1 plays a role in a variety of genomic processes, including the regulation of chromatin structure and transcription in response to specific cellular signals. PARP-1 also plays important roles in many stress-induced disease states. Potential therapeutic use of drugs that target PARP-1's enzymatic activity for the treatment of human diseases is the focus of intense research. Identification of PARP-1 inhibitors as correctors of F508del-CFTR trafficking may elucidate a novel target in the treatment of Cystic Fibrosis.

Aim: To determine the effect of BID and a panel of known PARP-1 inhibitors on the trafficking and function of F508del-CFTR.

Methods: We validated the compounds identified in the screen and their structural analogs by monitoring the appearance of complex glycosylated F508del-CFTR (Band C, ~170kda) and functionally, by its ability to transport halides. Appearance of Band C measured by Western blotting suggests increased trafficking and maturation along the secretory pathway. Halide transport measured by NaI Efflux and single chloride channel measurements by Ussing chamber in polarized human lung epithelial cells determine if the channels are functional at the cell surface.

Results: We found a 50% increase of complex glycosylated F508del-CFTR in BHKHis cells upon treatment with BID in the 1-0.1 μM range. The effect was dose-dependent. We confirmed increased trafficking in human airway epithelial cell line CFBe41o-F508del-CFTR, observing a 25% increase in Band C upon treating with BID in the 1-0.1μM concentration range. Treating with BID also resulted in a 3-fold increase in F508del-CFTR mRNA, measured by qRT-PCR at the corrective concentration in polarized CFBe41o- cells. In addition, we detected a 2.5 fold increase in NaI efflux in BHK(His) F508del-CFTR cells treated with BID for 24 hours. We identified a series of PARP-1 inhibitors sharing 70% structural similarity to our parent compound. Subsequent screening by Western blotting identified a potent PARP-1 inhibitor, PARPinh1 as a F508del-CFTR trafficking corrector. We detected a ~20% increase in Band C in airway epithelial cells in the 1-0.1uM range, within the IC50 for PARP-1 inhibition. NaI efflux assays in BHK cells treated with PARPinh1 showed a significant increase in channel activity relative to untreated F508del-CFTR (3 fold). Single channel measurements by Ussing chamber in polarized CFBe41o- F508del-CFTR show a 3 fold increase in current in PARPinh1 treated cells. Other known PARP-1 inhibitors, structurally unrelated to BID, have also been validated as correctors of F508del-CFTR trafficking.

Conclusion: Our study identifies novel small molecule F508del-CFTR trafficking correctors belonging to the PARP-1 inhibitor family. This suggests a novel target pathway for Cystic Fibrosis regulation and modulation.

Production of a large genomic CFTR construct inserted into a phage-based artificial chromosome (PAC) of potential interest for CF gene therapy

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Cystic fibrosis (CF) is a recessive disorder, which implies that a single copy of the normal *CFTR* gene is sufficient for normal function. This makes CF an attractive candidate disease for gene therapy. However, this approach has not yet met the expectations of a promising new treatment, since so far major difficulties have been encountered. These are mostly associated with: i) poor efficiency of vector delivery; ii) lack of persistent expression of the transgene; and iii) absence of tissue specificity. For successful CF gene therapy to be achieved, these hurdles need to be overcome. Vectors carrying a complete *CFTR* genomic *locus*, including the *CFTR* promoter and native regulatory sequences in introns and outside of the primary transcript can mediate stable and tissue-specific expression, avoiding gene silencing which typically occurs for cDNA constructs. We have previously reported the production of a large genomic construct containing about half of the *CFTR* gene *locus* (60 kb upstream region towards intron 9, construct CGT21) and demonstrated that it was stably propagated in lung sarcoma cells, where it was expressed and correctly spliced [1]. Moreover, efficient transfer into mammalian cells of this large chromosome-based vector, as well as alpha satellite DNA cloned in P1-based artificial chromosome (PAC), was achieved using a genetically engineered invasive *Escherichia coli* vector and efficiently generated human artificial chromosomes (HACs) *de novo*.
Here, we aimed to construct an optimized *CFTR locus* containing all *CFTR* exons. To this end, the production of large quantities of intact DNA is required. PAC vectors carried in *E. coli* and stored in agarose plugs are a good means of preserving large, intact DNA constructs which, after *Asc*I digestion and undergoing PFGE, allow for the digested bacterial DNA and broken molecules to run out of the plugs, being the intact vectors kept inside the plugs for further use. Such stores of structurally [2] and functionally characterized molecules were used here to reconstruct the complete CFTR *locus*. We ligated a corrected exon 10/intron junction fragment with two PACs carrying each half of the *CFTR* genomic sequence and obtained a clone of interest. Analysis done so far of the DNA stores prepared from this clone and of 3 subcolonies, revealed that the insert has the expected size and exon content all along the different regions of the *CFTR* genomic sequence, as well as exon 10 and the PAC vector fusions. Overall, these results indicate a high cloning stability of the optimized *CFTR locus* and suggest that the production of large stores of functional constructs for therapeutic purpose is feasible.

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Dysfunction of mitochondria Ca^{2+} uptake in cystic fibrosis airway epithelial cells.

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In the genetic disease cystic fibrosis (CF) mutations of cystic fibrosis transmembrane conductance regulator gene (CFTR), among them the deletion of phenylalanine at position 508 (F508del-CFTR), lead to chloride impermeability in many exocrine glands (salivary, airways, pancreatic) associated with dehydration and reduced volume of the final secretory fluid (**Riordan et al., 1989**). In homozygous F508del-CFTR airway epithelial cells, the histamine Ca^{2+} mobilization is abnormally increased. The ER Ca^{2+} release and plasma membrane Ca^{2+} influx seem to be responsible of this abnormal Ca^{2+} signalling (**Antigny et al., 2007**). Mitochondria are known to play key roles in the Ca^{2+} homeostasis in several cells types **(Carafoli, 1987; Gunter and Pfeiffer, 1990).** Previous and pioneer works revealed that mitochondria are dysfunctional in CF fibroblast cells and that the major site of increased intracellular Ca^{2+} in CF is mitochondrial (**Shapiro and Lam, 1987; Shapiro, 1989**).

In the present work, we studied the mitochondria morphology, the mitochondria membrane potential ($\Delta\Psi_{mit}$) (with TMRE probes) and the rate of caspase 3 activity in human CF tracheal gland cells compared to non CF human tracheal gland cells. Additionally, we examined the CF and non CF mitochondria Ca^{2+}-power-buffering using Rhod2-AM probes to load mitochondria. Mitochondria Ca^{2+} uptake was stimulated by histamine-ER Ca^{2+} release. Our findings show:

- **(v)** -using transmission electron microscopic analysis, we show that the mitochondria network is dispersed and fragmented in CF cells compared to non CF cells where mitochondria are extended and continuous.
- **(vi)** -a mitochondria membrane depolarization in CF cells, independently of specific CFTR activity (no effect of CFTR-$_{inh-172}$).
- **(vii)** -mitochondria Ca^{2+} buffering is decreased in CF mitochondria compared to non CF mitochondria, due to a dysfunction of mitochondrial Ca^{2+} uniporter.
- **(viii)** -apoptosis inducers in non CF mitochondria provoke a $\Delta\Psi_{mit}$ collapse and a decrease of mitochondria Ca^{2+} uptake to the same level as for CF mitochondria.

These results demonstrate existence of a mitochondria dysfunction in human CF epithelial cells. Our data suggest a pre-apoptotic state of the human CF epithelial cells compared to non-CF cells. This mitochondria Ca^{2+} uptake defect in CF cells participates certainly to the abnormal Ca^{2+} mobilization in CF cells. These observations show that F508del-CFTR mutation have many impact in cell physiology.

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CFTR and Tight Junctions in Cultured Normal Bronchial Epithelial Cells

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INTRODUCTION: Airway epithelial salt and water transport, which is defective in CF, is of central importance and can take place through paracellular and transcellular pathways. This transport depends critically on ENaC and CFTR operating in concert with the paracellular pathway. Fluid absorption is mainly controlled by the transport of Na^+ through ENaC while fluid secretion is regulated by movement of Cl^- and HCO^-_3 through CFTR. The movement of counter ions and fluid takes place predominantly through leaky tight junctions (TJ). The present study reports that the transepithelial resistance is higher in CF airway epithelia compared to healthy epithelia. A similar pattern was seen when CF-cells were compared to their corrected counterparts, which were made healthy with respect to CFTR. Thus, the corrected CF-cells had a lower resistance than the non-corrected cells. Our hypothesis is that there is a relationship between CFTR and the TJ protein complex.

MATERIAL AND METHODS: The normal airway epithelial 16HBE14o$^-$, the cystic fibrosis CFBE41o- and corrected CFBE41o- (CFBE41o pCep4) cell lines were cultured under isotonic conditions. Hygromycin B was added to CFBE41o pCep4 as selector for the vector. To some of the 16HBE14o- cultures a selective inhibitor of CFTR, CFTR inh172, or the stimulators IBMX + forskolin was added. Transepithelial resistance was measured as indicator of the tightness of the cultures. The permeability of TJ was investigated by transmission electron microscopy, with lanthanum nitrate added to the luminal side of the epithelium and classified as either: 1) no penetration of lanthanum tracer at all, “intact TJ” 2) penetration of lanthanum for a short distance into the TJ, “weakened TJ” and 3) penetration of the lanthanum through the entire TJ, “open TJ”.

RESULTS: Adding CFTR inh172 or IBMX + forskolin to 16HBE14o- cultures was accompanied by a immediate effect, less than 1 hour, on TEER, with a rise during exposure to the inhibitor and a decrease during exposure to the stimulators. After long term exposure, up to168 hours to the CFTR inhibitor, TEER increased with increased exposure time. This effect was prolonged in time proportional to the time of exposure. Ultrastructural analysis showed that the permeability to lanthanum in TJ was higher in the leaky cultures, i.e., those exposed to IBMX + forskolin, compared to the tighter cultures, i,e., controls and cultures exposed to CFTR inhibitor.

CONCLUSION: 16HBE14o- exposed to CFTR inh172 showed an increase in TEER comparable to the TEER in CFBE41o- cultures. The response was immediate but also prolonged over time. When CFTR was stimulated, i.e. the 16HBE14o- cultures were exposed to IBMX+forskolin, there was a significant decrease in TEER compared to unstimulated cultures. These results indicate that there is communication between CFTR and the TJ protein complex and that the TJ may be altered in CF cells.

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