2004 - ECFS Conference
New Frontiers in Basic Science of Cystic Fibrosis

Hotel dos Templários, Tomar - Portugal

Chairpersons: David N. Sheppard and Margarida D. Amaral

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2004 ECFS Conference
New Frontiers in Basic Science of Cystic Fibrosis

(Tomar – Portugal, 30 April – 3 May 2004)
Programme

FRIDAY, 30. 04. 2004		
13.00 - 15.00	***Registration / Light meal / Poster set up***	**Underground Floor Hall / Calçada Room** *(Underground Floor)*
15.00 - 15.30	**Official opening of the meeting**	G Döring (Tübingen Germany) MD Amaral, Lisboa (Portugal) DN Sheppard, Bristol (UK)
15.30 - 16.30	**Opening lecture** **R Frizzell, Pittsburgh (USA)**	**Infante II Room** *(Underground Floor)*
16.30 - 17.00	***Coffee break* + Poster viewing**	**Calçada Room** *(Underground Floor)*
17.00 - 19.00	**Symposium 1 – Genomics** **Chair: A Harris, Oxford (UK**	**Infante II Room** *(Underground Floor)*
17.00 - 17.10	Introduction	A Harris, Oxford (UK)
17.10 - 17.30	HNFα is involved in tissue-specific regulation of CFTR gene expression	A Harris, Oxford (UK)
17.30 - 18.00	Molecular basis of pre mRNA splicing defects in CFTR exons	F Pagani, Trieste (Italy)
18.00 - 18.20	Gene expression	L Clarke, Lisboa (Portugal)
18.20 - 18.35	Screening of CFTR gene rearrangements in French and Italian CF patients	C Bombieri, Verona (Italy)
18.35 - 18.50	Levels of normal CFTR transcripts in 5T and G576A carriers and patients	A Ramalho, Lisboa (Portugal)
18.50 - 19.00	General discussion	
20.00 - 21.30	***Dinner***	**Grão Mestre Room** *(Ground Floor)*
SATURDAY, 01. 05. 2004		
07.30 - 9.00	***Breakfast***	**Breakfast Room** *(Ground Floor)*
09.00 - 11.00	**Symposium 2 – Novel Therapeutic Strategies (Pharmacology)** **Chair: M Hug (Freiburg, Germany)**	**Infante II Room** *(Underground Floor)*
09.00 - 9.05	Introduction	M Hug (Freiburg, Germany)
09.05 - 9.25	Approaches to pharmacological treatment of CF	G Roomans, Uppsala (Sweden)
9.25 - 9.45	Development of a drug therapy to correct CFTR dysfunction	B Dormer, Cardiff (UK)
9.45 - 10.05	Identification of new drugs for the modulation of chloride transport in CF. Advance in the HTS programme	O Zergarra-Moran, Genova (Italy)
10.05 - 10.25	Searching for the CFTR-openers binding site	O Moran, Genova (Italy)
10.25- 10.55	Purinergic regulation of the epithelial Na^+ channel ENaC via hydrolysis of PIP_2	K Kunzelman, Regensburg (Germany)
10.55 - 11.00	General discussion	
11.00 - 11.30	***Coffee break* + Poster viewing**	
11.30 - 13.00	**Symposium 3 – Models of Airway Disease** **Chair: B Dormer, Cardiff (UK)**	**Infante II Room** *(Underground Floor)*
11.30 - 11.40	Introduction	B Dormer, Cardiff (UK)
11.40 - 12.00	ENaC-mediated airway Na^+ hyperabsorption causes cystic fibrosis -like lung disease in mice	M Mall, Chapel Hill (USA)
12.00 - 12.20	Potential of stem cells for airway regeneration	J Dorin, Edinburgh (UK)
12.20 - 12.40	Biogenesis and trafficking of CFTR	J Collawn, Birmingham (USA)
12.40 - 12.50	Characterization of a new polarized epithelial cell model to study CFTR	F Mendes, Lisboa (Portugal)

10.50 - 13.00	General discussion	
13.00 - 14.30	***Buffet Lunch***	**Grão Mestre Room** *(Ground Floor)*
14.30 - 17.00	***Break – Visit to Christ's Convent***	***Walk or Small Train***
17.30 - 20.00	**Symposium 4 – Cell Physiology** **Chair: D Sheppard, Bristol (UK)**	**Infante II Room** *(Underground Floor)*
17.30 - 17.35	Introduction	D Sheppard, Bristol (UK)
17.35 - 18.05	CFTR activators	T-C Hwang, Columbia (USA)
18.05 - 18.25	Acidic pH potentiates the activity of wild-type CFTR, but not the CF-associated mutants G551D and G1349D	J-H Chen, Bristol (UK))
18.25 - 18.45	Novel regulation of CFTR by external chloride	M Gray, Newcastle (UK)
18.45 - 19.05	Bicarbonate and proton secretion by airway serous cells	M. Hug, Freiburg (Germany)
19.05 - 19.30	Is CFTR a regulator or is any chloride channel a regulator of ENAC?	G Nagel, Frankfurt (Germany)
19.30 - 19.55	ND PK AMPK CFTR - What are they doing together?	A Mehta, Dundee (UK)
19.55 - 20.00	General discussion	
20.30 - 21.30	***Dinner***	**Grão Mestre Room** *(Ground Floor)*
SUNDAY, 02. 05. 2004		
07.30 - 9.00	***Breakfast***	**Breakfast Room** *(Ground Floor)*
09.00 - 11.00	**Symposium 5 – CFTR Structure, Folding & Processing** **Chair**: **MD Amaral, Lisboa (Portugal)**	**Infante II Room** *(Underground Floor)*
09.00 - 9.10	Introduction	MD Amaral, Lisboa (Portugal)
09.10 - 9.40	The structure of CFTR nucleotide binding domain 1	H Lewis, San Diego (USA)
09.40 - 10.00	CFTR: a dimer with a central pore	H Schillers, Münster (Germany)
10.00 - 10.20	Role of glycosylation and calnexin in the ER quality control of CFTR	C Farinha, Lisboa (Portugal)
10.20 - 10.40	Analysis of differentially displayed proteins in HeLa cells expressing CFTR and F508del-CFTR	A Edelman, Paris (France)
10.40 - 10.50	Rescue of cystic fibrosis mutations in the first nucleotide-binding domain by revertant mutations (G550 and 4RK)	M Roxo-Rosa, Lisboa (Portugal)
10.50 - 11.00	General discussion	
11.00 - 11.30	***Coffee break* + Poster viewing**	**Calçada Room** *(Underground Floor)*
11.30 - 13.00	**Symposium 6 – Modifier Genes** Chair: JJ Cassiman, Leuven (Belgium)	**Infante II Room** *(Underground Floor)*
11.30 - 11.40	Introduction	JJ Cassiman, Leuven, (Belgium)
11.40 - 12.00	Modifier genes	B Tümmler, Hannover (Germany)
12.00 - 12.20	Genetic modifiers of the CF phenotype in Belgian and Czech CF patients	H Cuppens, Leuven (Belgium)
12.20 - 12.30	General discussion	
13.00 - 14.30	***Buffet Lunch***	**Grão Mestre Room** *(Ground Floor)*
14.30 - 16.30	**Symposium 7 – CFTR-opathies** **Chair: B Tümmler, Hannover (Germany)**	**Infante II Room** *(Underground Floor)*
14.30 - 14.40	Introduction	B Tümmler, Hannover (Germany)
14.40 - 15.10	CFTR and its diseases	G Cutting, Baltimore (USA)
15.10 - 15.30	Genotypes determine only part of the phenotype	JJ Cassiman, Leuven (Belgium)
15.30 - 15.50	CFTR-related disorders in enterocytes and erythrocytes	H DeJonge, Rotterdam (The Netherlands)
15.50 - 16.10	CFTR and renal cyst growth: implications for autosomal dominant polycystic kidney disease	H Li, Bristol (UK)
15.10 - 16.20	CFTR gene mutations in infertile Portuguese patients with congenital absence of the *vas deferens*	A Grangeia, Porto (Portugal)
16.20 - 16.30	General discussion	

16.30 – 17.00	***Coffee break* + Poster viewing**	***Calçada Room*** *(Underground Floor)*
17.00 - 19.00	**Special Interest Group I – Proteomics & Interactomics** **Chairs: A Edelman, Paris (France)** **D Penque, Lisboa (Portugal)**	**Infante II Room** *(Underground Floor)*
17.00 - 17.15	Introduction	A Edelman, Paris (France)
17.15 - 17.45	Overview	D Penque, Lisboa (Portugal)
17.45 - 19.00	Discussion	
17.00 - 19.00	**Special Interest Group II – Host-Bacterial Interactions** **Chair: M Conese (Milan) Italy**	**Convento Room** *(Underground Floor)*
17.00 - 17.30	Introduction and Overview	M Conese (Milan) Italy
17.30 - 17.45	Neutrophils isolated from CF F508del-homozygotes do not demonstrate a primary defect in superoxide anion production and are not basally primed	DJ McKeon, Cambridge (UK)
17.45 - 18.00	Effects of reduced oxygen concentration on antibiotics sensitivity for *Pseudomonas aeruginosa* in cystic fibrosis	GS Ooi, Southampton (UK)
18.00 - 19.00	Discussion	
19.30 –	***Departure to Social Function***	
MONDAY, 03. 05. 2004		
07.30 - 9.00	***Breakfast***	**Breakfast Room** *(Ground Floor)*
09.00 - 11.50	**Symposium 8 – Gene Therapy** **Chair: C Boyd, Edinburgh (UK)**	**Infante II Room** *(Underground Floor)*
09.00 - 9.20		C Boyd, Edinburgh (UK)
09.20 - 9.40	Gene transfer to the airway epithelium by HIV-based vectors	M Conese, Milan (Italy)
09.40 - 10.00	Human artificial chromosomes: seeing viable stability	D Schindelhauer, München (Germany)
10.00 - 10.20	SFHR-mediated modification of CFTR *in vitro* and *in vivo*	D Grünert, San Francisco (USA)
10.20 - 10.40	Entry of plasmid DNA into the mammalian nucleus	F Munkonge, London, (UK)
10.40 - 10.50	General discussion	
10.50 - 11.00	***Short break***	
11.00 - 12.00	**Closing Lecture** **B Scholte, Rotterdam (The Netherlands)**	**Infante II Room** *(Underground Floor)*
12.15 -	***End of the Meeting – Shuttle departure to Lisboa airport***	

POSTERS

P1. **Screening of CFTR Gene Rearrangements in French and Italian CF Patients**
C. Bombieri, O. Raguénès, M.P. Audrézet, A. Bonizzato, C. Castellani, J.M. Chen, P.F.Pignatti, C.Férec

P2. **Levels of Normal CFTR Transcripts in 5T and G576A Carriers and Patients**
A. S. Ramalho, C. Barreto, H. Rocha, V. Serra, D. Penque, P. Pacheco, P. Loureiro, M. Mall, M. D. Amaral

P3. **Adjacent Genes from the Human CFTR-Region Associate Independently with Functionally Appropriate Nuclear Regions**
D. Zink, M.D. Amaral, A. Englmann, S. Lang, L.A. Clarke, C. Rudolph, F. Alt, K. Hochreuther, C. Braz, N. Sadoni, J. Rosenecker, D. Schindelhauer

P4. **Searching for the CFTR-Openers Binding Site**
O. Moran, O. Zegarra-Moran

P5. **Characterization of a New Polarized Epithelial Cell Model to Study the Cystic Fibrosis Transmembrane Conductance Regulator**
F. Mendes, J. Wakefield, M.D. Amaral MD, Z. Bebok, D. Penque

P6. **Expression, Maturation and Function of CFTR are Altered by Genistein Treatment**
A. Schmidt, L.K. Hughes, J. Althaus, J.S. Lunn, H. Li, Z. Cai, M. Roxo-Rosa, D. Penque, M.D. Amaral, D.N. Sheppard

P7. **Rescue of Cystic Fibrosis Mutations in the First Nucleotide-Binding Domain by Revertant Mutations (G550 and 4RK)**
M. Roxo-Rosa, M. Neto, C.M. Soares, Z. Cai, D. N. Sheppard, D. Penque, M. D. Amaral

P8. **Use of Human-Murine CFTR Chimeras to Investigate the Regulation of CFTR Cl^- Channels**
T. S. Scott-Ward, E. S. Dawson, A. Doherty, H. Davidson, D. J. Porteous, D. N. Sheppard, C. Boyd

P9. **Molecular and Functional Characterization of Missense Mutations on the CFTR Gene**
G. González-Gené, D. de Semir, A. Escalada, C. Solsona, V. Nunes, S. Larriba, J. M. Aran, T. Casals

P10. **CFTR and Renal Cyst Growth: Implications for Autosomal Dominant Polycystic Kidney Disease**
H. Li, D.N. Sheppard

P11. **CFTR Gene Mutations in Infertile Portuguese Patients with Congenital Absence of the *Vas Deferens***
A. Grangeia, F. Niel, F. Carvalho, S. Fernandes, A. Ardalan, E. Girodon, J. Silva, M. Sousa, A. Barros

P12. **Neutrophils Isolated from Cystic Fibrosis F508del-Homozygotes do not Demonstrate a Primary Defect in Superoxide Anion Production and are not Basally Primed**
D. J. McKeon, K. Cadawallader, A. Condliffe, E. R. Chilvers, D. Bilton.

P13. **Effects of Reduced Oxygen Concentration on Antibiotics Sensitivity for *Pseudomonas aeruginosa* in Cystic Fibrosis**
G. S. Ooi, G. Jones, R. L. Mehta

P14. **Increased Interleukin-9 Gene Expression Associated with Chronic *Pseudomonas aeruginosa* Infection**
H. L. Wallace, P. S. McNamara, C. A. Hart, P. McCormack, D. Heaf, B. F. Flannagan, R. L. Smyth, K.W. Southern

P15. **Low Temperature Effect on the Entire Protein Expression Profile of BHK Cells Expressing wt- or F508del-CFTR**
A. Simas, M. Roxo-Rosa, G. da Costa, A. V. Coelho, D. Penque

Symposium 1 – Genomics

Chair: Ann Harris, Oxford (UK)

S1.1 HNF1α is Involved in Tissue-Specific Regulation of CFTR Gene Expression

Nathalie Mouchel, Sytse A. Henstra, Victoria A. McCarthy, Sarah H. Williams,
Marios Phylactides and Ann Harris

Paediatric Molecular Genetics, Weatherall Institute of Molecular Medicine, Oxford University, Oxford. OX3 9DS. UK

Regulation of expression of the cystic fibrosis transmembrane conductance regulator gene (*CFTR)* gene is complex. The *CFTR* mRNA and protein show patterns of expression *in vivo* that are tightly regulated both temporally, during development, and spatially in different tissues. CFTR is abundant in epithelia within the pancreatic duct and the crypts of small intestine and colon and is expressed at comparatively low levels in the airway epithelium. Motifs in the basal promoter do not confer accurate temporal and spatial expression on transgenes and very little is known about elements that regulate *CFTR* expression. Present attempts at gene therapy for CF rely on vectors containing promoters from other genes that do not produce accurate tissue specificity or appropriate levels of regulated expression of the introduced *CFTR* genes. Our long term aim is to characterise genetic elements that contribute to tissue-specific regulation of *CFTR* expression, identity the transcription factors (TFs) involved, and characterise the mechanism(s) of regulation. We previously screened 400kb encompassing *CFTR* and flanking regions for DNase I hypersensitive sites (DHS), which are often associated with regulatory elements. DHS were identified 5' and 3' to the gene and within introns 1, 2, 3, 10, 16, 17a, 18, 20 and 21. Several DHS are correlated with tissue-specific *CFTR* expression. The nature and precise mechanisms of action of potential regulatory elements located at each of these DHS sites remains to be elucidated. Our current model proposes that spatial and temporal regulation of *CFTR* expression is mediated by interaction of multiple regulatory elements in chromatin.

To further refine this model, regions of *CFTR* that exhibit DHS were evaluated for binding to transcription factors we investigated transcription factor binding to the core elements in the DHS in introns 10, 17a and 20 of the *CFTR* gene, which were most prominent in the Caco2 colon carcinoma cell line. Evaluation of cross-species homology between sheep and human *CFTR* showed a high degree of conservation between several predicted regulatory elements enabling finer mapping of the functionally important sequences. We identified multiple binding sites for hepatocyte nuclear factor 1 (HNF1) in the DHS core. HNF1, which is expressed in many of the same epithelial cell types as CFTR and shows similar differentiation-dependent changes in gene expression, bound to these site *in vitro*. Over-expression of heterologous HNF1α augmented *CFTR* transcription *in vivo*. In contrast, antisense inhibition of *HNF1α* transcription reduced CFTR mRNA levels. *Hnf1α* knockout mice showed lower levels of cftr mRNA in their small intestine in comparison to wild type mice. This is the first report of a transcription factor that contribute to tissue-specific regulation of *CFTR* expression.

S1.2 Molecular Basis of Pre mRNA Splicing Defects in CFTR Exons

Franco Pagani

International Centre for Genetic Engineering and Biotechnology, Trieste, Italy

In CFTR gene, mutations or polymorphic variants at non-canonical RNA splicing regulatory elements (exonic or intronic) may unexpectedly result in aberrant splicing events and variable phenotypic expression of CF. In CFTR exons 9 and 12, systematic site-directed mutagenesis and *in vivo* functional splicing assay studies with hybrid minigenes have identified Composite Exonic Regulatory Elements of Splicing (CERES) that contain overlapping enhancer and silencer functions. Exonic variations in these elements can both increase or decrease exon skipping. As the sequence composition of CERES overlaps with the codon function, some of the exonic variations that induce aberrant exon skipping are at the third position of codon usage and do not change the amino-acid code. These results indicate that even the most looking polymorphism in a CFTR exon cannot be ignored as it may affect the splicing process.

On the other hand, the difference in severity of CF phenotype may result from different amounts of aberrant splicing that are modulated by the concentration of regulatory splicing factors. Phenotypic variability in the concentration of these regulatory factors among individuals and tissues may lead to variable amounts of exon skipping and consequent loss of function. In CFTR exon 9 we have identified several general regulatory splicing factors with inhibitory (SR proteins and hnRNPA1) or enhancer (TIA-1) splicing activity and a specific inhibitory splicing factor, TDP43. This RNA binding protein specifically binds to the Ugm polymorphic repeat at the 3' end of intron 8 and in this way promotes aberrant exon 9 skipping. In functional splicing assay *in vivo*, modulation of intracellular concentration of TDP43 by overexpression or antisense inhibition resulted in exclusion or inclusion of exon 9, respectively. Using an *in vitro* splicing assay, Ugm sequences promote splicing inhibition, depletion of TDP-43 from the nuclear extracts results in activation of splicing and this activation is abolished by addition of recombinant TDP-43. These results demonstrate that direct binding TDP-43 to the UG repeats in the CFTR intron 8 is responsible of CFTR exon 9 splicing inhibition.

The study of the basic mechanisms involved in the splicing recognition of CFTR exons with the identification of regulatory splicing factors and critical *cis*-acting RNA elements, is of utmost importance in developing innovative therapeutic strategies to correct aberrant splicing

S1.3 Gene Expression Profiles in Cellular Models of Cystic Fibrosis

Luka A. Clarke[1], Carla Braz[1] and Margarida D. Amaral[1,2]

[1]Department of Chemistry & Biochemistry, Faculty of Sciences, University of Lisboa, Portugal
[2]Centre of Human Genetics, National Institute of Health, Lisboa, Portugal

Our aim is to identify marker genes showing robust differential expression across a range of human Cystic Fibrosis (CF) cell lines and native tissues.
In preliminary experiments we have used small-scale "human starter" microarrays (MWG-Biotech, Germany) containing 400 oligonucleotide probes for 160 genes, to measure differential expression in three pairs of human cell lines with one member of each pair derived from CF tissue: CFTE-14/HBE-16 (tracheal/bronchial epithelial), 6CF-SME/Calu-3 (bronchial submucosal epithelial) and CFPAC/CAPAN (pancreatic duct epithelial).
Total RNA was extracted from the cells and double-stranded DNA was transcribed using an oligo d(T) primer incorporating a T7 promoter site, which was then used for amplification of cRNAs incorporating Cy3 and Cy5 fluorochrome-labelled UTPs. Cy3/5-labelled cRNA pairs were then mixed (1:1) and hybridized to starter arrays. For each cell line pair, six arrays were used (CF cell line-Cy3/non CF cell line-Cy5: 3 arrays, CF cell line-Cy5/non CF cell line-Cy3: 3 arrays; *i.e.*, the experiments were performed in triplicate with a colour swop), and statistical analysis of mean log ratios of fluorescence intensities was undertaken with a Bayesian method using the Normal distribution.
Several significantly regulated genes were identified for each paired CF cell line, some with documented roles in CF pathology. In summary, 1) in CFTE-14 vs. 16-HBE, 8 genes were up- and 5 genes down-regulated, 2) in 6CF-SME vs. Calu-3, 12 genes were up- and 13 genes down-regulated, and 3) in CFPAC vs. CAPAN, 15 genes were up- and 7 genes down-regulated. None of the genes were common to all three pairs, but several regulated genes were shared by two cell line pairs, including some involved in cell proliferation, DNA replication, lung inflammation, cytoskeletal development, lipid metabolism, intracellular signaling, glutathione metabolism and protein synthesis.
We are currently expanding our data by using whole genome 40K microarrays (MWG-Biotech) to analyze changes in gene expression in the following pairs of cells: 1) CFBE-41o/16-HBE (both bronchial epithelial), 2) 6CF-SME/Calu-3 and 3) pooled nasal epithelial cell samples from F508del-homozygous CF patients and age/sex matched non-CF controls.
Ultimately we hope to generate a short (~10-20) list of very consistent, strongly regulated genes that may be relevant to CF pathophysiology for use as reliable genetic markers for CF. These will ultimately be used to design oligonucleotide probes for a novel magnetoresistive sensor-based microchip array for high throughput CF diagnosis.

S1.4 Screening of CFTR Gene Rearrangements in French and Italian CF Patients

Cristina Bombieri[1], O. Raguénès[2], M. P. Audrézet[2] , A. Bonizzato[3] , C.Castellani[3], J.M.Chen[2], Pier Franco Pignatti[1] and Claude Férec[2]

[1]Section of Biology and Genetics, Univ. of Verona, Italy
[2]Inserm Unit 613, University Hospital, Brest, France
[3]CF Center of Verona, Verona, Italy.Verona, Italy

Despite the extensive efforts over the past years, a significant percentage of CF alleles still bear undetected CFTR mutations. The more than 1000 CFTR mutations identified so far are mainly single nucleotide substitutions, microdeletions and microinsertions. By contrast, only a few large deletions have been found so far in the CFTR gene. For this reason, it has been suggested that genomic rearrangements could account for a significant percentage of the unidentified disease alleles. These mutations, if present at the heterozygous state, elude the conventional PCR-based screening methodologies commonly used, including direct sequencing, and denaturing high performance liquid chromatography (DHPLC). This leads to an underestimation of gross gene mutations in CF, also limiting the study of the mechanisms underlying large genomic rearrangements involving the CFTR gene.

A systematic screening for large rearrangements of the CFTR gene was performed in French and Italian CF patients using quantitative multiplex PCR amplification of short fluorescent fragments (QMPSF). We analysed two well-characterised cohorts of 39 French and 25 Italian CF patients: all had classical CF and carried at least one unidentified allele after complete CFTR gene screening by DGGE or DHPLC analysis. A first study was performed on the French cohort of CF patients: 8 different rearrangements of the CFTR gene in 8/49 of the previously unidentified alleles (17%) were identified and characterised: 5 of them were novel mutations (Hum Mut 23:343,2004).

To confirm these results, we enlarged the study on a second cohort of Italian patients. Two rearrangements were identified and characterised in 5/26 of the previous unidentified Italian alleles (19%): an ins/del mutation involving exon 1, previously described in the French cohort, was found in 2 alleles, and a deletion spanning the exons 17a to 18 (3120+1Kbdel8.6Kb), previously described in Palestinian CF patients of different ethnic subgroups, was found in 3 alleles. Moreover, the possible deletion of exon 3 and the possible deletion of exon 11 were found in 2 more alleles: at present the characterization of these mutations is going on.

These studies represent a further significant step in understanding the molecular pathogenesis of the disease and have revealed amazing new aspects of the diverse mechanisms underlying large genomic rearrangements that cause human disease.

S1.5 Levels of Normal CFTR Transcripts in 5T and G576A Carriers and Patients

Anabela S Ramalho[1], Celeste Barreto[2], Herculano Rocha[3], Virgílio Serra[3], Deborah Penque[1], Paula Pacheco[1], Pedro Loureiro[1], Marcus Mall[4] and Margarida D Amaral[1,5]

[1]Centre of Human Genetics, National Institute of Health, Lisboa, Portugal
[2]Hospital de Santa Maria, Lisboa, Portugal
[3]Hospital Maria Pia, Porto, Portugal
[4]Universitäts-Kinderklinik Freiburg, Freiburg, Germany
[5]Dept Chemistry and Biochemistry, Faculty of Science, University of Lisboa, Portugal

Previous studies in patients carrying mutations that lead to a drastic reduction in the level of normal CFTR transcripts (included in class I, previously class V) showed that 5-8% of normal CFTR mRNA is sufficient to confer a milder clinical CF phenotype (1,2). For the design of CF therapies aiming to restore lung function by increasing the level of normal CFTR (gene therapy, namely), it is important to determine the level of normal CFTR transcripts that is sufficient to completely avoid CF. The objective of the current study is thus to determine such levels in individuals who carry polymorphisms/mutations that cause reductions in the level of normal CFTR transcript, but who do not have CF or have a borderline phenotype.

We quantitatively analysed CFTR transcripts from nasal and colonic epithelia from a group of non-CF individuals and CF patients carrying polymorphisms/gene variants resulting in the skipping of exons 9 or 12, namely: T5 in intron 8 (n=4) or the missense mutation G576A (n=5), respectively (3,4). Total RNA was extracted and RT-PCR amplification performed in the region of exons 8-10 with one Fam-labelled primer as before (2). For RT-PCR in the region of exons 10-13, we used primer C16B (5'-TTTCCTGGATTATGCCTGGCAC-3') and Fam-labelled B2L described elsewhere (5). RT-PCR products were analysed by a previously described sensitive and reproducible method (2). Transepithelial cAMP- and carbachol-induced Cl^- secretion was also determined in rectal biopsies from some of these patients, using a perfused micro Ussing chamber as described (6).

As expected, the percentage of exon 9^- transcripts resulting from the T5 allele is much higher than from the F508del allele. The T5 polymorphism in cis with I148N (exon 4) mutation seems to attenuate exon 9 skipping, but the resulting protein appears to be dysfunctional, as determined by Ussing chamber measurements. In a patient with T5 in cis with S1235R, the percentage of full-length CFTR transcripts is about 6%, whereas in a T5 carrier (parent of a CF child) it is ~10 %. The latter figure should be very close to the threshold of normal CFTR transcripts level necessary and sufficient to avoid CF.

References:
1. Highsmith *et al* (1994) *N Engl J Med* **331**, 974-980.
2. Ramalho *et al* (2002) *Am J Respir Cell Mol Biol* **27**, 619-627.
3. Chu *et al* (1991) *EMBO J* **10** 1355-1363.
4. Pagani *et al* (2003) *Human Molecular Genetics* **12**, 1111-1120.
5. Chalkey *et al* (1991) *J Med Genet* **28**, 777-780.
6. Mall *et al* (2004) *J Cystic Fibrosis* **3** S1. In press.

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Symposium 2 – Novel Therapeutic Strategies (Pharmacology)

Chair: Martin Hug Freiburg (Germany)

S2.1 Approaches to Pharmacological Treatment of Cystic Fibrosis

Godfried M. Roomans[1], Benjamin Gaston[2], Zhanna Servetnyk[1], Inna Kozlova[1] and Anca Dragomir[1]

[1]Department of Medical Cell Biology, University of Uppsala, Uppsala, Sweden
[2]Department of Pediatrics, University of Virginia School of Medicine, Charlottesville, VA, USA

Several pharmacological strategies are currently used to overcome the ion transport defect in cystic fibrosis (CF). One strategy is to inhibit the breakdown of F508del-CFTR by interfering with the folding of CFTR. It is also possible to stimulate CFTR or its mutated forms with e.g., xanthines, genistein, and various other compounds. We have recently shown that genistein, already at concentrations below 10 μM, can evoke Cl^- efflux in CF airway epithelial cells. Many potential activators are now being found as a result of high throughput processes. Some clinical tests have been done on 4PBA, S-nitrosoglutathione (GSNO) and genistein and it appears that these drugs are reasonably well tolerated, but their efficiency in treatment of CF has not yet been demonstrated. An alternative strategy is to compensate for the defective Cl^- transport by CFTR by stimulation of other Cl^- channels. Another alternative strategy is to attempt to maintain hydration of the airway mucus by inhibiting Na^+ uptake by the epithelial Na^+-channel (ENaC) using amiloride (-analogues).

We are currently investigating GSNO, an endogenous NO donor, levels of which are decreased in the airway of CF patients. GSNO has previously been shown to increase maturation of F508del-CFTR. The effect of GSNO on mutated CFTR was investigated in the bronchial epithelial cell line CFBE and in the submucosal airway gland cell line CFSME. Immunocytochemistry and confocal microscopy of CFTR showed that GSNO treatment (60 μM for 4 hours, with changes of GSNO every 30 min) increased the amount of apically localized protein. X-ray microanalysis showed that GSNO treatment significantly increased both basal Cl^- efflux and efflux in the presence of forskolin/IBMX. Studies with the fluorescent dye MQAE showed that GSNO treatment significantly increased cAMP-dependent efflux both in CFBE cells and in CFSME cells. After GSNO treatment, basal efflux increased proportionally with cAMP-activated Cl^- efflux. Basal efflux was sensitive to both DIDS (unspecific blocker of Cl^- channels other than CFTR) and glibenclamide (specific blocker of CFTR), consistent with the idea that GSNO can both correct the trafficking defect and activate F508del-CFTR. Hence, GSNO is an interesting candidate for pharmacological treatment of CF.

We have also investigated the effect of colchicine on CF airway epithelial cells, since treatment with this drug has been reported to have beneficial effects in CF patients. Colchicine-resistant cells were selected by growing in medium containing nanomolar concentrations of the drug. Colchicine-resistant clones had higher expression of multidrug resistance proteins compared to untreated cells. CFTR labeling by immunocytochemistry showed no significant changes. Intracellular Cl^- concentration and basal efflux in the colchicine-treated CF cells increased significantly compared with the untreated cells, while the cAMP-stimulated Cl^- efflux was unchanged. This suggests that colchicine promotes Cl^- efflux via alternative channels.

Finally, methods for collection and analysis of airway surface fluid from mouse airways were developed, in order to have a test system for animal experiments. Preliminary experiments provide proof of principle that the ionic composition of the ASL can be affected by pharmacological treatment.

S2.2 Development of a Drug Therapy to Correct CFTR Dysfunction

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The overall objective is to develop a drug therapy for CF targeted at the basic defect. Drug treatment is a proven route for therapy for many other diseases and the potential for oral administration means that it would target all affected CF cells. Our work suggests that to treat CF airways and lung disease it is necessary to correct the underlying submucosal glands that are inaccessible to nebulised treatments.
The aim is to identify pharmacological agents that move F508del-CFTR to the membrane in native cells from CF patients and activate CFTR Cl- transport and protein secretion function in CF gland cells.
Nasal epithelial cells, obtained by brushing from CF individuals, were used to determine CFTR location by immunofluorescence and confocal imaging. CFTR-mediated mucin secretion was measured by pulse–chase labelling of submandibular acini and measurement of release of ^{3}H-labelled mucins. CFTR Cl^- transport was measured using an ^{125}I efflux assay.
We have discovered two classes of drugs that correct the CFTR defect in native CF cells. MPB compounds which activate CFTR and move F508del-CFTR to the apical membrane [1] and PDE5 inhibitors which correct the CFTR-mediated protein secretion defect [2] and increase F508del-CFTR trafficking in nasal epithelial cells from CF patients. PDE5 inhibitor analogues were used to investigate whether Cl^- transport and protein secretion functions of CFTR are linked. Data showed that all nine analogues increased wild type CFTR mediated protein secretion (192% basal, $p<0.05$), only five increased CFTR-mediated Cl^- transport (1.7 to 3.3-fold). The studies suggest that CFTR regulates protein secretion by a direct action on exocytosis, not necessarily requiring CFTR Cl^- channel activation.
Our studies have shown that both F508del/F508del and single copy F508del CF individuals have mislocalised CFTR. Thus for eight F508del/F508del individuals 74±5 % of cells showed abnormally located CFTR, near to the nucleus and 9±1 % apical CFTR compared to six F508del/X CF individuals having 77±9 % of cells with near nuclear CFTR and 5±2 % apical CFTR. The results indicate that one copy of F508del-CFTR interferes with the trafficking of another mutant CFTR or that there is unequal expression of the two forms.
The most striking results towards development of a drug treatment for CF patients have shown that the PDE5 inhibitor sildenafil (Viagra®) had a dramatic effect in increasing trafficking of F508del-CFTR in F508del/F508del and F508del/X CF individuals. Thus following sildenafil (150μM) treatment, the number of CF cells having F508del-CFTR at an apical location was increased from 10 ± 1% to 32 ± 5% ($p<0.05$) and the number having an abnormal intracellular location was decreased from 65 ± 8% to 34 ± 2% ($p<0.05$). Sildenafil treatment also resulted in the appearance of CFTR Cl^- transport in CF15 cells in response to the CFTR agonists forskolin and genistein. Sildenafil did not affect the location of other normal cell proteins, indicating selectivity of action. The data suggest direct interaction of sildenafil with F508del-CFTR causing rescue of the mutant protein. The finding that a drug in clinical use corrects F508del-CFTR location is a major step toward developing a drug therapy for CF.

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S2.3 Identification of New Drugs for the Modulation of Chloride Transport in CF. Advance in the HTS Programme

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The objective of the HTS programme is to identify small molecules that restore normal chloride transport in cells expressing cystic fibrosis (CF)-causing mutant CFTR protein. From a functional point of view, most mutations cause CF either by reducing the CFTR channel function (gating defect) or by causing a protein misfolding and, therefore, an arrest of normal protein processing with almost no CFTR protein delivered to the plasma membrane (trafficking defect). The most frequent CF mutation, F508del, displays both defects. It is believed that restoring CFTR chloride channel activity would improve the clinical conditions of CF patients. Our drug discovery programme is organized in successive steps that start at the UCSF center with the primary screening of a chemical library using a fluorescent assay. To this aim we used Fisher rat thyroid cells cotransfected with the halide-sensitive yellow fluorescent protein (YFP) and wild type or mutant CFTR. Once hits are identified, sublibraries are created and screened. To define the potency and specificity of active drugs a secondary analysis using electrophysiological methods is then done at the Gaslini laboratory on transfected cell lines and cultured human bronchial epithelia. We have screened 60,000 compounds to identify activators of wild type CFTR, 150,000 compounds for F508del potentiators (drugs able to correct the gating defect) and 150,000 compounds for F508del correctors (drugs able to correct the trafficking defect). The results of these screenings yielded seventeen potent activators of wild type CFTR, some of them acting at nanomolar concentrations. Fourteen of these compounds neither increase cAMP concentration nor inhibit cell phosphatases, suggesting a direct action on CFTR. A few of these compounds activate also G551D mutant, though with much weaker affinity. The screening also identified various chemical classes of F508del potentiators, some of them with very high potency such as tetrahydrobenzothiophene, displaying Kds as low as 60 nM. Putative correctors that seem to improve the trafficking of F508del protein are also under evaluation. A specific inhibitor of CFTR could be extremely important for studying the channel function, therefore we also screened a library of 60,000 compounds to look for inhibitors. A potent and specific CFTR inhibitor, with an affinity in the order of 300 nM, has been identified and characterized. An interesting information that is coming out from this study, is that dose-response relationships for many of the activators/potentiators are different for wild type and mutant CFTR. This information might be used to identify the regions of the CFTR protein involved in activators binding. Moreover, the different affinity of activators for wild type and mutant protein indicates that mutant-specific treatment should be proposed for CF.

We acknowledge the financial support of the Cystic Fibrosis Foundation Therapeutics, Italian Cystic Fibrosis Foundation and Telethon-Italy.

P 4

S2.4 Searching for the CFTR-Openers Binding Site

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Several mutations causing cystic fibrosis (CF) produce a severe reduction of the chloride transport by a defect on the CFTR gating. The use of substances that could activate the defective chloride channels, CFTR-openers, has been suggested as possible CF-therapy. We have studied a series of CFTR-openers identified by high throughput screening (Galietta *et al*., 2001, *J. Biol. Chem*., 19723). The apparent affinity of openers was estimated by measuring the short-circuit current in epithelia formed by Fisher Rat Thyroid stable transfected with wild type or mutant (G551D, G1349D) CFTR. Modification of the apparent affinity of CFTR-openers by mutations nucleotide binding domains (NBD's) strongly suggest that the binding site is in these regions. Thus, we undertake a modelling strategy to identifying a putative binding site in the NBD's of the CFTR. Structure of each human NBD was predicted by homology with murine NBD1 structure. To construct the NBD1-NBD2 complex, monomers where then overlaid to the conserved regions of ABC-transporters NBD's crystallised in the "head-to-tail" conformation. ATP was included in the model, in the same position as in NBD-templates. Because the murine NBD1 crystal does not form "head-to-tail" dimers, the nucleotide (ATP, ADP or PBP) binding site is completed by the first 38 residues (strand S1, and helices H1b and H1c) and the 24 last residues (helix H9b), instead of the normal NBD complementary subunit. To conserve the "head-to-tail" conformation, we had to remove these regions from the human NBD's model. Binding sites for CFTR-openers were estimated by different molecular docking strategies. Docking trials converged to 3 positions that are characterised by a low-energy interaction between ligand and receptors, where all openers accommodate. We empirically evaluated the binding free energy (ΔG_{THEOR}) for each CFTR-ligand at each candidate-binding site, in wild type and mutant models. Binding free energy was also estimated from the dissociation constants obtained experimentally (ΔG_{EXP}). Comparison of ΔG_{THEOR} and ΔG_{EXP} resulted in a remarkable good correlation coefficient ($r = 0.91$) for one of the putative sites, while a smaller correlation coefficient resulted for the other two locations ($r = 0.53$ and $r = 0.29$). The most probable binding site for CFTR-openers is located in the interface between NBD1 and NBD2. The site is a hydrophobic pocket in NBD1, where the CFTR-opener is in contact with Walker-B, Q-loop and Switch-II regions. The binding site is completed with some residues of the LSGGQ signature and Q-loop of the NBD2. None of the studied mutation positions would be directly involved to the binding site, and they probably change the CFTR-opener affinity by destabilising the neighbour residues that actually form the putative binding site.

This work was partially supported by Italian Cystic Fibrosis Foundation.

S2.5 Purinergic Inhibition of the Epithelial Na^+ Channel ENaC via Hydrolysis of PIP2

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Stimulation of purinergic receptors inhibits amiloride sensitive Na+ transport in epithelial tissues by an unknown mechanism. Since previous studies excluded a role of intracellular Ca2+ or protein kinase C, we examined if purinergic regulation of Na+ absorption occurs via hydrolysis of PIP2 and a change of ENaC activity. Inhibition of amiloride sensitive short circuit currents (Isc-Amil) by ATP in native tracheal epithelia and M1 collecting duct cells was suppressed by high affinity binding of neomycin to PIP2 and recovery from ATP - inhibition was abolished by the phosphatidylinositol-3-kinase inhibitor wortmannin. Stimulation by ATP depleted apical membranes of PIP2, which was co-immunoprecipitated with the β-subunit of ENaC. Mutations in the PIP2 binding domain of β-ENaC but not γ-ENaC decreased ENaC – currents after expression in Xenopus oocytes, without affecting surface expression. Collectively, these data supply strong evidence for a novel and physiologically relevant ENaC regulation in epithelial tissues: While membrane localization of ENaC is controlled via C – terminal regulation, N – terminal binding of β- ENaC to PIP2 determines the activity of ENaC channels. Compounds that interfere with PIP2 synthesis and thus reduce the concentration of PIP2 in the luminal membrane of airway epithelial cells may provide a new avenue for the pharmacotherapy of the ion transport defect in the airways of cystic fibrosis patients.

Symposium 3 – Models of Airway Disease

Chair: Bob Dormer, Cardiff (UK)

S3.1 ENaC-Mediated Airway Na^+ Hyperabsorption Causes Cystic-Fibrosis like Lung Disease in Mice

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Chronic lung disease remains the major factor contributing to mortality and morbidity in cystic fibrosis (CF) patients. Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene result in defective epithelial cAMP-dependent Cl^- secretion and increased airway Na^+ absorption. The mechanistic links between these altered ion transport processes and the pathogenesis of CF lung disease, however, are unclear. To test the hypothesis that accelerated Na^+ transport alone can produce CF-like lung disease, we generated mice with airway-specific overexpression of epithelial Na^+ channels (ENaC).
To achieve this goal, we used the CCSP promoter to target expression of each ENaC subunit (α, β and γ) to the mouse lower airways. Expression of transgenic ENaC subunits was evaluated by RT-PCR, *in situ* hybridization, and immunohistochemistry, and expression was found to be confined to airway epithelia. Surprisingly, overexpression of β-ENaC alone was sufficient to cause a significant increase in Na^+ transport across freshly excised airway tissues, whereas overexpression of α- or γ-ENaC had no effect on Na^+ absorption.
To determine the effect of increased Na^+ absorption on ASL homeostasis and mucus clearance, we measured the height of the periciliary liquid layer (PCL), the mucus concentration (% solids), and mucociliary clearance (MCC). We found that PCL height and MCC were significantly reduced, and % solids significantly increased, in β-ENaC overexpressing mice compared to wild-type littermate controls.
Defective mucus transport caused a severe spontaneous lung disease sharing key features with CF. β-ENaC overexpressing mice showed the expected Mendelian distribution of genotypes and normal airway histology at birth, but exhibited significant postnatal pulmonary mortality (40% survival at day 30). Histopathologic evaluation revealed marked mucus retention and goblet cell metaplasia throughout the airways of deceased β-ENaC transgenic mice, suggesting that death was caused by asphyxia as a result of severe mucus plugging. Studies on surviving β-ENaC transgenic mice demonstrated that airway Na^+ hyperabsorption and mucus obstruction was associated with sterile neutrophilic inflammation and poor bacterial clearance.
Collectively, this novel animal model demonstrates that increased airway Na^+ absorption *in vivo* causes airway surface liquid volume depletion, increased mucus concentration, delayed mucus transport, and mucus adhesion to airway surfaces. As a result, β-ENaC transgenic mice develop a severe spontaneous lung disease sharing common features with CF and chronic bronchitis. We expect that this animal model will allow an *in vivo* evaluation of various factors contributing to the complex pathophysiology of CF lung disease, and of novel therapeutic interventions in CF and possibly other chronic airway diseases characterized by airway inflammation and mucus obstruction, e.g., cigarette smoke-induced chronic bronchitis. Amongst other novel therapies, our data suggest that such strategies should include those to restore proper ASL volume to airway surfaces. Supported by DFG (MA 2081/2-1), CFF RDP and NIH SCOR

S3.2 Potential of Stem Cells for Airway Regeneration

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The airway in CF respiratory disease is compromised by epithelial damage as a consequence, predominantly, of bacterial infection and inflammatory cell invasion. Therapy of the disease has concentrated on aggressive antibiotic regimes and gene therapy delivered directly to the respiratory tract. Over the past few years, the astonishing pluripotency of adult marrow derived stem cells has been documented and their potential to mediate gene delivery and repair various organs has been considered. A major limitation is the fact that the level of contribution *in vivo* to cells in non-haematopoietic tissues is low, although this level does appear to be exacerbated by tissue damage. It is still not clear whether the phenomenon arises from truly pluripotent stem cells in the marrow, by "transdifferentiation", or by cell fusion involving myeloid cells. In this presentation, the most recent literature in this field will be examined with particular reference to the respiratory tract. In addition our own results using human cord blood cells injected into lethally irradiated SCID mice, which reveals the presence of human cells that do and do not express haematopoietic markers in the lung and no evidence of cell fusion will be presented. We have also analysed lungs from irradiated mice transplanted with genetically marked donor cells and report the level of donor cells in the airway. The majority of these express the universal haematopoietic cell marker CD45. The lack of ability of marrow SP cells to contribute *in vitro,* ex *vivo* or *in vivo* to the respiratory tract of mice will also be discussed.

S3.3 Biogenesis and Trafficking of CFTR

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The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-dependent protein kinase A (PKA)-activated chloride channel that is found on the apical surface of a number epithelial cell types. One unusual feature of CFTR is that during biogenesis as much as 75% of the wild type protein is degraded by the endoplasmic reticulum (ER)-associated degradative (ERAD) pathway, implying that CFTR is intrinsically unstable. F508del, the most common mutation in the CFTR gene, results in a protein-folding defect and the mutant protein is completely degraded by ERAD. Previous studies on CFTR maturation utilized heterologous, over-expression systems because of the limited amounts of CFTR synthesized endogenously in epithelia. In the studies presented here, we show that in an airway epithelial cell line, Calu-3, and in a colonic epithelial cell line, T84, all newly synthesized wild-type CFTR is converted to the maturely glycosylated form. Further, we show that inhibition of the proteasome by ALLN stabilizes CFTR B band in heterologous cells, but has no effect on B band in Calu-3 and T84 cells, suggesting that the proteasome plays no role in degradation of newly synthesized wild type CFTR in these epithelial cells. Wild type CFTR maturation appears to be the same under both polarized and non-polarized conditions in Calu-3 cells. We also examined CFTR trafficking at the cell surface and found that wild type CFTR internalization is dramatically slower in polarized Calu-3 cells compared to non-polarized Calu-3 and HeLa cells. Further, disruption of the actin cytoskeleton enhances wild type CFTR internalization in polarized Calu-3 cells, suggesting that there is a tethered pool of CFTR at the apical surface. Comparison of wild type and F508del CFTR endocytosis and recycling in HeLa cells suggests that F508del CFTR internalizes at the same rate as the wild type protein, but fails to recycle efficiently. Our results illustrate three points. First, wild type CFTR biogenesis is efficient in Calu-3 and T84 cells and we predict this will be true for other epithelial cell types that endogenously express CFTR. Two, the surface pool of CFTR is more stable at the apical surface than might be expected; suggesting that part of the surface pool is tethered to the actin cytoskeleton. And third, the defect in the F508del-CFTR is due to a failure to recycle efficiently, suggesting that F508del has lost the ability to interact with a protein in the recycling pathway (loss of function) or now interacts with factors that prevent recycling (gain of function). Currently we are examining these possibilities using a proteomics approach.

P 5

S3.4 Characterization of a New Polarized Epithelial Cell Model to Study the Cystic Fibrosis Transmembrane Conductance Regulator

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The majority of studies on the localization, trafficking and recycling of CFTR are performed in non-epithelial or non-polarized epithelial cells. However, polarized and non-polarized epithelial cells possess distinctly organized and regulated membrane trafficking pathways and thus, the choice of an appropriate polarized cell system is critical for the investigation of CFTR trafficking pathways. Since an adequate polarized human airway epithelial cellular model expressing mutant CFTR is not currently available, we have established and are currently characterizing a new cell model: Madin-Darby canine kidney type II (MDCK II) cells stably expressing wild-type (wt) or mutant CFTR proteins. MDCK II cells, that do not express CFTR endogenously, form a highly polarized monolayer of epithelial cells when growing on filters and have been widely used to study membrane traffic.

We have developed stable MDCK II cell lines expressing human wt-CFTR or F508del-CFTR. MDCK II cells were infected with lentiviral vectors containing the human wild-type or F508del-CFTR cDNA as described (1, 2). By RT-PCR with 2 pairs of primers (mammalian- or human-specific), we detect CFTR transcripts in the 3 cell lines tested: the parental MDCK II, MDCK wt-CFTR and MDCK F508del-CFTR. Sequencing of the PCR products revealed that in the transfected cell lines CFTR transcripts detected were of human origin, whereas in the parental line CFTR transcripts detected (in very low level) corresponded to the endogenous expression of canine CFTR. Western blot analysis combined with digestion with glycosidases showed that wt-CFTR expressed in these cells is fully glycosylated, as demonstrated by the typical 2 band pattern (the mature glycosylated band C and the core-glycosylated band B) whereas F508del-CFTR is only detected as an immature form (band B). No CFTR protein could be detected in MDCK parental cells by Western blot. When these cell lines were grown on filter inserts, allowing polarization (typically TER $\geq$600 $\Omega.cm^2$), expression levels of wt- and F508del-CFTR increased when compared to non-polarized cells.

We are further characterizing these new cell lines by immunofluorescence studies and by iodide efflux assays to measure CFTR Cl^- channel activity.

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Symposium 4 – Cell Physiology

Chair: David N. Sheppard, Bristol (UK)

S4.1 - CFTR Activators

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CFTR is a chloride channel activated through the classical cAMP-protein kinase A (PKA) pathway. In the past ten years, evidence has emerged that the cAMP-dependent CFTR activity can be further enhanced by a large group of compounds with little structural similarities. These compounds include isoflavonoids (e.g., genistein), benzimidazolone analogs (e.g., NS004), xathines (e.g., IBMX), and pungent alkaloids (e.g., capsaicin). Among them, genistein is considered as a prototype since extensive studies of how it acts on CFTR have been reported. We showed that genistein does not activate CFTR in the absence of cAMP stimulation and that the magnitude of enhancement is inversely proportional to the open probability (*Po*) of CFTR. This latter property of genistein makes it an ideal candidate for future drug development in cystic fibrosis. Indeed, the activity of some disease-associated CFTR mutants (e.g., G551D, ΔF508) can be drastically enhanced by genistein. All these CFTR activators seem to act through a common mechanism since single-channel kinetic studies show similar effects of these compounds on CFTR gating, i.e., an increase of the opening rate and a decrease of the closing rate. Recently, we found that capsaicin, the major pungent ingredient in chili pepper, may act as a partial agonist for genistein. At a saturating concentration, capsaicin's enhancement effect on CFTR is ~60% of that elicited by genistein. The maximally enhanced CFTR currents with genistein are partially inhibited by capsaicin. The molecular target for the CFTR activators may be the CFTR protein itself since these chemicals can potentiate PKA-activated CFTR currents in excised inside-out membrane patches. However, the binding site for CFTR activators may reside in the cytoplasmic domains of CFTR since they act preferentially from the cytoplasmic side of the membrane. The fact that these chemicals also potentiate a CFTR mutant whose regulatory (R) domain is completely removed indicates that the binding site is not located in the R domain. The enormous structural diversity of CFTR activators suggest that "the binding site" for these compounds should not be considered as a well-defined binding pocket. We propose that binding of the CFTR activators at the NBD1/NBD2 dimer interface could explain both the structural diversity and their dual effects on opening and closing steps of the gating transition.

S4.2 Acidic pH Potentiates the Activity of wild-type CFTR, but not the CF-Associated Mutants G551D and G1349D

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Gating of the cystic fibrosis transmembrane conductance regulator (CFTR) is primarily controlled by ATP binding and hydrolysis at two nucleotide-binding domains (NBDs). We previously demonstrated that acidic intracellular pH (pHi) alters CFTR gating to increase open probability (Po). To investigate this effect of acidic pHi, we studied wild-type and mutant CFTR Cl- channels using excised inside-out membrane patches. The pipette (external) solution contained 10 mM Cl^- at pH 7.3. The bath (internal) solution contained 147 mM Cl^-, PKA (75 nM) and ATP (0.03-10 mM) at 37°C. To adjust the bath solution to pH 8.3 and pH 6.3, we used Tris and H_2SO_4, respectively. To ensure that [MgATP]i was identical at each pHi, different amounts of ATP, calculated using the Bound-and-Determined computer program, were added to each pHi solution.

At each pHi, the relationship between Po and [MgATP]i (n = 6-7) was best fit by a Michaelis-Menten function. However, at pH 6.3 the Km value was decreased compared with that at pH 7.3 (pH 6.3, Km = 36 μM; pH 7.3, Km = 90 μM), while the maximum Po (Pomax) was increased (pH 6.3, Pomax = 0.71; pH 7.3, Pomax = 0.61). These data suggest that acidic pHi might alter both the ATP affinity and ATPase activity of the NBDs. To study the effects of intracellular pH on ATP binding, we used Mg^{2+}-free solutions that abolish ATP hydrolysis. Under these conditions, Po was greatly decreased at pH 7.3, but less reduced at pH 6.3 ($n = 5$-6; $p < 0.05$). Moreover, acidic pHi failed to enhance the activity of the CFTR mutant K1250M, which disrupts the ATPase function of NBD2 (pH 7.3, Po = 0.32 ± 0.05; pH 6.3, Po = 0.21 ± 0.02; $n = 5$; $p < 0.05$). These data suggest that acidic pHi might enhance channel gating by promoting ATP binding to the NBDs. To better understand this mechanism, we investigated the role of the LSGGQ motifs in each NBD using the CF-associated mutants G551D and G1349D, which interfere with ATP binding. At pH 6.3, both G551D and G1349D failed to enhance CFTR activity (G551D, pH 7.3, NPo = 0.029 ± 0.009; pH 6.3, NPo = 0.021 ± 0.006; n = 6; G1349D, pH 7.3, NPo = 0.096 ± 0.040; pH 6.3, NPo = 0.095 ± 0.037; n = 5). These data suggest that the potentiation of CFTR activity at acidic pHi involves ATP binding and that the LSGGQ motifs participate in this effect. We speculate that the ATP binding sites of CFTR likely contain pH-sensitive amino acid residues.

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S4.3 Novel Regulation of CFTR by External Chloride

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We have previously shown using fast whole cell patch clamp recordings (fWCR) that raising extracellular HCO3- inhibits CFTR currents in native guinea-pig pancreatic duct cells (1). Our data showed that increasing the concentration of HCO3- in the luminal fluid caused an inhibition of both inward and outward CFTR currents. Inhibition of outward current (Cl- influx) was expected as external [Cl-] was replaced by the less permeant HCO3- ion. However, the inhibition of inward current (Cl- efflux) was surprising as the intracellular Cl- concentration should remain constant because of the large Cl- reservoir in the pipette, and thus currents would not have been predicted to alter from simple theories of ion flow through channels. Thus replacing external Cl- with HCO3- resulted in 'trans-inhibition' of Cl- efflux through CFTR. The inhibitory effect of HCO3- was concentration-dependent and about 70% of the CFTR conductance was inhibited in the presence of 140 mM HCO3-. CFTR inhibition was not caused by changes in either extracellular or intracellular pH, or in either the pCO2 or CO32- content of the HCO3- rich solutions. We proposed that this effect of external HCO3- on CFTR was a novel, negative feedback mechanism, for the control of HCO3- secretion in the pancreas (1). We have now extended these studies to investigate whether this effect is also observed with human CFTR and the anion-dependence of the effect. All experiments were performed on CHO cells stably expressing wild type human CFTR using fast and slow whole cell patch clamp current recordings. The replacement of extracellular Cl- with equimolar HCO3-, NO3- , Gluconate- Aspartate- or Br- resulted in the equal inhibition of the outward and inward currents through CFTR. Identical inhibition was observed with iso-osmolar mannitol replacement, suggesting a change in the extracellular [Cl-] alone is sufficient to produce the inhibitory effect. Comparable results were obtained using slow WCR, however the concentration response curve was significantly different over a range of external [Cl-]. For example when extracellular Cl- was lowered from 155.5 mM to 116.5, 91.5 or 71.5 mM (mannitol replacement) the mean percent inhibition of inward current during fast WCR were 37 ± 3.6, 44 ± 8.7 and 59 ± 7.6, respectively (n=4-6) compared to 12 ± 4.4, 7 ±1.6 and 24 ± 2.3, respectively, during slow WCR (n=5), implicating a cytosolic factor in the inhibitory response. Increasing the internal [Ca2+] from the control value of 1 nM to 100 nM during fast WCR reduced the degree of inhibition to levels comparable with slow WCR. The mean % inhibition of the inward current was 9 ± 8, 24 ± 3.5 and 31 ± 5 at 116.5, 91.5 and 71.5 mM external [Cl-], respectively (n=4-7). The addition of 1 mM bromotetramisole (a protein phosphatase inhibitor) to the pipette solution during fast WCR also alleviated the block to levels comparable with slow WCR, 29 ± 8.3 (n=4) at 71.5 mM Cl-. Combining increased [Ca2+] and bromotetramisole had no additional inhibitory effect. These data suggest that intracellular [Ca2+] and the phosphorylation state of CFTR can modulate the inhibitory effect of extracellular Cl- replacement on Cl- efflux through CFTR. Physiologically, we propose that inhibition of CFTR occurs as luminal HCO3- concentration rises, and that this mechanism has a key role to play in regulating epithelial HCO3- and fluid secretion. It may also be important generally in the regulation of the pH at epithelial surfaces.

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S4.4 Bicarbonate and Proton Secretion by Airway Serous Cells

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Airway serous cells secrete electrolytes, peptides and mucins upon stimulation with cholinergic and ß-adrenergic agonists. Bicarbonate (HCO_3^-) ions, and protons (H^+ ions) are transported during secretion and thereby influence mucus composition. In our present study we have addressed the question, which transporters are responsible for the extrusion of HCO_3^- and H^+ ions by airway serous cells. To functionally understand transport of these ions we performed microfluorimetric measurements of the intracellular pH (pH_i) using the serous cell line Calu-3 and isolated porcine submucosal gland (pSMG) cells. For the investigation of HCO_3^- exit, we exposed Calu-3 or pSMG cells to gradients for Cl^- and HCO_3^- and measured respective changes in pH_i. Replacement of extracellular Cl^- by gluconate in the presence of HCO_3^- resulted in a modest increase in pH_i suggesting the presence of a Cl^-/ HCO_3^- exchanger. Stimulation with Forskolin decreased pH_i. However, when Cl^- was replaced in the presence of Forskolin, a marked alkalinization was observed that could not be inhibited by a classical inhibitor of anion exchangers, DIDS. In contrast, addition of Glibenclamide, an inhibitor of the CFTR Cl^- conductance, abolished the alkalinization induced by Cl^- replacement. To study H^+ transport mechanisms cells were acidified by loading the cells with NH_4^+ . Using this approach we have previously demonstrated that Calu-3 and pSMG cells utilize a Na^+ dependent H^+ exchanger that could be inhibited with the compound HOE694, a selective Blocker of NHE-1. However, even in the complete absence of Na^+ or at maximal concentrations of HOE694 a partial recovery of pH_i after acid load could still be observed. Equimolar replacement of extracellular Na^+ by K^+ resulted in a modest increase in the rate of pH_i recovery after acid load suggesting the presence of a K^+ dependent exchanger. The effect of high extracellular K^+ could not be mimicked by depolarization of the membrane using Ba^{2+}. RT-PCR experiments using isoform specific primer sets detected mRNA for the non-gastric (kidney) but not the gastric isoform of the H^+/K^+ ATPase in Calu-3 cells and 16-HBE human bronchial epithelial cells. In a further series cells were cultured on permeable supports and exposed to an antibody against the N-terminal region of the non-gastric isoform of the H^+/K^+ ATPase. Calu-3 cells showed clear immunolocalization of this protein in the apical and lateral membranes. In 16-HBE cells staining for H^+/K^+ ATPase was predominantly detected in the lateral membranes.
We conclude that airway epithelial cells utilize a non-gastric ATPase for H^+ ion secretion. HCO_3^- extrusion is stimulated by Forskolin and requires the activity of CFTR. The parallel assembly of CFTR with the H^+/K^+ ATPase could serve the purpose of pH-neutral electrolyte secretion.

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S4.5 Is CFTR a Regulator or is any Chloride Channel a Regulator of ENAC?

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The autosomal recessive genetic disease cystic fibrosis (CF) is characterized by thick mucus in the airways and progressive destruction of the lung. Clearly, in certain CF epithelia, like the pancreas, salt and fluid secretion are severely reduced, whereas it is well established for the CF sweat gland that salt absorption is reduced, due to a reduced chloride permeability.

The cause of the thick viscous mucus in CF airways is less clear. A popular hypothesis is that, contrary to the sweat duct, an increased absorption (hyperabsorption) of salt and water in CF airways leads to dehydrated mucus. How did this theory evolve? Amiloride, the specific inhibitor of the epithelial sodium channel (ENaC), showed an increased potential shift in CF airway tissue compared to healthy airways. From these potential measurements it was concluded that in CF airways ENaC is more active and that sodium (chloride) absorption must be increased. However, it has to be pointed out that from potential measurements no straightforward conclusions may be drawn about ion transport.

Cloning of the gene that is causing CF by positional cloning identified the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) as being defective or lacking in CF. Overwhelming evidence proved that CFTR is an anion channel that is activated by protein kinases. The chloride channel function of CFTR could nicely explain the earlier observations in CF sweat duct but provided no explanation for the postulated hyperabsorption of sodium in CF airways.

An explanation for apparently increased sodium absorption in CF airways was put forward when heterologous expression experiments with coexpressed CFTR and ENaC seemed to indicate that CFTR is inhibiting ENaC whereas defective CFTR had no effect on ENaC. Recent experiments in our three independent laboratories however showed that these results could be seriously flawed by neglecting the influence of a series resistance. No effect of CFTR conductance on ENaC activity could be observed when we minimized series resistance. Only if a series resistance was limiting the measured conductance, ENaC conductance seemed reduced upon activation of CFTR. Not surprisingly, apparent ENaC inhibition is less pronounced if CFTR mutants with reduced conductance are tested upon their effect on ENaC under these conditions.

Recently it was suggested that CFTR and other chloride channels may inhibit ENaC by an increase of cytoplasmic chloride concentration. Because some of these results were derived from potential measurements, it is important to emphasize that potential measurements can only give indirect clues on transport and that changes in membrane conductance for one ion may change voltage effects of an unrelated ion conductance. In well-controlled excised-patch experiments on *Xenopus* oocyes, we indeed found no influence of cytoplasmic chloride concentration on ENaC.

Consistent with some *in vivo* measurements performed in lung, we propose that absorption of salt in that tissue simply results from apical sodium and chloride conductances, both being able to limit absorption of the other. From that perspective, neither CFTR nor other chloride channels deserve to be called regulators of ENaC.

S4.6 ND PK AMPK CFTR - What Are They Doing Together?

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The histidine phosphorylated form of the ubiquitous enzyme nucleoside diphosphate kinase (NDPK) can phosphorylate dinucleotides (such as GDP) into trinucleotides (eg GTP). NDPK phosphotransferase activity can add high energy phosphate to GDP whilst the latter is bound to some G proteins but also undertakes a related role in dynamin-mediated endocytosis by providing high local concentrations of GTP to that protein. NDPK is also a protein histidine kinase controlling DNA repair and inter alia, intermediary fatty acid metabolism.

- We have previously shown that at the apical membrane of airway epithelia, the *ex vivo* phosphorylation of nucleoside diphosphate kinase (NDP kinase, NDPK) is stimulated by $[Cl^-]$ but inhibited by $[Na^+]$. We now show that NDPK regulation fails in CF and involves a second protein kinase, AMPK activated kinase. AMPK is a regulator of CFTR as proposed by Foskett and Hallows.
- In cytosol-free membranes from mouse tracheal epithelium and human bronchial epithelial cell lines where CFTR is mutated or missing, we observe CFTR mutation-dependent, impaired NDP kinase phosphorylation associated with attenuation of membrane-localised dinucleotide phosphotransferase activity. This suggests that NDPK can be regulated in a membrane environment.
- We show that the catalytic subunit of the cellular metabolic energy sensor AMP-activated protein kinase (AMPKα) binds to and regulates NDPK (H1-isoform) histidine phosphorylation. AMPK inhibits CFTR, as shown by Hallows and Foskett, and we dissect the significance of the AMPK-NDPK interaction in wild-type and CF human bronchial epithelial cell lines (16HBE14o-, CFBE41o- respectively gift of Dieter Gruenert) by pharmacologically activating AMPK and measuring phosphotransferase activity of the apical membrane.
- We applied 5-aminoimidazole-4-carboxamide-1β-D-ribofuranoside (AICAR, 0.6 mM), a cell permeant activator of AMPK that generates ZMP, an analogue of AMP. We found that AICAR results in a time-dependent increase in both NDP kinase A phosphorylation and phosphotransferase activity in the wild type but not CF cells. Since ZMP can have non-AMPK actions, we specified the findings by using metformin, a recently described activator of AMPK that acts by a ZMP-independent pathway. Metformin also resulted in increased phosphotransferase activity following AMPK activation in wild-type but not CF cells.

Overall, our results suggest that NDPK and AMPKα form a functional complex in wild type but not CF cells. To study this further we also show that AMPK and NDPK fail to co-localise in murine CF tissues and that CF null mouse tracheas show excess NDPK protein (but not mRNA). This suggests that in CF, dephosphorylated NDPK accumulates which could disrupt its normal roles in regulation. We propose that CFTR could form a platform for the interaction between NDPK and AMPK thereby conferring AMPK-dependent regulation of NDPK.

This work was supported in part by the CF Trust, Wellcome Trust, and Anonymous Trust. The work in Cambridge was supported by the MRC and the CF Trust.

Symposium 5 – CFTR Structure, Folding & Processing

Chair: Margarida D Amaral, Lisboa (Portugal)

S5.1 The Structure of CFTR Nucleotide Binding Domain 1

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Human CFTR is a 1480 residue, multidomain, integral membrane protein that regulates chloride ion flow across the cell membrane. It is a member of the ATP-binding cassette (ABC) transporter family of proteins and consists of two membrane spanning domains (MSDs), two nucleotide-binding domains (NBDs), and a regulatory region (R) arranged in the order MSD1-NBD1-R-MSD2-NBD2[1]. The most common mutation in CFTR resulting in cystic fibrosis is deletion of phenylalanine 508 (F508del), which is located within the first nucleotide-binding domain (NBD1). A better understanding of the structure and function of NBD1 and the role of Phe508 may accelerate development of new approaches for the treatment of CF. We have undertaken the crystallographic structure determination of NBD1 to better understand its role in the function of CFTR, its phosphorylation and ATP binding properties, and functional effects of disease-causing mutations, such as F508del.

Given the uncertainty in the globular domain definition of CFTR NBD1, we chose to clone and express in *E. coli* many constructs in parallel covering residues from 363 to 686, and test these truncated forms of the protein for solubility. Concurrently, a pan-genomic approach was employed by working on NBD1s from ten organisms in parallel. High-level production of soluble proteins (>5mg/L) that are well behaved in solution was limited to a narrow globular domain definition. Optimal recombinant protein was obtained from one CFTR construct and this protein was successfully crystallized and its structure determined using x-ray crystallography [2].

As expected from sequence conservation, the structure of NBD1 resembles that of other ABC transporter NBDs. However, significant differences in NBD1 were also seen, including a loop insertion at the N-terminus of the domain. Interestingly, unhydrolysed ATP, bound to magnesium, was observed in the structure. While conventional interactions of NBD1 (Walker A, Walker B, etc.) were observed with the ATP phosphate groups[3], atypical packing was observed for the adenine base. Stacking by a conserved aromatic side chain, as observed in most other ABC structures, is not observed in the NBD1 structure. Rather, interaction with an aromatic reside in the N-terminal inserted loop is observed via a perpendicular π electron interaction.

The location of Phe508 is clearly observed in the structure, being exposed at the surface of the domain in a short coil region. Deletion of this residue may lead to misfolding of the protein due to steric constraints in the NBD1 fold, or a loss of critical interactions between NBD1 and the intracellular helical domain. Studies are continuing to determine the structure of NBD1 that include this important mutation as well as of the human NBD1 domain due to their greater relevance to drug discovery efforts.

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S5.2 CFTR: A Dimer with a Central Pore

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The cystic fibrosis transmembrane conductance regulator (CFTR) is a protein that belongs to the superfamily of ATP binding cassette (ABC) transporters. Dimerization of CFTR is necessary for full ion channel function although structural details of CFTR in native membrane are yet unknown. In order to identify CFTR in native plasma membrane we applied atomic force microscopy (AFM) to inside-out oriented membrane patches of CFTR-expressing Xenopus Laevis oocytes after cAMP stimulation. First, oocytes were injected with CFTR-cRNA and, three days later, voltage-clamped verifying successful CFTR expression and incorporation into the plasma membrane. Then, plasma membrane patches were isolated, placed inside-out on appropriate substrate and incubated with gold-labelled antibodies against the C-terminus of CFTR. Finally, the intracellular surface of the plasma membrane was scanned by AFM. In close vicinity to the immunogold labels we detected ring-like structures with bipartite symmetry. The substructure of the ring, formed by the extramembrane protein domains of CFTR, is consistent with the model of a CFTR dimer. Derived from AFM molecular mass analysis of the intramolecular domains we conclude that two CFTR molecules line up in parallel, tail by tail, forming a pore in its center. This molecular arrangement could represent the CFTR chloride channel configuration, operative in native plasma membrane. In a another approach, plasma membrane patches of human red blood cells were isolated, placed inside-out on appropriate substrate and incubated with QDot®-labelled antibodies against the C-terminus of CFTR. This allows identification of CFTR by fluorescence microscopy and particle counting by AFM. We found at least two populations of red blood cells in each individual: red blood cells without CFTR and red blood cells with up to 1000 copies CFTR per cell. On the basis of these observations we developed a functional blood test for the diagnosis of cystic fibrosis using hemoglobin release as the detection parameter.

S5.3 Role of Glycosylation and Calnexin in the ER Quality Control of CFTR

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A substantial amount of wt-CFTR and most F508del-CFTR are retained in the endoplasmic reticulum (ER) and undergo ER-associated degradation (ERAD) probably a consequence of partial or complete misfolding. Among the molecular chaperones proposed to play a role in the targeting of misfolded substrates to retrotranslocation and ERAD, calnexin (CNX), described to form complexes with both wt- and F508del-CFTR, has been the most often implicated (1,2). Other studies, however, have suggested that ERAD is independent of CNX or that association with CNX actually prevents ERAD (3,4).
ER glucosidases I and II sequentially remove two of the three glucose moieties present in the 14-unit glycan moiety added to glycoproteins in the ER. CNX then recognizes and interacts with monoglucosylated glycoproteins, through the resulting 12-unit glycan structure. By inhibiting glucosidases I and II, e.g. with castanospermin (CAS), glucose trimming is prevented as well as substrates recognition by CNX. In contrast, 1-deoxymannojirimycin (DMM), an inhibitor of mannosidases (also of the ER ones) prevents trimming of outer mannose residues of the Man9 moiety and thus formation of Man7-8 glycans which are less efficiently re-glucosylated by UDP-glycoprotein-glycosyltransferase (UGGT). CNX interaction of substrates is thus enhanced in the presence of DMM.
We have previously shown that, under CNX overexpression, the turnover of F508del-CFTR is significantly increased, whereas the turnover and maturation efficiency of wt-CFTR remain unchanged. Here, using those inhibitors (CAS and DMM) we looked at the effect of glucose and mannose trimming and thus of CNX binding on the turnover and processing of CFTR.
Pulse-chase assays followed by immunoprecipitation (IP) were performed in CHO cells stably expressing wt-CFTR or F508del-CFTR after treatment with CAS or DMM.
The turnover of immature wt-CFTR (band B) under CAS or DMM was found to be decreased, suggesting a role of CNX in wt-CFTR degradation. As the proportion of band B observed after treatment with these compounds (52%) was significantly higher than the proportion of total protein (band B plus mature protein, band C) observed without treatment (25%), each of these compounds must indeed prevent ERAD of wt-CFTR. The enhancing alpha-mannosidase-like protein (EDEM) is probably involved in this process as suggested by co-IP experiments. However, for F508del-CFTR although stabilization of the immature form was observed after treatment with DMM (12% after a 3h-chase vs 6% without inhibitor), no stabilization was observed under CAS. Results observed for DMM were also confirmed by usage of swainsonine and kifunensine described to specifically inhibit ER mannosidase I and II. CNX downregulation, by an RNAi approach, only impaired wt-CFTR processing, with no effect on F508del-CFTR. Studies with *in vitro* mutagenized CFTR lacking the consensus glycosylation sites are in progress.
Altogether, our results suggest that CNX-mediated ERAD occurs for both wt- and F508del-CFTR, but F508del-CFTR seems to also undergo ERAD through an alternative degradative pathway.

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S5.4 Analysis of Differentially Displayed Proteins in HeLa Cells Expressing CFTR and F508del-CFTR

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Cystic fibrosis (CF) is the most common genetic disorder in Caucasians caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene which encodes a cAMP-regulated Cl^- channel expressed in several epithelial cells. Although CF is a monogenic disease it is clear that epigenetic factors play an important role in the pathogenesis of CF patients. Several arguments also point to the role of modulator genes and/or interacting proteins in the pathogenesis of CF.
The aim of the present study was to identify the differentially expressed proteins derived from HeLa cells over-expressing WT-CFTR and F508del-CFTR using 2 dimensional gel electrophoresis and mass spectrometry. The total proteins from three cell lines (WT-CFTR, F508del-CFTR and pTracer (empty vector) HeLa were investigated. A new protocol for extraction of total proteins was developed. Proteins were detected using silver staining. Isoelectrofocusing was performed on acrylamid strips with pH range from 4 to 7, and SDS-PAGE on 10% polyacrylamide gels. Three spots were found differentially expressed between WT-CFTR and F508del-CFTR (overexpressed in F508del-CFTR and downregulated in WT-CFTR). Four different spots were analysed by mass spectrometry MALDI-TOF and nanoelectrospray ESI-MS and showed that the overexpresed proteins were keratins 8 and 18. The subsequent immunocytochemistry experiments using anti-CFTR and anti-keratin 8/18 showed differences in the intracellular network of keratins, and in distribution of CFTR F508del-CFTR. Diminishing the concentration of keratin 18 (by RNA interference experiments) or changing its intracellular localization by culturing the cells at low temperature showed that both, the intracellular concentration of keratin 18 and its intracellular distribution, are involved in the trafficking of CFTR/F508del-CFTR (Proteomics 2004, in press).

This work is supported by CEE grant QLG2-CT-2001-01335, a grant from Vaincre la Mucoviscidose. The 2D gels were performed in the Plateau Protéome of IRNEM, Paris, France

S5.5 Rescue of Cystic Fibrosis Mutations in the First Nucleotide-Binding Domain by Revertant Mutations (G550 and 4RK)

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R560T, A561E and V562I are described in the CFTR mutation database (1), located within the first nucleotide-binding domain (NBD1) of CFTR, between the signature and Walker B motifs, at a region that constitutes a hotspot for CF mutations (1). Using biochemical assays, we demonstrated that R560T and A561E disrupt the processing of the CFTR protein. In contrast, V562I seems to be as efficiently processed as wild-type CFTR.

The introduction of a negatively charged amino acid into the signature motif LSGGQ (G550E) was previously described to partially correct the F508del-CFTR trafficking defect in HeLa and Fischer rat thyroid (FRT) cells (2). In addition, simultaneous mutation to lysines in the endoplasmic reticulum (ER) retention signals (RXR motifs) present in CFTR (R29, R516, R555 and R766; designated 4RK) promotes the maturation of F508del-CFTR (3). Based on these data, we tested whether the G550E and 4RK variants also rescue the trafficking defect of the above processing mutations. Similarly to F508del-, the A561E-CFTR trafficking defect is reverted by either G550E or 4RK, being the mature form of CFTR (band C) clearly detected by Western blot. On the other hand, G550E does not revert the trafficking defect of R560T-CFTR. Interestingly, pulse-chase assays followed by CFTR immunoprecipitation show that band B of V562I-CFTR is stabilised even further by introduction of the 4RK, but is not stabilised by the introduction of G550E. We are also investigating the effect of the revertant mutations on the function of these CF mutants as chloride channels, by the iodide efflux assay.

To better understand the effects of revertant mutations, we employed a comparative model of the NBD1-NBD2 dimer to obtain a structural perspective. In this model, R560, A561 and V562 span the lower half of the D8 α-helix of NBD1, which ends at the amino acid residue 563, not far from the ATP binding site of NBD2. Results of our studies suggest that CFTR-V562I is not a mutant. They also suggest that CFTR-V562I, in the presence of the revertant mutations might be a better protein than wild-type CFTR to use for CF gene therapy.

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Symposium 6 – Modifier Genes

Chair: JJ Cassiman, Leuven (Belgium)

S6.1 Modifier Genes

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The substantial variability of the clinical manifestation of cystic fibrosis even amongst F508del-homozygous patients points to the substantial influence of factors other than CFTR on shaping CF disease. Data retrieved from the European CF Twin and Sibling Study indicate that genetic factors other than CFTR predominantly determine growth and gastrointestinal disease in CF whereas the CFTR mutation genotype and environmental factors are the key players for CF lung disease. The latter finding of the comparatively small genetic increment may partly explain the substantial controversy in the literature about the role of particular genes / polymorphisms on the outcome of CF lung disease. Moreover the frequently pursued approach of retrospective data mining for clinical features that segregate with a particular modifier genotype inherently carries the risk of false-positive associations.

We search for genetic modifiers in a preselected cohort of dizygous F508del-homozygous sibs and twins with contrasting extreme phenotypes. The association study is conducted on siblings to reduce the confounding influence of random environmental factors as compared to unrelated individuals. A candidate gene region is first scanned by microsatellite geno- and haplotyping and in case of a positive association thereafter tested for informative SNPs that segregate with the disease phenotypes 'concordant mild', 'concordant severe' and/or 'discordant'. An identified modifier should then be corroborated by testing for divergent phenotypes in bioassays. An example is the association of divergent marker haplotype at the CLCA locus on chromosome 1 that encodes putative calcium – activated chloride channels with the expression of DIDS – sensitive chloride secretory responses in intestinal current measurements of rectal suction biopsies.

Numerous *loci* with genes encoding ion conductances, elements of the CFTR network, and elements of the inflammatory and immune host responses, respectively, have meanwhile been detected to segregate with disease phenotype in the sib and twin cohort. The CFTR linkage group on chromosome 7q turned out to be a major determinant for concordance of disease in CF siblings.

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S6.2 Modulating Genes of the CF Phenotype

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It is clear that, besides the CFTR genotype, other genetic factors and environmental factors affect the CF phenotype. For several genes, an association with the CF phenotype has been reported. However, a modulating role of a genetic factor with disease, based on association studies, should be only treated as tentative until the finding of an association has been replicated in other studies. Therefore we investigated if we could confirm the association of all reported modifier genes in another group of CF patients. Moreover, by studying all these genes in a single group of patients, the combinatorial effect of all these modifier genes may be determined. Also we investigated if proteins that directly or indirectly interact with CFTR modulate CF lung disease.

The list of candidate modifiers includes mannose binding lectin protein, the α-ENaC subunit, the β-ENaC subunit, the γ-ENaC subunit, hBD1, hBD2, hBD3, hBD4, surfactant proteins A1 and A2, GSTM1, GSTM3, GSTP1, the β2-adrenergic receptor, TNFα, TGFβ1, syntaxin 1A, syntaxin 8, IL8, IL10, NHERF1, NHERF2, CAP70, the α1-subunit of AMPK, the α2-subunit of AMPK and the angiotensin I converting enzyme. The coding region and exon/intron junctions of hBD1, hBD2, hBD3 and hBD4 were completely analysed by sequence analysis. For the other genes, 7 multiplex single nucleotide primer extension assays were developed, in order to genotype the different SNPs in these genes. On average, about 7 SNPs can be analysed per multiplex assay. Genotyping for polymorphic loci in these genes was performed in 104 Belgian and Czech CF patients (homozygous for F508del), 200 control individuals, and dizygous twin and sibling pairs with extreme clinical phenotypes and their parents (European CF twin and sibling study). Proteins directly/indirectly interacting with CFTR were studied here for the first time, no association with disease was observed in the Belgian and Czech CF patients. For the majority of the genes for which an association has been described in the literature, no association could be confirmed in this study. In the 104 Belgian and Czech CF patients, only an association of MBL, SP-A2, TNFα, TGFβ1, GSTM1, and potentially IL8 and IL10, was found.

Symposium 7 – CFTR-Opathies

Chair: Burkhard Tümmler, Hannover (Germany)

S7.1 CFTR and its Diseases

Garry Cutting

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S7.2 Genotypes Determine only Part of the Phenotype

Jean-Jacques Cassiman

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The molecular defects of an exponentially increasing number of genetic diseases are being unraveled. From these studies it has become clear that in the overall majority of cases a simple relation between the gene, the position and nature of the mutation in the gene, and the final clinical phenotype or clinical course of the disease is not straightforward. A case in point is cystic fibrosis. More than 1000 mutations and over 200 polymorphisms have been described in the CFTR gene. Some of these have been tentatively classified as severe, others as mild. The border between the two is however very unsharp as is the distinction between polymorphisms and disease mutations. Individuals who apparently do not produce CFTR protein can still have a mild form of the disease, while others with classical symptoms do not even carry a mutation in the CFTR gene. Single organ CF can be due to mutations in the two CFTR alleles, but in many cases only one allele seems to be affected. CF related diseases, such as other forms of lung disease or pancreatitis carry CFTR mutations in a minority of the cases. Nevertheless the involvement of CFTR in these diseases is significant, compared to controls.

These findings confirm that there is no clear and simple relation between a gene, a mutation and a phenotype. This is not surprising since most cellular functions are the result of the action of a series of proteins, each with its own polymorphisms, that work in the same cascade or pathway or that can modulate the effect of other proteins at a distance through a different pathway.

The experience with many transgenic animals has shown that many knockout constructs give no clinical phenotype, suggesting that rescue pathways may prevent the appearance of a particular defect.

The CFTR gene is clearly a player in a multifactorial network. Its role is preponderant in CF but the impact of its mutations decreases as one moves away from classical CF.

S7.3 CFTR-Related Disorders in Enterocytes and Erythrocytes

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Bioelectric measurements in intestinal mucosa from CF patients and CFTR null mice have demonstrated a central role of the CFTR Cl^- channel in transepithelial salt and water secretion. Hyperactivation of intestinal CFTR channels by microbial toxins acting through cAMP (choleratoxin) or cGMP signals (heat-stable *E. coli* enterotoxin) results in secretory diarrhea, a major cause of infant mortality worldwide. In contrast, class II mutations in the CFTR gene, causing misfolding of the CFTR protein in the ER and enhanced ubiquitination and proteolysis in post-Golgi compartments (e.g. F508del), may result in intestinal obstruction (Meconium Ileus, DIOS) and malnutrition, a major pathophysiological feature of CF and the prime defect in CF mouse models.

Molecular cloning and functional studies in KO mouse models have enabled the identification of the signaling enzymes and ion transporters involved in enterotoxin-induced chloride secretion and inhibition of NaCl absorption, and have stimulated the search for anti-diarrheal drugs by high-throughput screening approaches, including inhibitors of intestinal isoforms of guanylyl cyclase (GC-C) and cGMP-dependent protein kinase (cGKII), and of CFTR itself. Sofar the most promising results have been reached with novel CFTR and cGKII inhibitors, acting as effective and non-toxic anti-secretory drugs in rodent models. Strategies to compensate for, or correct the intestinal secretory defect in CF include:

(i) down-regulation of intestinal NaCl absorption at the level of the Na^+/H^+ antiporter NHE3. Our recent studies in CF mice (both F508del and CFTR null) have revealed a ~40-50% loss of basal NHE3 function, associated with a reduction of NHE3 protein in the apical membrane; in contrast, the cAMP-inhibition of NHE3 is lost in CF mouse intestine, most plausibly due a defective CFTR-NHE3 interaction involving the tandem PDZ domain proteins NHERF1 and NHERF2. KO mouse models for these linker proteins are presently tested for their intestinal phenotype;

(ii) down-regulation of passive Cl^- absorption in CF jejunum resulting in blockade of glucose-stimulated NaCl absorption as reported in F508del CF patients (Russo MA et al. 2003; JCI 112: 118-123). Sofar we could not confirm the occurrence of a similar compensatory mechanism in our congenic FVB-F508del mice, offering a possible explanation for the high incidence of DIOS in this CF mouse strain (100% mortality within 2-3 days) in response to a new experimental diet.

(iii) pharmacological correction of the F508del folding defect and/or stabilization of rescued CFTR.

The F508del mouse has proven a valuable model for pre-clinical testing of such therapeutic strategies. *Ex vivo* incubation (8-16h) of muscle-stripped intestinal mucosa at low temperature (26ºC) resulted in a gain in cAMP-and cGMP-induced intestinal secretion up to wild-type levels, the appearance of CFTR protein on the crypt surface, and a partial conversion of immature, core-glycosylated CFTR into the mature protein. A similar gain of function was also reached upon short-term (4-6h) *ex vivo* exposure of F508del intestine to the proteasome inhibitor epoxomycin and the proteasome/calpain inhibitors MG-132 and ALLN. In contrast, the ER calcium pump inhibitors thapsigargin and curcumin, reported to rescue CFTR function in rectal and nasal mucosa of F508del mice after 3 days of oral treatment *in vivo* (Egan ME et al. 2004; Science 304: 600-602), failed to improve ileal Cl^- secretion in short-term *ex vivo* incubations. *In vivo* testing of proteasome inhibitors for their ability to rescue CFTR functions in F508del mice and to prevent intestinal malabsorption and obstruction, is in progress.

In contrast to the high levels of CFTR expression in enterocytes, very low levels have been detected by RT-PCR in human erythroblasts. Functional expression of CFTR in red blood cells is evidenced by (1) the failure of CF erythrocytes to perform deformation-and cAMP-induced ATP release, causing vasorelaxation defects; (2) a CF defect in the *Plasmodium falciparum*-activated inwardly rectifying chloride conductance, possibly involved in the ATP release mechanism (Verloo P et al. 2004; JBC 279: 10316-10322). The diagnostic value of the ATP release assay ("CAR") is presently tested in a large number of CF patients of different genotypes and in obligate carriers.

P 10

S7.4 CFTR and Renal Cyst Growth: Implications for Autosomal Dominant Polycystic Kidney Disease

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Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common single gene disorders to affect humans. The disease is caused by mutations in the polycystin proteins that initiate a cascade of events, which lead to the formation of multiple fluid-filled epithelial cysts (1). These cysts progressively destroy kidney function leading to renal failure.

Several lines of evidence suggest that the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel plays a key role in cyst formation and growth. First, immunocytochemical studies demonstrated that CFTR is located in the apical membrane of ADPKD cysts (2;3). Second, Cl^- currents with properties and regulation identical to those of CFTR have been identified in ADPKD epithelial cells using the whole-cell patch-clamp technique (3). Third, CFTR antisense oligonucleotides inhibited cAMP-stimulated fluid secretion by ADPKD epithelia (4).

In this study, we investigated the hypothesis that inhibitors of CFTR might retard cyst formation and growth. We used MDCK cells that form cysts when grown in collagen gels and inhibitors of the CFTR Cl^- channel. These inhibitors included the thiazolidinone $CFTR_{inh}$-172, a potent, specific inhibitor of CFTR (5) and the sulphonylurea, glibenclamide, the arylaminobenzoate, NPPB and the flavonoid, genistein, three non-specific CFTR blockers. As a control, we tested the effects of the disulphonic stilbene, DIDS that is without effect on CFTR when added to the extracellular side of the membrane.

To examine the role of CFTR in cyst formation, we seeded gels with MDCK cells and grew cysts in the absence and presence of $CFTR_{inh}$-172 (10 μM) for 6 days. $CFTR_{inh}$-172-treatment reduced both the number and volume of cysts formed ($P < 0.05$).

To examine the role of CFTR in cyst growth, we treated cysts with CFTR blockers for a 6-day period beginning on day 6. $CFTR_{inh}$-172 (10 μM), glibenclamide (100 M), NPPB (50 μM) and genistein (100 μM) all retarded cyst growth dramatically ($P < 0.01$). However, DIDS (200 μM) failed to diminish cyst growth ($P > 0.05$).

To understand the mechanisms by which CFTR blockers inhibit cyst growth, we tested their effects on cAMP-stimulated apical membrane Cl^- currents and cell proliferation. All the CFTR blockers tested inhibited cAMP-stimulated apical Cl^- currents, whereas DIDS (200 μM) was without effect. In contrast, both CFTR blockers and DIDS inhibited cell proliferation in the presence of forskolin (10 μM). We interpret our data to suggest that the CFTR Cl^- channel plays a key role in cyst formation and growth. Our data also suggest that CFTR blockers retard cyst growth mainly by preventing CFTR-induced fluid accumulation within the cyst lumen.

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S7.5 CFTR Gene Mutations in Infertile Portuguese Patients with Congenital Absence of the *Vas Deferens*

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Introduction: More than 95% of males with cystic fibrosis (CF) are infertile due to congenital absence of the *vas deferens* (CAVD). A high prevalence of cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations has been reported in isolated CAVD.
Objective: To first evaluate the frequency and type of CFTR mutations in 48 Portuguese CAVD patients.
Materials and methods: Patients were initially screened for the 31 most frequent CFTR mutations in the European population using a commercial kit (Abbott Diagnostics, Applied Biosystems). In patients with one or no CFTR mutations, the 27 CFTR exons and their flanking intron sequences were screened by Denaturing Gradient Gel Electrophoresis (DGGE) and denaturing High Performance Liquid Chromatography (dHPLC), followed by direct sequencing when an abnormal pattern was detected. Genotyping of the intron 8 splicing variants (IVS8-T) was performed using an ABI PRISM 310 Genetic Analyzer (GeneScan, Applied Biosystems).
Results: The initial screening identified 23 CFTR mutations in 21/48 (43.8%) patients, 2 (4.2%) with two mutations and 19 (39.6%) being carriers. DGGE and dHPLC analysis detected 15 further mutations, of which one is a novel missense mutation in exon 20 (P1290S). Whole gene screening thus increased the number of patients with at least one CFTR mutation to 28/48 (58.3%), 10 (20.8%) with two and 18 (37.5%) with a single mutation. IVS8-T analysis revealed 23/48 (47.9%) patients with the 5T allele, 3 (6.2%) in homozygozity and 20 (41.7%) in heterozygosity. The association of CFTR mutations with the 5T allele increased the total number of patients with at least one mutation to 34/48 (70.8%), 26 (54.2%) with two and 8 (16.7%) with a single mutation, leaving without diagnosis 14 (29.2%) CAVD patients.
Discussion: Portuguese CAVD patients show a similar type and frequency of CFTR mutations as previously described in other populations (1). The CFTR mutation detection rate was 14.5% higher using an extensive screening of all exons (58.3%) when compared to the routine screening (43.8%). Furthermore, screening of the whole CFTR gene allowed detection of a new missense mutation. The 5T allele was the most frequently mutation found and accounted for 27.1% of total alleles, with its allelic frequency in CAVD Portuguese patients being similar to other studies and significantly higher than in the general population ($P<0.001$) (2). The association between the 5T variant with CFTR mutations increased the mutation detection rate to 70.8%. The present data also provide further evidence that the association of CFTR mutations with the 5T variant might represent the most common genetic cause of CAVD. For assisted reproduction, these patients should be firstly screened for CFTR mutations, with preimplantation genetic diagnosis being offered if both members of the couple are carriers in order to avoid transmission of CF or infertility.

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Special Interest Group I – Proteomics

Chairs: A Edelman, Paris (France)
D Penque, Lisboa (Portugal)

Overview - Proteomics as a New Approach to Understand Cystic Fibrosis

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Cystic Fibrosis (CF) still remains the most common lethal recessive disorders in worldwide without an effective treatment. The cellular abnormalities that link CF genotype to CF cell/tissue phenotype and the influence of modifier genes and environmental factors especially in the lung disease process are not completely elucidated. Proteomics, large-scale studies of protein profile under given condition, offers an innovative approach to understanding CF pathogenesis and providing new means for therapy intervention.

Traditionally, proteomics employs two-dimensional electrophoresis for separating and visualization of proteins combined with mass spectrometry and bioinformatics tools for protein identification. In recent years, novel methodologies that circumvent the need for 2DE are being developed and made proteomics a powerful technology in the analyses of protein complexes.

Proteomics can be devoted to three main biological purposes: 1) spatial and temporal characterization of total protein expression in a cell/tissue in order, for example, to characterize the normal and pathological cell/tissue processes; 2) quantitative/qualitative comparative study of global changes in protein expression between normal and disease or treated and non-treated, to look for disease diagnostic/prognostic markers or toxic effects/responses, and 3) characterization of protein complexes providing functional identification of protein-protein interactions or DNA/RNA-protein interactions. Identification of such complexes and molecules that can regulate these interactions is of great interest since they may subsequently be used as targets for therapeutic drug screening.

The proteomics strategies based on those biological purposes that we have used to understand CF and eventually discover molecular targets for the development of new therapies for CF will be presented and discussed.

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Special Interest Group II – Host-Bacterial Interactions

Chair: Massimo Conese, Milan (Italy)

Overview – **Host-Bacterial Interactions in the Pathophysiology of Cystic Fibrosis Lung Disease**

Massimo Conese

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Mutations in the CFTR gene disrupt the cAMP-regulated chloride channel by CFTR and interfere with its regulation of other ion channels. Reduction or elimination of cAMP-mediated chloride transport through the CFTR channel is usually thought to reduce chloride and water secretion into the airway lumen and to cause hyperabsorption of sodium and water from the lumen, producing dehydrated and viscous secretions. Endobronchial colonization commonly occurs early in the life, with a number of organisms including *Staphylococcus aureus* and other microorganisms. Eventually, nearly all patients become infected with *Pseudomonas aeruginosa*, which is closely associated with progressive pulmonary deterioration. Chronic inflammation, characterized by a massive influx of neutrophils in the airways, contributes to obstructive lung disease and tissue destruction leading to bronchiectasis and respiratory failure. There are many gaps in this pathophisiologic process with different hypothesis that have tried to link diverse CFTR functions to the development of CF lung disease.

Two hypotheses have proposed to link CFTR, ion transport and lung disease (Wine J., J. Clin. Invest. 1999, 103: 309-312). The "high salt hypothesis" postulates that normal ASL has low levels of salt as a result of salt absorption in excess of water. In CF, salt is poorly absorbed resulting in excessively salt ASL. The "low volume hypothesis" postulates that normal ASL has salt levels approximately equal to plasma. In CF, the removal of CFTR's inhibition of EnaC results in abnormally elevated isotonic fluid absorption which depletes the ASL and leads to reduced mucociliary clearance. The first hypothesis has as a consequence that salt-sensitive antimicrobial molecules, particularly those belonging to the defensin family, may not function in the CF ASL milieu (Bals R. *et al.*., J. Clin. Invest. 1999, 103: 303-307). Recent studies raise concerns about the viability of this hypothesis.

First, a series of studies have not detected a difference in salt concentration between normal and CF airways. Moreover, it is not clear why failure of salt-sensitive defensins alone would predispose to broad-spectrum infections, given that these are minor components (~1000-fold less than lysozyme, for example) of a highly redundant antimicrobial system. The second hypothesis has as a consequence that, despite the failure to clear mucus from airway surfaces, goblet cells likely continue to secrete mucins and generates plaques and plugs on CF airway surfaces. The increased sodium transport requires increased mitochondrial activity and O_2 consumption. Thus, the combination of the increased O_2 consumption by the CF epithelium, coupled with the deep mucus plaques forming on airway surfaces that restrict O_2 diffusion, create steep O_2 gradients and hypoxic niches (Boucher R.C., Adv. Drug. Deliv. Rev. 2002, 54: 1359-1371). Bacteria can penetrate into thickened mucus plaques and evade host neutrophils and macrophages, which appear unable to penetrate thickened mucus. Also, it appears that *P. aeruginosa* can grow in hypoxic environments and exhibit adaptation consistent with biofilm formation, *e.g.* alginate production. Bacteria residing in anaerobic biofilms are particularly resistant to endogenous antimicrobial substances and antibiotics. Criticisms of the "low volume-anoxic mucus" hypotheses have arisen because of the lack of data in CF infants that shows reduced mucociliary clearance prior to the development of chronic lung disease.

Other hypotheses have implicated CFTR functions other than ion transport to lung disease. According to the "cell receptor" hypothesis, the CFTR itself has been proposed to serve as a receptor for *P. aeruginosa* (Pier G.B., Curr. Opin. Microbiol. 2002, 5: 81-86). Therefore, bacteria that evade other innate airway defenses may be cleared by epithelial phagocytosis with subsequent apoptosis and apoptotic bodies shedding. The loss of this clearance mechanism in CF would predispose to chronic endobronchial

infection. This hypothesis fails to explain the pathogenesis of CF lung disease prior to the onset of *P. aeruginosa* infection. Furthermore, it fails to explain why CF patients with Class IV mutations, who express CFTR in the apical membrane without channel function, have an infectious phenotype. Finally, this hypothesis should be demonstrated in hypersecretory models of mucins, since it has been shown that *P. aeruginosa* can bind to mucus plaques.

A pro-inflammatory tendency (i.e., "intrinsic hyperinflammation") has also been proposed to be important in the pathogenesis of CF lung disease (Chmiel J.F. *et al.*, *Clin. Rev. Allergy Immunol*. 2002, **23**: 5-27). This hypothesis proposes that CFTR dysfunction (*i.e.* accumulation in the endoplasmic reticulum) causes a cellular stress with NF-κB activation and IL-8 basal secretion. IL-8 is the major chemokine attracting polymorphonuclear neutrophils (PMNs). Activated PMNs secrete proteases, enzymes and reactive oxygen species (ROS), inducing damage to the respiratory epithelium. Neutrophil elastase degrades elastin, collagen and proteoglycans, and it is a potent secretagogue and enhances macromolecular secretion from serous gland cells. Elastase cleaves immunoglobulins, complement component and opsonic receptors on the surface of phagocytes. This would bring to a vicious cycle of infection and inflammation. However, most recent data indicate that the CF lung is sterile and uninflamed at birth and remains uninflammed until the onset of postnatal infection.

In late phases of the disease, the role of adaptive immunity is still not resolved. The neutrophil-dominated inflammatory response is unusual for a chronic-type inflammation, which is typically characterised by the mononuclear infiltrates and granulomatous tissue. Besides the vicious cycle of infection and inflammation proposed above, it could be that a defect in adaptive immunity mechanism cannot resolve the inflammation (Conese M. *et al.*, J. Cystic Fibrosis 2003, 2: 129-135). Macrophages are activated by bacterial products and produced both primary pro-inflammatory cytokines and cytokines activating T helper cells. Th cell responses can be either Th1 (distinguished by IFN-γ production, low antibody response and macrophage activation) or Th2 (characterized by IL-4, Il-5 or IL-10 production and high antibody response): While Th1 response is targeted to cell-mediated immunity, against intracellular bacteria, Th2 cells activate humoral immune responses. In CF there is a predominant Th2 response and the reasons for this are unclear. It is probably for the lack of an inappropriate Th1 response that the massive infliltrate of the CF lung is made up by neutrophils.

Finally, other researchers have found that CF PMNs show peculiar functional features. CF PMNs have increased respiratory burst, secrete higher levels of LTB4, another PMN chemoattractant, show decreased mobilisation of opsonin receptors, which mean less phagocytosis capacity, and increased production of elastase. Since these features are presented by blood PMNs, it is possible that CF PMNs are primed in vivo by the pro-inflammatory cytokines or that CFTR mutations determine this phenotype (Witko-Sarsat V. *et al.*, *Med. Inflamm* 1999, **8**: 7-11). However, so far only CFTR mRNA and not protein has been demonstrated in neutrophils. The "activated" PMNs, once transmigrated to the lung, could be more harmful than protective.

P 13

Effects of Reduced Oxygen Concentration on Antibiotics Sensitivity for *Pseudomonas aeruginosa* in Cystic Fibrosis

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Introduction. The majority of cystic fibrosis (CF) patients suffer from chronic respiratory infections with *Pseudomonas aeruginosa* (*PsA*). In non-CF clinical settings, a strong correlation exists between results of *in vitro* sensitivity tests and outcome of antibiotic therapy. However in CF patients with PsA infection, *in vitro* susceptibility testing results correlate less well with outcome of therapy. Recent evidence suggests that *PsA* resides as a biofilm in an anaerobic microenvironment within airway mucus, which has a gradient of reducing concentration of oxygen, with the lowest concentration being at the base of the mucus layer. In contrast, aerobic conditions are used for standard *in vitro* testing. The aim of this study is to investigate the effects of reduced oxygen concentration on *in vitro* susceptibility for *PsA* to a number of antibiotics commonly used to treat infectious exacerbation of CF.

Method. Sixty-two stored *PsA* isolates from 25 CF patients previously treated at Southampton General Hospital were used against as panel of antibiotics: Gentamicin, Ceftazidime, Ciprofloxacin, Meropenem, and Tobramycin at varying oxygen concentrations (3%, 5%, 12% and 21%). The target oxygen concentration was achieved using an anaerobic jar (Oxoid, UK) and a microaerophilic workstation (Don Whitley Scientific). The isolates were tested using anti-microbial disc diffusion test as outlined by the British Society of Antimicrobial Chemotherapy and the diameter of the zone of inhibition was measured using an electronic calibre. The minimum inhibitory concentration (MIC) was then calculated.

Results. Minimum inhibitory concentration (MIC) to gentamicin, ciprofloxacin and tobramycin increased, as the oxygen concentration decreased. Fifty percent of *PsA* isolates were less sensitive to gentamicin ($P < 0.001$), 56% to ciprofloxacin ($P < 0.001$) and 90% to tobramycin ($P < 0.001$). Although 54% of *PsA* were less sensitive to both ceftazidime and meropenem, the change was not significant. Both linear and exponential trends were observed in gentamicin, ciprofloxacin and tobramycin as the oxygen concentration changes. This study also revealed that some isolates exhibit morphological change as the oxygen concentration reduced.

Discussion. The reduced oxygen concentration significantly reduces the antibiotic sensitivity of *PsA* to gentamicin, ciprofloxacin and tobramycin. Ceftazidime and meropenem that are beta lactam were not significantly affected by changing oxygen concentration. Ceftazidime and meropenem remain sensitive regardless the change in oxygen tension.

Symposium 8 – Gene Therapy

Chair: C Boyd, Edinburgh (UK)

S 8.1 Improving Delivery of Large Plasmids

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We are investigating the use of CFTR genomic context vectors (GCVs) for correcting the CF ion transport defect. By including natural control elements, GCVs (in contrast to current vectors based on CFTR cDNA) may provide long-term, tissue-specific expression. GCVs are large and their size (> 100 kb) precludes efficient delivery by most existing synthetic delivery systems. Much effort is therefore being expended into improving the design of both DNA constructs and synthetic carriers. We have demonstrated that KLN 47, a lipophosphoramide containing an arsonium cation, is able to transfer a large GCV into epithelial cells almost as efficiently as a small plasmid (4.7 kb). We also show that the nature of the polar head group is crucial to the efficiency of gene transfer and that, despite the introduction of an arsonium cation, no significant additional toxicity is induced.

S8.2 Gene Transfer to the Airway Epithelium by HIV-Based Vectors

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Phase I gene therapy trials for the treatment of CF lung disease have demonstrated that cystic fibrosis transmembrane conductance regulator cDNA transfer to respiratory epithelial cells is feasible, but a clinical effect is far to be obtained. In addition, duration of gene expression has been shown to be limited in time. This means that more efficient gene transfer vectors and with a long-lasting gene expression profile should be investigated for CF. Most of the gene transfer systems used in CF patients (cationic liposomes and adenoviruses) results only in episomal maintenance of the vector DNA. A new approach to gene therapy of CF is to target lung airway epithelium with integrating vectors. HIV-1-derived lentiviral (LV) vectors seem to be promising vehicles for gene transfer since they integrate in the host genome, transduce non-dividing cells and ensure long-term expression of the transgene. Previous studies have shown that LV vectors efficiently transduce airway epithelia *in vivo* from the apical surface, only when tight junctions are disrupted by chemical/physical agents. Our study aims to evaluate the gene transfer mediated by a high efficient, last generation LV vector *in vivo* and *in vitro* models of polarised epithelium. A LV vector (PPT-GFP) pseudotyped with the envelope of the vesicular stomatitis virus (VSV-G), carrying the green fluorescent protein (GFP) under the CMV promoter and the central polypurine tract (cPPT) element, was injected intratracheally in C57BL/6 mice at the dose of 4.5x106 TU. Mice were sacrificed after 48 hours, lungs were removed and fixed in paraformaldehyde; cryosections were stained with an antibody raised against GFP. Results show that PPT-GFP transduced the murine airway epithelium both at the level of bronchial and alveolar compartments. These data were confirmed in the alveoli by epifluorescence. Since heparan sulfate (HS) has been described to mediate infection of VSV-G-pseudotyped retroviral vectors and HIV-1, the expression of this proteoglycan by human respiratory cells and its involvement in LV-mediated gene transfer were studied. Human bronchial epithelial cells (16HBE) and CF tracheal epithelial cells (CFT1) were analysed for HS expression by flow cytometry and confocal microscopy. HS was expressed by 85-88% of cells and showed a preponderant baso-lateral localisation in polarised epithelial cells. Pre-incubation of the LV vector with heparin determined a dose-dependent inhibition of CFT1 cell transduction efficiency up to 65% of untreated vector. Our study shows that 1) HIV-1-based LV vectors can transduce murine airways, without pre-treatment with agents which disrupt the epithelial barrier integrity and 2) HS might be the receptor involved in VSV-G-pseudotyped LV-mediated gene transfer. Future experiments will include the study of entry mechanism (intra- or para-cellular) into polarised respiratory epithelia and the role of HS and/or other receptors in LV-mediated gene transfer. Our data suggest that a gene therapy approach to CF lung disease based on LV integrating vectors, avoiding further damage to the airways, might be feasible.

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S8.3 Human Artificial Chromosomes: Seeing Viable Stability

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There are not many choices if aiming at the stable inheritance and expression of a gene in a growing and regenerating somatic cell population. One either integrates a gene into a host chromosome, or one provides the sequences required to form a chromosome *de novo* using transfer of human artificial chromosome DNA. In both cases the genes develop their functional state within a chromosomal chromatin context. Genes which are not part of a chromosome can be transferred quite efficiently into the cell generation currently present, but the genes are lost over the course of time. In addition, the vast majority of transiently transferred gene molecules functions only very weakly, so that large numbers are required to detect the expression of, for example a green fluorescence marker gene (ref 1). A simple cell culture lipofection experiment using detection of such a marker gene present on human artificial chromosome constructs now led a step further and demonstrated that early detection, which likely reflects the transient expression state from large numbers of constructs, is contraproductive in terms of *de novo* chromosome formation and stable inheritance. Seeing the expression marker early after lipofection obviously impairs subsequent stable clone and artificial chromosome formation, as 40 out of 41 early expressors ceased growth at the 1-8 cell stage. In contrast, a number of viable clones, which did not express visible amounts before day 4-6 (8-32 cell stage), turned out to develop stable and visible marker gene expression. FISH analysis of those viable clones demonstrated the stable inheritance of low copy human artificial chromosomes.

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S8.4 SFHR-Mediated Modification of CFTR *in vitro* and *in vivo*

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The identification and sequencing of the gene responsible for cystic fibrosis (CF), the cystic fibrosis transmembrane conductance regulator (CFTR), has led to a myriad of analytical and therapeutic efforts to better understand and treat CF. Therapeutically, there has been a great deal of emphasis on the development of genetic strategies to ameliorate and ultimately "cure" CF. Most of these strategies have focused on the complementation of the mutant CFTR with the wild-type CFTR "mini-gene" cDNA derived from the CFTR mRNA. These studies have achieved a certain degree of positive results, but have also encountered numerous obstacles impeding the development of an effective therapy. An alternative to the cDNA-based complementation strategies, has been a more direct approach aimed at "correcting" or "repairing" the mutant genomic CFTR sequences. One of these sequence-specific genomic DNA modification strategies, small fragment homologous replacement (SFHR) has employed small DNA fragments (SDF) or large oligonucleotides to facilitate sequence-specific modification of mutant (F508del) CFTR both *in vitro* and *in vivo*. Initial *in vitro* studies in airway epithelial cells indicated not only genomic DNA sequence modification, but also SDF-induced changes in CFTR mRNA and CFTR dependent chloride (Cl^-) transport. Quantitative analysis indicated an SFHR-mediated repair frequency in the neighborhood of 1-10% both at the DNA and functional level. Further studies have been undertaken to evaluate the potential for SFHR-mediated modification *in vivo.* Studies were carried out in normal mice (using SDFs to introduce a F508 deletion into the mouse (m) CFTR) and indicated SDF-induced modification of mCFTR mRNA in the mouse lung after direct instillation. Preliminary studies in CF (F508del) mice also suggested that multiple applications of wild-type CFTR SDF to the nasal mucosa could improve the cAMP-dependent Cl^- ion transport characteristics of the CF mouse mucosa.

The therapeutic potential of pluripotent stem cells has suggested a role for these cells in the regeneration of epithelium damaged by CF pathology. Initial studies carried out in mouse embryonic and bone marrow-derived stem (ES and BMS) indicate that mCFTR can be modified through the introduction of sequence-specific SDFs. Studies using F508del heterozygote ES cells indicated both the correction of the F508del mutation in the ES cell clone and the absence of random integration of the SDF. Analysis of wild-type ES cells transfected with a F508del-SDF showed the presence of the SDF derived sequence in the mRNA and showed the production of CFTR protein comparable to that found in the F508del CF mouse. In light of these studies and other preliminary studies showing the localization of BMS cell to the damaged mouse lung, one can envision applying SFHR to ES and/or BMS cells derived from CF patients. Autologous transplantation of these cells that could potentially repair CF epithelial tissue with cells expressing wild-type CFTR.

S8.5 Entry of Plasmid DNA into the Mammalian Nucleus

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We have shown that the import of plasmid DNA (pDNA) into the nucleus of digitonin-permeabilized HeLa cells is time- and energy-dependent, wheat-germ agglutinin (WGA) inhibitable and dependent on the re-addition of cytosol. Using pDNA-affinity chromatography, we have now isolated cytosolic shuttle proteins that `piggyback' pDNA into the nucleus. Depletion of the pDNA-binding proteins resulted in a reduction in transport of fluorescent tagged pCMV□-DTS import substrate into nuclei of digitonin-permeabilised cells. Proteins in the import active fraction were analysed by mass spectrometry with subsequent protein database searches. Eight of the proteins localise to both the cytoplasmic and nuclear compartments, as well as possess DNA-binding and nuclear localising sequences (NLS) and therefore represents putative pDNA shuttle proteins. Recombinant shuttle proteins have now been taken forward to a full functional characterisation. In summary, these studies utilise a proteomic approach to identify a subset of cytosolic proteins that shuttle pDNA into the nucleus with a long-term aim of identifying their corresponding DNA-binding sites. These binding motifs should offer a stable and versatile framework for the design of plasmids that recognize desired target sites on cyto-nucleoplasmic shuttle proteins.

P 3

Adjacent Genes from the Human CFTR-region Associate Independently With Functionally Appropriate Nuclear Regions

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Mammalian gene loci display a non-random positioning within cell nuclei, which is related to their functional regulation. In particular, association with the nuclear periphery and pericentromeric heterochromatin seems to play a role in the regulation of transcription and recombination (1,2). Also, the positioning of gene loci at specific regions of the respective chromosome territory appears to play a role in their functional regulation (3). Currently, it is not clear how the association of gene loci with specific nuclear domains relates to their organization within chromosome territories.

We investigated nuclear positioning and transcriptional regulation of the functionally unrelated genes GASZ, CFTR, and CORTBP2, mapping to adjacent loci on human chromosome 7q31. The analysis was performed with different human cell lines and primary cell types. In 10 out of 11 cases where one of the genes investigated was not transcribed it was found associated with the nuclear periphery and with perinuclear heterochromatin. In contrast, in their actively transcribed states the gene loci always associated with euchromatin in the nuclear interior. Furthermore, the results showed that GASZ, CFTR, and CORTBP2 associated independently from each other with these functionally distinct chromatin fractions localizing different nuclear regions. While the nuclear localization of CFTR was changed by altering its transcription levels, the transcriptional status of CFTR was not changed by driving this gene into a different nuclear environment. This implied that in case of CFTR the transcriptional activity affected the nuclear positioning, and not vice versa. Together, the results show for the first time that small chromosomal sub-regions can display a highly flexible nuclear organization, which is regulated mainly at the level of individual genes in a transcription dependent manner.

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Expression, Maturation and Function of CFTR are Altered by Genistein Treatment

P 6

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The most common cystic fibrosis (CF) mutation F508del disrupts the folding of the cystic fibrosis transmembrane conductance regulator (CFTR), preventing the delivery of the protein to the apical membrane where it functions as a regulated Cl^- channel. The demonstration that benzo(c)quinolizinium compounds act as pharmacological chaperones to rescue the cell surface expression of F508del (Dormer *et al J Cell Sci* 114, 4073-4081, 2001) suggests that other CFTR modulators might rescue the apical membrane expression of F508del. A potent modulator of the CFTR Cl^- channel is the flavonoid genistein: genistein (30 μM) stimulates CFTR activity, whereas genistein (100 μM) inhibits channel activity (Wang *et al J Gen Physiol* **111**, 477-490, 1998). In this study, we tested the effects of genistein on the expression, maturation and function of CFTR using BHK cells expressing wild-type (BHK-WT CFTR) and F508del CFTR (BHK-F508del). We incubated cells with genistein (30 and 100 μM) for either 2 or 24 h at 37 °C before assaying CFTR activity using the iodide efflux technique. As controls, we incubated cells at 27 °C. At 37 °C, forskolin (10 μM) and genistein (50 μM) stimulated an efflux of iodide from BHK-WT CFTR cells (n = 7), but not BHK-F508del cells (n = 4). At 27 °C, BHK-F508del cells generated an efflux of iodide, although the magnitude of the response was greatly reduced and the onset markedly delayed compared with that of BHK-WT CFTR cells (n = 7). Following incubation of BHK-F508del cells with genistein (30 or 100 μM) for either 2 or 24 h at 37 °C, forskolin (10 μM) and genistein (50 μM) failed to stimulate an efflux of iodide from BHK-F508del cells (n = 3 - 5). Consistent with these data, we observed no maturation of F508del-CFTR, following incubation of BHK-F508del cells with genistein (30 μM) for periods of time between 2 and 12 h (by Western blotting) and 24-72 h (by immunocytochemistry). However, preliminary results suggest that incubation of BHK-WT CFTR cells with genistein (30 - 100 μM) for 2 - 12 h alters the maturation of wild-type CFTR. Furthermore, incubation of BHK-WT CFTR cells with genistein (100 μM) for 2 h at 37 °C caused a large decrease in the magnitude of iodide efflux stimulated by forskolin (10 μM) and genistein (50 μM) (peak efflux: control, 37.4 ± 6.5 nmol min^{-1}; genistein (100 μM), 15.9 ± 2.1 nmol min^{-1}; $P < 0.05$; n = 5 - 7), without altering the onset of the response. We conclude that genistein fails to rescue the cell surface expression of F508del expressed in BHK cells. However, genistein might alter the expression and/or function of wild-type CFTR at the cell surface.

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Use of Human-Murine CFTR Chimeras to Investigate the Regulation of CFTR Cl^- Channels

P 8

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The single-channel properties of murine CFTR, while broadly similar, exhibit some important differences to those of human CFTR. To identify the protein regions responsible, we constructed human-murine CFTR chimeras by replacing nucleotide-binding domain 1 (NBD1), NBD2 and the R domain of human CFTR with the equivalent regions of murine CFTR to form the chimeras hmNBD1, hmNBD2 and hmRD, respectively. When expressed transiently in CHO cells, CFTR chimeras formed regulated Cl^- channels. In the presence of ATP (0.3 mM) and PKA (75 nM) at –50 mV, the open probabilities (P_o) of CFTR chimeras (e.g. hmNBD2, $P_o = 0.46 \pm 0.02$; $n = 5$) were similar to that of human CFTR ($P_o = 0.43 \pm 0.05$; $n = 5$), but distinct from that of murine CFTR ($P_o = 0.09 \pm 0.01$; $n = 4$). ADP (0.3 mM) inhibited strongly CFTR chimeras, human and murine CFTR with equipotency ($P > 0.05$; $n = 5$-6). In contrast, the effects of pyrophosphate (PP_i; 5 mM) differed between human CFTR, murine CFTR and the CFTR chimeras. Like its effects on human CFTR (control, 100%; PP_i, 165 ± 5%; $n = 6$; $P < 0.01$), PP_i (5 mM) stimulated markedly hmRD (control, 100%; PP_i, 175 ± 8%; $n = 6$; $P < 0.01$). However, PP_i (5 mM) failed to stimulate murine CFTR (control, 100%; PP_i, 103 ± 15%; $n = 6$; $P > 0.5$) and hmNBD2 (control, 100%; PP_i, 99 ± 2%; $n = 6$; $P > 0.5$) and only weakly stimulated hmNBD1 (control, 100%; PP_i, 125 ± 10%; $n = 4$; $P < 0.05$). These results suggest that PP_i might interact with both NBDs to regulate CFTR channel gating, with NBD2 possibly playing the dominant role.

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Molecular and Functional Characterization of Missense Mutations on the CFTR Gene

P 9

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Introduction: More than 1300 mutations have been described on the *CFTR* gene (www.genet.sickkids.on.ca/cftr) but their relationship to the cystic fibrosis (CF) phenotype has only been established for a few of them. Approximately 45% of these mutations are missense leading to an aminoacid change in the CFTR protein.

Objectives: The aim of this study was to assess the effect on the processing and activity of the CFTR protein of 8 missense mutations found in the Spanish population.

Methodology and results: Using site-directed mutagenesis on the pCMVNot6.2 plasmid (kindly provided by Dr. Rommens) which contains the wild-type *CFTR* cDNA, we have generated 4 mutations on the NH_2 terminal intracellular domain (P5L, S50P, E60K, R75Q) and 4 mutations on the first transmembrane domain (TM1) (G85E, G85V, Y89C, E92K). The automatic sequencing of the complete *CFTR* cDNA of the mutant plasmids showed the presence of the generated mutations. In order to choose the most efficient transfection vector leading to low cellular toxicity, a comparative study using pEGFP-N1 plasmid encoding the enhanced green fluorescent protein (EGFP) was performed with 3 non-viral transfection vectors: Polyfect, PEI and GenePorter2 on HEK293 cells. Flow cytometry of transfected cells revealed Polyfect as the most suitable vector. We have assessed the pattern of maturation of the wild-type, F508del and the CFTR mutant proteins at 48 hours post-transfection by western-blot. P5L and R75Q mutants had an electrophoretic pattern similar to the wild-type CFTR showing both B band (partially glycosylated) and C band (mature protein). However, the amount of completely glycosylated protein was lower in both mutants. The remaining 6 mutations produced only partially glycosylated proteins.

The whole-cell patch-clamp technique is being used to evaluate the chloride transport of the CFTR mutant channels. The initial electrophysiological recordings reveal that the P5L and the R75Q mutant channels have forskolin induced Cl^- current which are in agreement with the presence of the mature protein observed in western-blot experiments.

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P 14

Increased Interleukin-9 Gene Expression Associated with Chronic *Pseudomonas aeruginosa* Infection

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The pro-inflammatory cytokine Interleukin-9 (IL-9) has been identified as a key component in severe bronchiolitis (*Lancet*. 2003;**363**:1031-7), and has been shown to increase mucus production and the release of IL8 in the airways (*Am J Respir Cell Mol Biol*. 2000; **22**:649-56). The role of IL9 in the pathogenesis of cystic fibrosis (CF) lung disease is unclear, as is the relationship between airway infection and the extent of inflammation. We aim to examine IL-9 gene expression and bacterial load in the lower airways of young people with varying degrees of CF lung disease.

Induced sputum technique was used to obtain lower airway secretions. RNA was isolated from sputum samples and IL-9 expression measured using RNAse protection assay. Expression levels were qualitatively determined as high, intermediate or low relative to the level of control transcript in each sample. Bacterial load was determined by preparing serial dilutions and culturing on appropriate media. Bacterial colonies were enumerated and subcultured for identification by standard methods. Preliminary data were obtained from four young people with CF whose clinical status is described in the table below.

Patient 1 had the highest bacterial load of 5.36×10^8 cfu/ml sputum and a high level of IL-9 expression. Patients 2 and 3 had bacterial loads of 4.22×10^8 cfu/ml and 2.0×10^6 cfu/ml, respectively, and showed intermediate levels of IL-9 expression. Patient 4 grew *Haemophlius influenzae* (1.33×10^5 cfu/ml), did not grow *P. aeruginosa* and had the lowest levels of IL-9 expression.

Further samples are undergoing analysis, however these preliminary data suggest a significant correlation between the extent of IL-9 expression and *P. aeruginosa* load in induced sputum samples.

Patient	Sex	Age	FEV_1 (% pred)	CXR score	Sputum producer	Number of *PsA* cultures in past 24 months
1	M	17.3	83	6	yes	12
2	F	17.4	46	11	yes	33
3	M	16.7	86	3	yes	25
4	F	16.6	84	0	no	2

Low Temperature Effect on the Entire Protein Expression Profile of BHK Cells Expressing wt- or F508del-CFTR

P 15

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Previous studies have shown that low temperature can overcome the processing defect of F508del-CFTR and activate cAMP-dependent Cl^- channels in CF cells. Although some explanations have been proposed for this observation, the mechanisms involved in the restoration of F508del-CFTR function by low temperature remains to be elucidated.

Using a proteomics approach, we have investigated and compared the effects of low temperature on the entire protein expression profile of BHK cells stably expressing wt- or F508del-CFTR. Our objective is to identify a group of proteins that are differentially expressed under low temperature conditions suggesting their potential involvement in the restoration of normal protein trafficking to the F508del-CFTR mutant.

We analysed by two-dimensional (2D) SDS-PAGE the total protein extracts of BHK cells expressing wt- or F508del-CFTR before or after incubation at 26 ºC for 24 h or 48 h. Cells were radiolabelled with 100 μCi/ml [^{35}S]-methionine for 3 h just before the end of the low temperature treatment. Cells were then lysed directly in IEF lysis buffer and the total protein extracts ($0.8x10^6$/gel) were applied to 18 cm non-linear pH 3-10 or pH 4-7 gradient strips (Amersham Bioscience). After IEF, the strips were applied to 8-16% (w/v) SDS gradient polyacrylamide gels for the second dimension run for protein separation according to their molecular mass. The proteins were visualised by autoradiography, high-resolution maps were built and differentially expressed protein spots identified by computer-based analysis (Melanie software). For some of the detected protein spots qualitative/quantitative differences are found by matching the proteomes of those two cell lines, grown at 37 ºC (controls) or after incubation at low temperature (26 ºC) for 24 h or 48 h. The major and reproducible differences are: the expression of 9 protein spots induced in BHK F508del-CFTR. Of these 9 protein spots, 4 spots decrease their expression after incubation at 26 ºC for 24 h; another 4 spots decrease their expression only after 48 h of incubation; and one spot remains unchanged even for longer incubation at low temperature. Interestingly, in BHK wt-CFTR some of these 9 protein spots remain unchanged or are up or down regulated after incubation at 26 ºC. One protein spot that is exclusively expressed in BHK F508del-CFTR is also slightly reduced after low temperature treatment. There are 2 protein spots that are not expressed in BHK-F508del either growing at 37 ºC or after incubation at 26 ºC. Finally, there are many protein spots that are up or down regulated equally in both cell lines after low temperature treatment.

The complete identification and functional characterization of these differently expressed proteins are in progress and may provide new insights into the mechanisms responsible for the rescue of F508del-CFTR trafficking by low temperature.

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