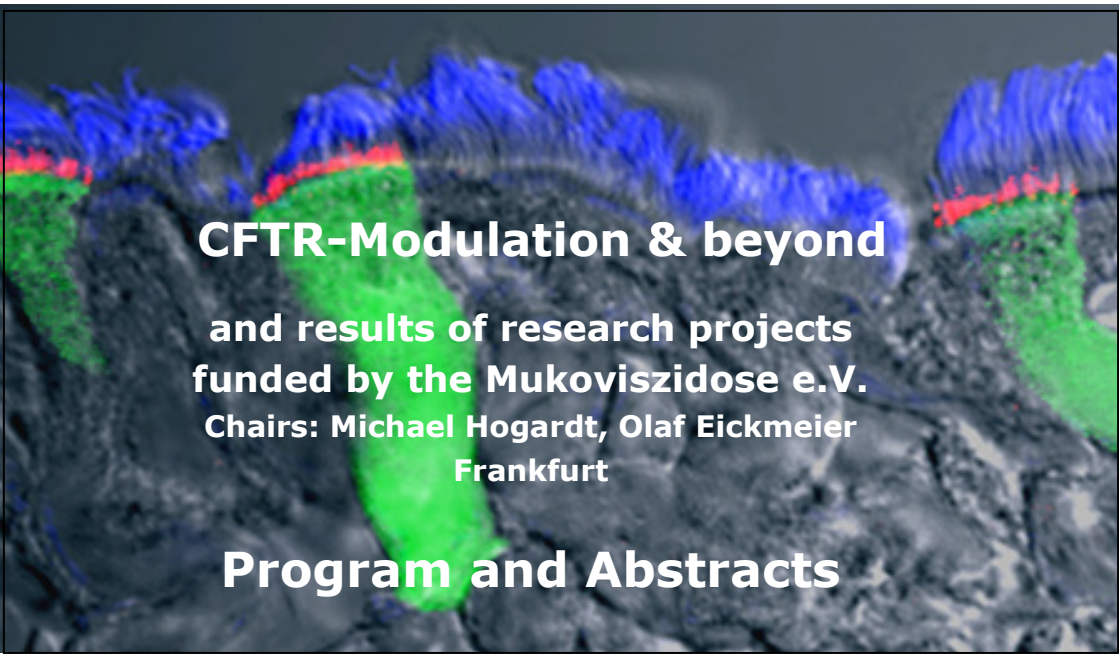
15th Scientific Meeting

24th – 25th September 2015
Waldthausen/Mainz



CFTR-Modulation & beyond

and results of research projects funded by the Mukoviszidose e.V.

Chairs: Michael Hogardt, Olaf Eickmeier
Frankfurt

Program and Abstracts

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Program: CFTR Modulation & beyond

Thursday, 24 September 2015

Session1

Moderation:	Anna-Maria Dittrich (Hannover) Olaf Eickmeier (Frankfurt)
12:30 pm	**Opening of the meeting** Manfred Ballmann, Chairman of the supervisory board Mukoviszidose Institut gGmbH
12:40 – 1:15 pm	**Opening Talk: Search for novel correctors and potentiators of mutant CFTR** Luis Galietta (Genua)
01:20 – 01:55 pm	**Clinical trials with CFTR modulators** Silke van Koningsbruggen-Rietschel (Cologne)
02:00 – 02:15 pm	**MBW and MRI as sensitive markers of stable CF lung disease and at exacerbation in children and adolescents** (selected abstract) Mirjam Stahl (Heidelberg)
02:20 – 02:55 pm	**Do CFTR modulators correct bicarbonate transport in CF?** Hugo de Jonge (Rotterdam)
03:00 – 03:35 pm	**Translational measures to assess efficacy of CFTR modulators** Nico Derichs (Berlin)
03:40 – 03:55 pm	**Intestinal Current Measurement Detect Activation of Mutant CFTR in Cystic Fibrosis Patients with the G551D Mutation Treated with Ivacaftor** (selected abstract) Simon Gräber (Heidelberg)
04:00 – 04:15 pm	**Effects of ivacaftor on inflammatory mediators in the upper airways' epithelial lining fluid of CF patients with G551D** (selected abstract) Christin Arnold (Jena)

04:15 – 04:45 pm	**Discussion & Coffee Break**
04:45 – 05:20 pm	**Towards therapeutic targeting of dysfunctional miRNA expression in the CF lung** Catherine Greene (Dublin)
05:25 – 05:50 pm	**Evaluation of anti-inflammatory substances targeting Th17-dependent respiratory inflammation in CF** Anna-Maria Dittrich (Hannover)
05:55 – 06:10 pm	**Sleeping Beauty (SB) transposon mediated expression of transgenes in airway epithelial cells** (selected abstract) Sahra M. Johler (Munich)
06:15 – 06:30 pm	**Generation of disease-specific iPSCs and development of transgenic reporter cell lines for cystic fibrosis disease modelling and drug screening** (selected abstract) Madline Schubert (Hannover)
06:35 – 06:50 pm	**Generation and functional characterization of epithelial cells with stable expression of SLC26A9 Cl- channels** (selected abstract) Johanna J. Salomon (Heidelberg)
06:50 – 07:15 pm	**Discussion & Coffee Break**

Keynote Session

Moderation:	Helge Hebestreit (Würzburg) Olaf Sommerburg (Heidelberg)
07:15 – 08:15 pm	**Advances and challenges in pharmacological rescue of CFTR** Martina Gentzsch (Chapel Hill)
8:30 pm	**Dinner**

Friday, 25 September 2015

Session 2

Moderation:	Michael Hogardt (Frankfurt) Olaf Sommerburg (Heidelberg9
08:00 – 08:35 am	**Moving forward with CF gene therapy** Stephen Hyde (Oxford)
08:40 – 09:15 am	**New mechanisms of CF airways inflammation in the absence of bacterial infection** Marcus Mall (Heidelberg)
09:20 – 09:35 am	**Neutrophil elastase activity on the surface of sputum neutrophils is associated with pulmonary hyperinflation and airflow obstruction in cystic fibrosis** (selected abstract) Susanne Dittrich (Heidelberg)
09:40 – 10:05 am	**Microevolution of *Pseudomonas aeruginosa* in cystic fibrosis lungs** Nina Cramer (Hannover)
10:10 – 10:25 am	**Physico-chemical compatibility of mixed inhalation solutions for nebulizers** (selected abstract) Hannah Walz-Jung (Mainz)
10:30 – 11:00 am	**Discussion & Coffee Break**
Moderation:	Manfred Ballmann (Siegen) Michael Hogardt (Frankfurt)
11:00 – 11:25 am	**Modulation of the Hsp90-Aha1 Chaperone System to correct the folding deficiency of the CFTRdeltaF508 protein in Cystic Fibrosis** Wolfgang Obermann (Bochum)
11:30 - 11:45 am	**Silent but visible: a common silent polymorphism in CFTR alters physiological function and global protein conformation** (selected abstract) Robert Rauscher (Hamburg)

11:50 – 12:05 am	**Allele specific NF-kB and STAT4 disruption on a regulatory SNP modifies CF disease outcome** (selected abstract) Chidiebere Awah (Hannover)
12:10 – 12:40 am	**Closing talk:** **Development of nanobodies against CFTR** Cédéric Govaerts (Brussels)
12:45 am	**Closing of the meeting**

Table of Content

CFTR Modulation & beyond

Session 1

Search for novel correctors and potentiators of mutant CFTR

Authors: N. Pedemonte, E. Caci, E. Pesce, V. Tomati, A. Gianotti, P. Scudieri, G. Ottonello, L. Goldoni, S. Venzano, T. Bandiera, L. J. V. Galietta

F508del, the most frequent mutation among patients with cystic fibrosis (CF), causes a defective maturation of CFTR protein. The maturation defect can be treated with chemical compounds known as *correctors*. F508del and other mutations like G551D also show a gating defect that requires another type of compounds called *potentiators*. Present correctors only show partial efficacy since F508del mutation causes multiple defects to CFTR protein. New potentiators are also required because combination of present potentiators (e.g. VX-770/Ivacaftor) with correctors (e.g. VX-809/Lumacaftor) does not seem to achieve the required effect on F508del-CFTR, i.e. maximization of mutant protein mutation. This negative effect may be due to an undesired destabilizing effect of potentiators on F508del mutant. Our project aims at identifying new correctors and potentiators for the functional rescue of mutant CFTR protein.

We screened a selected chemical library with 11,334 compounds using two different cell types (FRT and CFBE41o-) expressing CFTR with the F508del mutation. For the screening, we used two separate functional assays designed to identify correctors and potentiators. By high-throughput screening of the chemical library, we have found: i) 104 hits active as potentiators among which 5 compounds with activity comparable to that of VX-770; ii) 5 new correctors with activity on both FRT and CFBE41o- cells. All compounds have been confirmed and characterized with dose-response experiments and cytotoxicity tests. Recent tests on primary bronchial epithelial cells from CF patients have evidenced two classes of correctors and one class of potentiators with significant rescue activity on CFTR-dependent chloride secretion.

The active compounds identified so far will be a starting point for the synthesis of novel potentiators and correctors having improved potency and efficacy.

The project is supported by Italian Cystic Fibrosis Foundation.

Do CFTR modulators correct bicarbonate transport in CF?

Author: Hugo de Jonge

Accumulation of viscid mucus in the lung, intestine, pancreas, hepatobiliary tract, and reproductive tract is a hallmark of CF and is the primary cause of defective mucociliary clearance in the airways and of luminal obstruction in the GI and reproductive tract. Recent studies in CF animal models (mouse1-3, pig4, rat5) indicate that normal mucus release in intestinal and tracheal epithelium requires concurrent bicarbonate (HCO_3^-) secretion and that, in the absence of bicarbonate, mucins released by goblet cells in response to cholinergic stimulation remain densily packed and attached to the epithelial surface. Moreover the pancreatic phenotype of CF patients segregates well with mutations in the CFTR gene that severely disrupt CFTR-dependent HCO_3^- secretion6 , and CFTR mutations that impair the ability of the CFTR channel to switch from a Cl^- conductive into a HCO_3^- conductive mode upon activation of the WNK/SPAK signaling pathway increase the risk for pancreatitits and simusitis7,8. However, in contrast to the wealth of information about the chloride transport defect in native epithelia from CF mice and CF patients emerging from in vivo and ex vivo assays (e.g. sweat test; nasal potential difference, NPD; intestinal current measurements in human rectal biopsies, ICM), there is as yet a paucity of data about bicarbonate secretion and mucus release in native epithelia from healthy individuals and CF patients. A therapeutically relevant key question, i.e. are clinically applied CFTR modulators including the corrector VX-809 (lumacaftor) and the potentiator VX-770 (ivacaftor), in addition to their known ability to improve transepithelial Cl^- secretion, able to correct defects in HCO_3^- secretion and mucin release is not addressed in depth in current literature. For example it is not known whether direct binding of these modulators to the CFTR protein, while restoring CFTR trafficking or gating, also restores the ability of WNK/SPAK to switch CFTR from the Cl^- into the HCO_3^- conductance state, or the ability of CFTR to interact physically with SLC26 Cl^-/HCO_3^- exchangers through STAS-R domain coupling.

We aim to fill this knowledge gap by studying features of HCO_3^- transport, including the impact of CFTR mutations and its restoration by clinically applied CFTR modulators, in readily accessible human tissue, i.e. rectal biopsies and 3D and 2D cultures of rectal and ileal organoids obtained from HCs and CF patients. Major advantages of the use of

(sterile) organoids in addition to native epithelium in our studies are (i) their unlimited capacity to passage and expand without loss of genetic and functional properties, and to re-grow from frozen stocks, making the studies less dependent on the availability of fresh biopsies; (ii) the absence of confounding effects of inflammation or microbial infection, such as hyperplasia of goblet cells; this abnormality is very prominent in native CF mouse intestine but lost at the level of the organoids; (iii) the ease and high capacity of the microscopical swelling assay (FIS) 9; (iv) their ability to form polarized monolayers covered by a secreted mucus layer when grown on Transwell membranes10, allowing electrical and pH-stat measurements of Cl- and HCO3- secretion. Preliminary results of this new project will be presented at the meeting.

1.Garcia MAS, Yang N, Quinton PM 2009. J Clin Invest 119: 2613-22; 2. Gustafsson JK, Ermund A, Ambort D, Johansson MEV et al 2012. J Exp Med 209:1263-72; 3. Liu J, Walker NM, Ootani A, Strubberg AM, Clarke LL 2015. J Clin Invest 125: 1056-68; 4. Hoegger MJ, Fischer AJ, McMenimen JD, Ostedgaard LS, Zabner J, Stolz DA, Welsh MJ 2014. Science 345: 818-22; 5. Tuggle KL, Birket SE, Cui X, Hong J, Warren J, Reid L, Chambers A et al 2014. PLoS One. doi: 10.1371; 6. Choi JY, Muallem D, Kiselyov K, Lee MG, Thomas PJ, Muallem S 2001. Nature 410: 94-97; 7. Park HW, Nam JH, Kim JY, Namkung W, Gray MA, Kim KH, Lee MG 2010. Gastroenterology 139: 620-31; 8. LaRusch J, Jung J, General IJ, Lewis MD et al 2014. PLOS Genetics 10 (7): e1004376; 9. Dekkers JF, Wiegerinck CL, De Jonge HR, Bronsveld I et al 2013. Nature Med 19: 939-945; 10. VanDussen KL, Marinshaw JM, Shaikh N et al 2015. Gut 64: 911-20.

Intestinal Current Measurements Detect Activation of Mutant CFTR in Cystic Fibrosis Patients with the G551D Mutation Treated with Ivacaftor

Authors: S. Y. Gräber, M. J. Hug, O. Sommerburg, S. Hirtz, J. Hentschel, A. Heinzmann, C. Dopfer, A. Schulz, J. G. Mainz, B. Tümmler, M. A. Mall

Introduction: Recent studies suggest that sensitive outcome measures of in vivo CFTR activity may be important to accelerate clinical development of emerging CFTR modulator therapies for cystic fibrosis (CF). Intestinal current measurements (ICM) detect pharmacological activation of mutant CFTR ex vivo. However, whether ICM can detect rescue of mutant CFTR function in CF patients remains unknown. The

aim of our study was to determine if ICM is sensitive to detect rescue of CFTR Cl- channel function in CF patients with the G551D-CFTR mutation after ivacaftor initiation.

Methods: Rectal biopsies were obtained from 37 non-CF controls and 13 CF patients with the G551D mutation before and after initiation of ivacaftor. CFTR Cl- channel function was determined from bioelectric responses to cAMP-dependent stimulation in micro-Ussing chambers.

Results: Before ivacaftor therapy, ICM detected variable residual cAMP-induced Cl- secretory responses in rectal tissues from G551D CF patients that corresponded to ~13 % of non-CF controls. After ivacaftor initiation, Cl- secretory responses in tissues from G551D CF patients were significantly increased compared to baseline (ΔIsc = 42.7, IQR 27.8 – 63.6 µA/cm2; P < 0.001) corresponding to a level of 52% of non-CF controls.

Conclusion: ICM is sensitive to detect in vivo rescue of CFTR function in individual G551D CF patients treated with ivacaftor. These results support the development of ICM as an outcome measure of in vivo rescue of mutant CFTR activity that may facilitate precision medicine with emerging CFTR modulators for CF patients with a spectrum of CFTR mutations including the common mutation F508del.

Effects of ivacaftor on inflammatory mediators in the upper airways´ epithelial lining fluid of CF patients with G551D (preliminary results)

Authors: C. Arnold, J. Hentschel, K. Hünninger, U. C. Hipler, T. Lehmann, J. F. Beckl, J. G. Mainz

Introduction: New surrogate parameters are required to assess effects of CFTR correctors and potentiators, as FEV1 provides restricted sensitivity. Assessment of inflammation in epithelial lining fluid (ELF) is most interesting but lower airway sampling by BAL is limited for invasiveness. Electrophysiological assessment, in intestinal or upper airway mucosa, which equally reveals defective CFTR channels, is limited for complexity. The aim of the present study is to assess the potential to discriminate CFTR modulator effects in ELF non-invasively sampled from the upper airways by nasal lavage (NL). First results shall be compared to clinical and, exemplarily to electrophysiological findings.

Methods: 17 CF patients with one G551D mutation (median age 16yr, range 0-42yr) are attended at the Jena University Hospital CF Center. 10/17pts receive ivacaftor. We sampled ELF by NL in all of them and compared results to NL of 38 healthy controls and 45 CF patients without CFTR-potentiator therapy (median age 14yr, range 2-75yr). In 5/17pts with G551D NL was serially sampled (home based) prior and during new ivacaftor therapy up to every second day and immediately frozen until assessment of inflammatory mediator concentrations by ELISA and multiplexed immunoassays (Neutrophil Elastase (NE), Interleukin (IL)-1ß, IL6, IL8). In 4/17pts results were correlated to electrophysiological data (intestinal current measurement) prior and during the treatment.

Results: Altogether, more than 250 NL samples were collected from 11 G551D patients. Among these more than 130 NL samples of 5 patients were collected at close intervals prior and during ivacaftor therapy. Ivacaftor improved sweat chloride, lung function and bodyweight in most patients. Measurements of the inflammatory mediator concentrations are presently performed. In the NL samples from 10 G551D-patients assessed until now IL-8 and NE declined by median from 109 pg/mL to 92 pg/mL respective from 99 ng/mL to 90 ng/mL. Further detailed analyzes of presently collected samples will be provided together with comparison to non-G551D CF patients and healthy controls, as well as relation to clinical findings.

Conclusion: ELF has successfully been collected by NL by all included patients. The method is suitable for day to day home based sampling when combined with direct freezing. The non-invasively approach is promising for assessment of therapeutic effects of CFTR modulators on airway inflammation.

Towards therapeutic targeting of dysfunctional miRNA expression in the CF lung

Author: C. Greene

The cystic fibrosis lung is a complex milieu comprising multiple factors that coordinate its physiology. MicroRNAs (miRNAs) are small non coding RNAs that have emerged as major regulators of the protein content of a cell. In the most part, miRNAs negatively regulate target mRNA expression. It is becoming increasingly clear that miRNAs play a key role in the development and manifestations of CF lung disease. Their

involvement in the pathogenesis of CF lung disease stems from the fact that their expression is altered in vivo in the CF lung due to intrinsic and extrinsic factors; defective chloride ion conductance, endoplasmic reticulum stress, inflammation, and infection have been implicated as stimuli that can alter endogenous miRNA expression in this setting. Considering their central role in the regulation of gene expression, miRNAs represent therapeutic drug targets. Overexpression and inhibition strategies exist to modulate a cell's miRNA content. Beyond this nanoparticles or microparticles can be used to encapsulate these miRNA modulators for delivery to airway epithelial cells and immune cells, respectively. This presentation will describe how miRNAs are involved in the regulation of aspects of innate immunity and inflammation, what happens when this goes awry in cystic fibrosis and discuss the current state-of-the-art and challenges associated with developing miRNA-targeted therapeutics.

Sleeping Beauty (SB) transposon mediated expression of transgenes in airway epithelial cells

Authors: S. M. Johler, J. Rejman, O. Walisko, A. Munder, W.-M. Weber, B. Tümmler, C. Rudolph, Z. Ivics, J. Rosenecker

Introduction: Cystic Fibrosis (CF) – a rare genetic disease for which until now only symptomatic therapy is available. The current gene therapy approaches resulted only in low efficiency and a transient protein expression, therefore novel strategies for efficient and permanent expression of the transgene are required. For this purpose, employing the Sleeping Beauty (SB) transposon system in combination with in vitro transcribed (IVT) mRNA is a novel and promising strategy which aims at permanent insertion of the wild-type CFTR gene into respiratory epithelia. This innovative and promising strategy is based on the combination of a plasmid-based transposon (pDNA) including the CFTR gene with stabilized, non-immunogenic mRNA (SNIM® RNA) encoding the SB transposase which is capable of inserting the CFTR expression cassette into the genome, leading to an efficiently and properly regulated transposition. For this purpose, several technical aspects have to be developed to establish this novel technology in the future in a clinical setting: i) investigate the stability of nucleic acids during nebulization since this way would be the most convenient and efficient means of delivery nucleic acids to the airways of patients; ii) demonstrate proof of concept for the combined mRNA/SB based expression cassette in vitro

iii) analyse long-term expression of mRNA/SB based expression in airway epithelia.

Methods: Ad i) analyse delivery of nucleic acids into the lung, nebulization experiments with a PARI Boy® Nebulizer were performed. IVT metridia luciferase mRNA and green fluorescent protein (GFP) mRNA complexed with PEI branched or Lipofectamine 2000 were nebulized and used to transfect human bronchial epithelia cells (16HBE and BEAS-2B). Transfection efficiency was evaluated by measuring luciferase activity and numbers of GFP positive cells, accordingly. Ad ii) In order to find the right ratio for pDNA and mRNA encoding the SB transposase, 4 cell lines (16HBE, BEAS-2B and mouse cell lines mSEC1 CF and mSEC1 Non-CF) were transfected with 250 ng pDNA carrying a luciferase transgene and different amounts of mRNA. Ad iii) Luciferase activity in supernatants was measured at indicated time points over time in airway epithelial cells.

Results: Ad i) We tested two different kinds of non-viral IVT mRNA complexes, mRNA polyplexes as well as mRNA lipoplexes, and could demonstrate stability of IVT mRNA during nebulization. Although the stability is confirmed with both complexes, cationic lipids show a higher transfection potential compared to polymers such as branched PEI. Ad ii) By delivery of pDNA, carrying a luciferase transgene, together with IVT mRNA encoding the SB transposase which allows a targeted integration control, we have established a long-term transgene expression system and optimized ratios for pDNA and IVT mRNA. Ad iii) High levels of luciferase activity were detectable as long as 20 days post transfection, a ratio of 1:50 between donor plasmid and SB mRNA is the most efficient.

Conclusion: Ad i) We could demonstrate that IVT mRNA can be nebulized and is not destroyed by the nebulization process; Ad ii) combination of a plasmid-based transposon (pDNA) including a transgene with stabilized, non-immunogenic mRNA (SNIM® RNA) encoding the SB transposase is capable of inserting a transgene into the genome of respiratory epithelial cells.

Generation of disease-specific iPSCs and development of transgenic reporter cell lines for cystic fibrosis disease modelling and drug screening

Authors: M. Schubert, S. Merkert, A. Haase, L. Engels, R. Haller, N. Lachmann, T. Moritz, J. de la Roche, B. Tümmler, L. Galietta, U. Martin

Cystic fibrosis (CF) is a genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene coding for a cAMP-activated chloride-channel expressed in different epithelia. So far, immortalized cell lines overexpressing mutant CFTR-variants have been used to screen compound libraries. In fact, CFTR-protein-modulators have been identified, but show modest effects at best. Obviously, the complexity of the mutant CFTR-maturation and turnover kinetics including the influence of genetic modifiers require the use of advanced personalized cellular models that closely recapitulate the properties of the most clinically affected organs. To address these unmet needs we focus on the generation of induced pluripotent stem cell (iPSC) lines from CF-patients homozygous for F508del mutation with mild and severe phenotype and with known intragenic recombination. Therefore, CF-iPSCs were generated via reprogramming of $CD34^+$ cells isolated from small volumes of non-mobilized peripheral blood. The resulting CF-iPSCs were analysed regarding their karyotype, pluripotency status and potential to differentiate. Moreover, different transgenic iPSC and embryonic stem cell (ESC) lines were generated overexpressing a halide sensitive yellow fluorescent protein (YFP) monitoring CFTR-function [1], in combination with the overexpression of an artificial CFTR or an endogenous CFTR-Tomato-fluorescence-reporter.

Several CF-iPSC lines were established and characterized in detail. The generated YFP-reporter cell lines showed stable transgene expression also during *in vitro* differentiation. Preliminary measurements to test the CFTR dependent halide sensitivity of our transgenic cell lines confirmed the functionality of the overexpressed YFP reporter, which was comparable to a positive control cell line. Differentiation of YFP-expressing CFTR-Tomato-reporter iPSCs towards cholangiocytes revealed $YFP^+/Tomato^+$ cells, displaying CFTR channel specific response after Forskolin application. Hence, the stable integration of the halide reporter into CF-patient-specific iPSCs in combination with integration of the

CFTR-Tomato-reporter should enable disease modelling of F508del-based CF with regard to the individual genetic context and the implementation of high-throughput screening for novel correctors and potentiators of CFTR-trafficking mutations.

[1] Galietta LJ, Haggie PM, Verkman AS. Green fluorescent protein-based halide indicators with improved chloride and iodide affinities. FEBS letters. 2001;499(3):220-4. Epub 2001/06/26

Generation and functional characterization of epithelial cells with stable expression of SLC26A9 Cl- channels

Authors: J. J. Salomon, S. Spahn, X. Wang, J. Füllekrug, C. A. Bertrand, M. A. Mall

Previous in vivo results revealed that the epithelial Cl- channel SLC26A9 is essential for preventing airway obstruction associated with IL13-induced airway inflammation (Anagnostopoulou et al. 2012). Two mutations in the SLC26A9 gene were recently found in patients with bronchiectasis (Bakouh et al. 2013). These results suggest that SLC26A9 may be a promising alternative Cl- channel in cystic fibrosis (CF). However, the picture of understanding the regulation of SLC26A9 Cl-channels at the epithelium is far from being complete. We therefore generated and characterized a novel epithelial cell line with stable expression of SLC26A9. As an in vitro epithelial cell model, Fisher rat thyroid (FRT) cells were used and stably transduced with a HA-tagged SLC26A9 construct. The expression was confirmed using RT-PCR and Western blotting. Whole-cell patch clamp experiments were performed. Transepithelial Cl- currents were measured by applying a Cl- gradient in Ussing chambers. The effect of knockdown of osmotic-stress sensing kinases (e.g. WNKs) on Cl- currents was studied. SLC26A9 expression in transduced FRT (FRT-SLC) cells was found at high levels on mRNA and protein level. Biotinylation assays detected SLC26A9 at the cell membrane. Whole-cell patch clamp recordings confirmed a significantly augmented Cl- current in FRT-SLC cells ($Im=-249\pm65$ pA, $P<0.05$), when compared to control ($Im=-86\pm16$ pA). Transepithelial measurements revealed that the basal short circuit current (Isc) was significantly increased in FRT-SLC (12.3 ± 2.0 µA/cm², $P<0.01$) compared to control-transfected FRT (FRT-CTL; 3.9 ± 0.5 µA/cm²) cell monolayers. CAMP (IBMX/forskolin)-stimulated Cl- secretion was significantly increased in FRT-SLC compared to FRT-CTL cells ($\Delta Isc=4.9\pm0.5$ vs.

ΔIsc=0.7±0.2 μA/cm², P<0.01). In RNAi studies, knock-down of the kinase WNK1 in FRT-SLC epithelial monolayers diminished the basal Isc to 81.6±7.4% compared to control-treated FRT-SLC cells. We established a novel stable HA-tagged SLC26A9-expressing FRT epithelial cell line which provides a valuable cell model for further SLC26A9 functional assays. SLC26A9 contributes to constitutive and cAMP-stimulated Cl- currents that are regulated by WNK1. This model may facilitate identification of activators of SLC26A9 Cl- channels that may compensate deficient Cl- transport in CF patients and may serve as a novel therapeutic target in CF.

Keynote Session

Advances and challenges in pharmacological rescue of CFTR

Author: M. Gentzsch

The development of drugs that directly enhance CFTR function offers an exciting approach for pharmacological treatment of cystic fibrosis (CF). Potentiator ivacaftor (VX-770, trade name Kalydeco) is the first FDA-approved CFTR modulator for treatment of CF patients with a G551D CFTR mutation. A major goal of CF research is to develop therapies that replicate the remarkable success that VX-770 achieved in G551D CF patients, for all CF individuals. Orkambi, a therapy that combines VX-770 with corrector lumacaftor (VX-809), has recently been approved by the FDA for treatment of CF patients that are homozygous for the misfolding CFTR mutation, ?F508. This corrector-potentiator treatment was slightly beneficial in clinical trials, but less effective than VX-770 for G551D patients. Our studies conducted in ?F508/?F508 human bronchial epithelial (HBE) cultures chronically treated with VX-809 and VX-770 indicated a decrease in VX-809-corrected ?F508 CFTR function due to destabilization by VX-770. Thus, chronic treatment with CFTR potentiators and correctors may require careful consideration of dosing, and development of new potentiators that do not interfere with CFTR stability.

Most CF patients carry the ?F508 mutation; however, ~2,000 different CFTR mutations have been identified. Therefore, the precise identification of therapies that effectively modulate CFTR in patients with rare CFTR mutations is essential. We tested the suitability of VX-809 and VX-770 for rescue of several rare CF mutations by Ussing chamber and Western blot analyses. HBE cells from CF patients carrying R117H, classified as a conductance mutation, showed benefit from chronic treatments with VX-809 or VX-770 and even larger CFTR-mediated responses when used together, suggesting that this protein misfolds but is more stable than ?F508. P67L is also classified as a CFTR conductance mutant but in cells from patients with a P67L mutation we again observed misprocessing and correction of this defect by VX-809. VX-770 was most effective at lower concentrations and displayed a strong synergistic effect with VX-809, resulting in CFTR function similar to what is observed with normal CFTR. HBE cells bearing the CFTR misprocessing mutation, N1303K, did

not show marked rescue with VX-809, unlike ?F508. CFTR nonsense mutations, G542X and W1282X, did not produce CFTR protein even with VX-809 treatment. The rare mutation, S1251N, has a gating defect and the FDA has approved use of VX-770 for patients with this mutation. We found that in HBE cells expressing S1251N, the most effective improvement occurs with VX-770 at lower concentrations and combined with VX-809. Taken together, different classes of CF mutations respond differently to therapies.

Strikingly, there is not only heterogeneity of responses to therapies between different mutations, but also variability among patients with identical mutations. In vitro experiments with cultured heterologous-expressing cell lines and in silico calculations of ??G (free energy difference; ??G = ?G mutant – ?G wild type) may allow for assessment of stability of different CFTR mutants. However, these approaches do not consider the in vivo environment of endogenous CFTR that may be accountable for the dissimilar responses of mutations in the same class. Because cells from patients with identical CFTR mutations may show diverse drug responses, personalized treatment strategies are necessary. As primary CF HBE with specific mutations are limited by acquisition of donated lungs, we produced conditionally reprogrammed cells (CRC) to expand supplies of HBE and human nasal epithelial cells with defined CFTR mutations. CRC cultures retained CFTR function over multiple passages, allowing for investigation of genotype-specific rescue of CFTR by small-molecule therapies. We are also assessing CFTR function in a physiologically relevant ex vivo assay using 3D organoids readily prepared from bronchial and nasal tissues. These bronchospheres and nasospheres allow detection of acute volume changes in response to CFTR activation, quantified by measurements using live-cell imaging. The ultimate goal is the development of precise models that utilize cells acquired from CF patients to test different combinations of CFTR modulators for designing optimal treatment strategies tailored for each patient.

Research is currently underway to 1) develop novel models for drug evaluation and identify means to rescue rare CFTR mutations, 2) understand mechanistic details on how environmental factors affect CFTR rescue, 3) assess the significance of CFTR rescue for mucociliary clearance in CF airways, and 4) evaluate the diverse roles of CFTR and efficacy of CFTR modulators in different tissues. Knowledge obtained from these studies will be applied towards designing and optimizing assays with biologically relevant outcome measures for personalized

CFTR-targeting therapeutics, which will result in the development of successful treatment plans for each individual CF patient.

[Supported by Else Kröner-Fresenius-Stiftung, CF Foundation, and National Institutes of Health].

Session 2

Moving forward with CF gene therapy

Abstract written by U. Griesenbach; talk given by S. Hyde

Although a large number of CF gene therapy trials have been carried out over the last two decades, these have not addressed whether gene transfer to the lungs can ameliorate a clinically relevant endpoint. The UK CF Gene Therapy Consortium has developed a programme of both non-viral and lentivirus-mediated gene therapy for CF. The former has identified the optimal plasmid, liposome and delivery device. A Phase IIa open label single-dose safety study has identified a safe dose suitable for repeated administration. A multi-dose toxicology programme in mice has shown a cumulative effect of repeated administration, with 12 doses producing a uniform response equivalent to ~100% of endogenous Cftr mRNA levels. We next undertook a randomized, double-blind, placebo-controlled Phase IIb trial of the CFTR gene/liposome complex pGM169/GL67A. Subjects received 5 ml of nebulised pGM169/GL67A (Active) or saline (Placebo) at 28 ± 5 day intervals over 1 year. The primary endpoint was the relative (%) change in percent predicted FEV1.The pre-specified Per Protocol analysis (≥9 doses) included 54 receiving Placebo (62 randomized) and 62 receiving gene therapy (78 randomized). Subgroups of patients were enrolled for gene expression measurement in both nose and lower airway via bronchoscopy. The trial met its primary endpoint and results will be presented. The talk will also include an update on using lentiviral vectors for CF gene therapy, with a first-in-man trial scheduled for 2016.

The non-viral multidose trial is funded by the National Institute for Health Research's EME programme. Work leading up to the trial was funded by the UK CF Trust.

New mechanisms of CF airways inflammation in the absence of bacterial infection

Author: M. A. Mall

Recent studies in infants with cystic fibrosis (CF) detected neutrophilic inflammation and small airways mucus obstruction as early abnormalities that are often present in the apparent absence of bacterial infection. In

many organs, hypoxic cell death triggers sterile neutrophilic inflammation via Interleukin-1 receptor (IL-1R) signaling. Although hypoxia is common in airways from patients with cystic fibrosis (CF), its role in neutrophilic inflammation has not been studied. We recently demonstrated that hypoxic epithelial necrosis due to airway mucus obstruction precedes neutrophilic inflammation in Scnn1b-transgenic (Scnn1b-Tg) mice with CF-like lung disease. We therefore determined the role of epithelial necrosis and IL-1R signaling in the development of neutrophilic airway inflammation, mucus obstruction and structural lung damage in CF lung disease. To achieve this goal, we used genetic deletion and pharmacological inhibition of IL-1R in Scnn1b-Tg mice and determined effects on airway epithelial necrosis, levels of IL-1α, KC and neutrophils in bronchoalveolar lavage and mortality, mucus obstruction and structural lung damage. Further, we analyzed lung tissues from patients with CF and non-CF controls for the presence of epithelial necrosis. We found that lack of IL-1R had no effect on epithelial necrosis and elevated IL-1α, but abrogated airway neutrophilia and reduced mortality, mucus obstruction and emphysema in Scnn1b-Tg mice. Treatment of adult Scnn1b-Tg mice with the IL-1R antagonist anakinra had protective effects on neutrophilic inflammation and emphysema. Furthermore, numbers of necrotic airway epithelial cells were elevated and correlated with mucus obstruction in patients with CF. These results support an important role of hypoxic epithelial necrosis in the pathogenesis of neutrophilic inflammation independent of bacterial infection and suggest IL-1R as a novel target for anti-inflammatory therapy in CF and potentially other muco-obstructive airway diseases.

Neutrophil elastase activity on the surface of sputum neutrophils is associated with pulmonary hyperinflation and airflow obstruction in cystic fibrosis

Authors: A. S. Dittrich, N. Heath, M. Wiebel, F. Herth, C. Schultz, M. A. Mall

Introduction: Neutrophilic airway inflammation plays a central role in cystic fibrosis (CF) and previous studies identified free neutrophil elastase (NE) in bronchoalveolar lavage fluid and sputum as a key risk factor of early bronchiectasis and decline in lung function. However, the relevance of membrane-associated NE activity in the pathophysiology of CF lung disease is not well understood. In this cross-sectional, prospective study, we used ratiometric Foerster resonance energy

transfer (FRET) reporters to determine membrane-associated NE activity on the surface of neutrophils (NEmo-2) in CF sputum. NE activity levels were then correlated with measurements of pulmonary function.

Methods: Inflammatory cells and cell-free supernatants were isolated from spontaneous and induced sputum of patients with CF (n=45) and healthy non-smokers (control, n=9). For the quantification of membrane-associated NE activity, 20,000-30,000 cells were incubated with NEmo-2 for 10 min (T10). To control for NE-specific cleavage of NEmo-2, cells were pre-incubated with the NE inhibitor sivelestat (T0). NE activity was calculated as the ratio of donor and acceptor fluorescence measured by confocal microscopy and normalized to samples treated with sivelestat (T10/T0).

Results: In CF, absolute (CF: 4.84 [2.10 to 15.00] vs. control: 0.75 [0.36 to 1.09], 10^5/ml, $p<0.001$) and differential neutrophil counts (CF: 95.75 [92.50 to 97.25] vs. control: 36.35 [23.75 to 52.75], %, $p<0.001$) were significantly increased compared to control. CF was associated with elevated levels of membrane-associated NE activity on the surface of sputum neutrophils (CF: 1.61 [1.36 to 1.77] vs. control: 1.09 [1.05 to 1.15], norm. D/A-ratio, $p<0.001$). However, membrane-associated NE activity was not correlated with the total number (rho=0.15, p=0.34) or percentage of neutrophils in CF sputum (rho=0.29, p=0.06). Of note, membrane-associated NE activity correlated with both, airflow obstruction (FEV1% predicted: rho=-0.39, $p<0.01$ and FEV1/VC: rho=0.50, $p<0.001$) and pulmonary hyperinflation (residual volume: rho=0.57, $p<0.001$ and intrathoracic gas volume: rho=0.56, $p<0.001$).

Conclusion: We conclude that NE activity is elevated on the surface of neutrophils in CF airways. Our findings suggest that membrane associated NE activity may contribute to severity of lung disease and serve as a valuable biomarker in patients with CF.

Microevolution of *Pseudomonas aeruginosa* in cystic fibrosis lungs

Authors: N. Cramer, J. Klockgether, S. Fischer, B. Tümmler

Objectives: Serial *P. aeruginosa* isolates in half year intervals from 35 CF patients at our local CF clinic who became colonized with *P. aeruginosa* in the 1980s were selected. The microevolution of *P. aeruginosa* in CF lungs

was investigated in the six patients with the mildest and the six patients with the most severe course of their chronic *P. aeruginosa* infection.

Methods: Serial isolates were genotyped with a customized microarray. Sequential isolates of the initially colonizing clone were then subjected to whole genome sequencing by SOLiD5500 technology. Nucleotide variations compared to the PA14 genome were extracted, filtered, annotated and used for the reconstruction of clades. The 250 sequenced bacterial isolates were characterized in mutation rates, morphology, motility and secretion of virulence effectors.

Results: Exopolysaccharide biosynthesis, antimicrobial resistance and global regulators of lifestyle and metabolism are the most common functional categories whose genes were hit by mutations in the CF lungs. Microevolution was not uniform. The *P. aeruginosa* clone inhabiting severely affected lungs repetitively generated descendants with stop mutations or drastic amino acid changes in key genes of lifestyle, but these loss-of-function mutants were not recovered later. In contrast, *P. aeruginosa* clones predominantly acquired benign amino acid substitutions in patients who maintained a normal function of their chronically colonized lungs for up to 30 years.
Conclusion: Modes of microevolution of *P. aeruginosa* in CF lungs are associated with the severity of the chronic lung infection.

Physico-chemical compatibility of mixed inhalation solutions for nebulizers

Authors: W. Kamin, M. Federici, I. Krämer, Hannah Walz-Jung

Introduction: Many patients suffering from cystic fibrosis (CF) rely on inhalation therapy with nebulizers. Often they need to inhale several different drugs per day, including antibiotics. To save time an estimated 25 % of these patients mix the nebulizer solutions for simultaneous inhalation. This popular practice may cause problems in terms of physico-chemical compatibility and stability of the mixed drugs.

Methods: Physical compatibility of the inhalation mixtures was determined by visual inspection for any changes up to 24 hours after mixing as well as by measuring pH and osmolality.Mixtures were designated as physical incompatible when color or odor changes, haze or precipitation occurred. Chemical compatibilities were determined as follows:

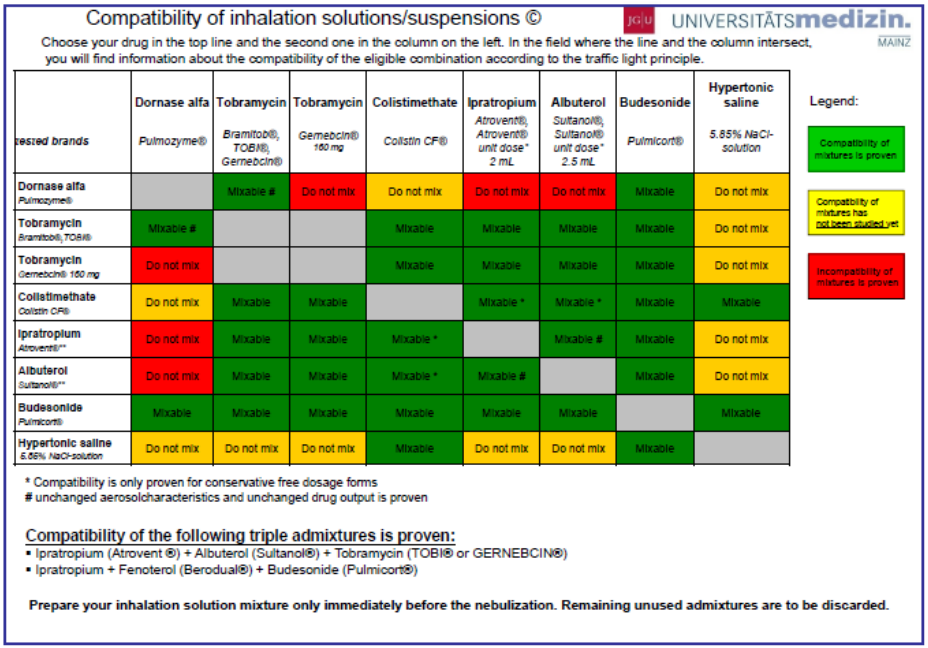
Dornase alfa	Kinetic colorimetric DNase activity assay

	Size-exclusion high performance liquid chromatography (SEC) Ultraviolet spectroscopy Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-Page) Tentacle strong cation-exchange chromatography
Tobramycin	Fluorescence immunoassay
Colistimethate	Microbiological assay of antibiotics (agar diffusion assay)
Budesonide	RP-HPLC: Stability-indicating high-performance liquid chromatography assay with ultraviolet detection
Ipratropium bromide	RP-HPLC
Albuterol	RP-HPLC

Results: Results of in vitro compatibility studies with CF relevant nebulizer solutions/suspensions are shown in our compatibility table. Tested solutions are mostly compatible with each other, but certain excipients contained in some of the drug products caused incompatibilities. Especially the activity of dornase alfa was impaired by sodium metabisulfite used as an excipient in Gernebcin®, by benzalkonium chloride used in Atrovent® and Sultanol®, and by disodium edetate used in Atrovent®. Concerning hypertonic saline we found compatibility with Colistimethate (Colistin CF®) and Budesonide (Pulmicort®).

Conclusion: More drugs, including the recently approved antibiotic aztreonam (Cayston®), need to be tested when mixed with 5.85% sodium chloride solution, since nebulized hypertonic saline has been established as an effective adjunctive therapy for respiratory symptoms in CF patients. Final recommendations for the inhalation of the tested mixtures cannot be made before clinical studies have been successfully performed.

References: Kamin W et al. Inhalation solution: Which ones may be mixed? Physico-chemical compatibility of drug solutions in nebulizers - Update 2013. J Cyst Fibros. 2014; 13(3): 243-50



Compatibility of inhalation solutions/suspensions ©

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Choose your drug in the top line and the second one in the column on the left. In the field where the line and the column intersect, you will find information about the compatibility of the eligible combination according to the traffic light principle.

tested brands	Dornase alfa *Pulmozyme®*	Tobramycin *Bramitob®, TOBI®, Gernebcin®*	Tobramycin *Gernebcin® 160 mg*	Colistimethate *Colistin CF®*	Ipratropium *Atrovent®, Atrovent® unit dose* 2 mL*	Albuterol *Sultanol®, Sultanol® unit dose* 2.5 mL*	Budesonide *Pulmicort®*	Hypertonic saline *5.85% NaCl-solution*
Dornase alfa *Pulmozyme®*		Mixable #	Do not mix	Do not mix	Do not mix	Do not mix	Mixable	Do not mix
Tobramycin *Bramitob®,TOBI®*	Mixable #			Mixable	Mixable	Mixable	Mixable	Do not mix
Tobramycin *Gernebcin® 160 mg*	Do not mix			Mixable	Mixable	Mixable	Mixable	Do not mix
Colistimethate *Colistin CF®*	Do not mix	Mixable	Mixable		Mixable *	Mixable *	Mixable	Mixable
Ipratropium *Atrovent®***	Do not mix	Mixable	Mixable	Mixable *		Mixable #	Mixable	Do not mix
Albuterol *Sultanol®***	Do not mix	Mixable	Mixable	Mixable *	Mixable #		Mixable	Do not mix
Budesonide *Pulmicort®*	Mixable	Mixable	Mixable	Mixable	Mixable	Mixable		Mixable
Hypertonic saline *5.85% NaCl-solution*	Do not mix	Do not mix	Do not mix	Mixable	Do not mix	Do not mix	Mixable	

Legend:

Compatibility of mixtures is proven

Compatibility of mixtures has not been studied yet

Incompatibility of mixtures is proven

* Compatibility is only proven for conservative free dosage forms
# unchanged aerosolcharacteristics and unchanged drug output is proven

Compatibility of the following triple admixtures is proven:
- Ipratropium (Atrovent ®) + Albuterol (Sultanol®) + Tobramycin (TOBI® or GERNEBCIN®)
- Ipratropium + Fenoterol (Berodual®) + Budesonide (Pulmicort®)

Prepare your inhalation solution mixture only immediately before the nebulization. Remaining unused admixtures are to be discarded.

Modulation of the Hsp90-Aha1 Chaperone System to correct the folding deficiency of the CFTRdeltaF508 protein in Cystic Fibrosis
Author: W. M. J. Obermann

Deletion of a single phenylalanine residue at position 508 of the protein CFTR (Cystic Fibrosis Transmembrane Conductance Regulator), a chloride channel in lung epithelium, is the most common cause for Cystic Fibrosis. As a consequence, folding of the CFTRdeltaF508 protein and delivery to the cell surface are compromised resulting in degradation of the polypeptide. Accordingly, decreased surface presence of CFTRdeltaF508 causes impaired chloride ion conductivity and is associated with mucus accumulation, a hallmark of Cystic Fibrosis. Molecular chaperones such as Hsp90 and its co-chaperone partner Aha1 play a key role in targeting folding deficient CFTRdeltaF508 for degradation. Thus, pharmacological manipulation to inhibit Hsp90-Aha1 chaperone complex formation appears beneficial to inhibit proteolysis of CFTRdeltaF508 and rescue its residual chloride channel activity.

Therefore we have screened a collection of 14,400 drug-like chemical

compounds for inhibitors of the Hsp90-Aha1 complex by Amplified Luminiscence Proximity Homogeneous Assay. Eleven true hits were identified out of which two showed promising results when we tested their ability to restore chloride channel activity in cellular systems that express the mutant CFTRdeltaF508 protein. One molecule was most effective and may therefore serve as a lead compound that can be further developed into a drug to treat cystic Fibrosis patients.

Silent but visible: a common silent polymorphism in CFTR alters physiological function and global protein conformation

Authors: S. Kirchner, R. Rauscher, L. Ostedgaard, I. Braakman, D. Sheppard, Z. Ignatova

Introduction: Synonymous single-nucleotide polymorphisms (sSNPs) in the coding sequence are considered neutral for protein function, as by definition they affect only codons, not amino acids. We investigated T2562G, one of the most common sSNPs in the cystic fibrosis transmembrane conductance regulator (CFTR) gene; this silent mutation is associated with the genetic disease cystic fibrosis (CF) and an increased risk of CFTR-related diseases.

Methods: We used Western Blots and Microarrays to determine the protein and tRNA levels in a human bronchial epithelial cell line and primary cells derived from CF-patients. Moreover we examined single channel and ussing chamber experiments to learn about CFTR-channel properties.

Results: Microarray experiments reveal that the mutant ACG codon is read by a raretRNA, suggesting that T2562G sSNP most likely alters the local ribosomal speed along mRNA. Increasing the cellular concentration of tRNAThr cognate to the mutant ACG codon rescued the folding and functional defects of T2562G-CFTR.

Conclusion: These findings reveal an unexpected mechanism for sSNP-associated diseases. The speed of proteinbiosynthesis affects CFTR folding and ultimately channel behaviour, thus sSNPs should be considered modulating rather than silent.

Allele specific master transcription factor complexes assembly or disruption on a regulatory FAS SNP modifies CF disease outcome

Authors: C. Awah, S. Tamm, S. Hedtfeld, D. Steinemann, B. Tümmler, F. Stanke

Cystic fibrosis is a life threatening disease affecting about 1:2,500 people among the caucasians. The patients with the same cystic fibrosis transmembrane receptor mutations have been shown to have different clinical outcome. We identified the FAS SNP rs7901656 that associates with the differential clinical outcome in CF patients. We have shown that some master regulator transcription factor complexes binds preferentially to the C-allele of rs7901656 FAS in a co-operative manner, while another master regulator transcription factor complex binds the T-allele of rs7901656 in a competitive manner. This preferential assembly of master transcription factors modulate FAS expression in allele specific manner in CF patients affecting disease outcome. We performed associations studies on EU CF Twin and Sibling cohort and found that the master transcription factor variation in patients homozygous CFTR508del affects the disease manifestation.

Our definition of how these master regulator transcription factor complexes are assembled or disrupted on the regulatory SNP offers a new therapeutic target for personalized medicine i.e. protective programmed cell death and modified response to FAS activation could enhance cytokine secretion to invading bacteria which could be of utmost importance to CF patients.

Closing talk

Development of nanobodies against CFTR

Authors: M. Sigoillot, D. François, M. Overtus, M. Grodecka, L. He, J. R. Riordan, J. Steyaert, T. Laeremans, C. Govaerts

The deletion of the residue phenylalanine 508 (F508) in the first nucleotide binding domain (NBD1) of CFTR prevents folding of the whole protein leading to its clearance by the quality control system and defects in channel gating. It is established that F508 mutation affects NBD1 thermodynamic stability and possibly the interface between the NBD and the TM domain. Thus, chaperones that target this region are the most likely to recover protein stability, a first step towards recovering transport activity. On the other hand, current corrector molecules stemming from high throughput screening do not appear to significantly improve DeltaF508-CFTR thermostability, although this property appears to be a key feature in the molecular origin of the disease. Therefore, there is a need to develop CFTR-specific chaperones with the ability to improve the thermostability of DeltaF508 mutant.

We are developing our research project aims at developing small antibodies fragments named nanobodies as therapeutic tools against cystic fibrosis to study and possibly act on CFTR. Nanobodies are recombinant single domain antibodies of naturally occurring heavy-chain- only antibodies found in camelids and they present a number of remarkable properties such as high affinity, high stability and ability to recognize specific conformational epitopes. Here, we report the identification and characterization of nanobodies with CFTR-stabilizing properties that could act as correctors for the DeltaF508 mutant and help overcome the functional deficiency. We immunized llamas with several forms of CFTR, namely purified full length CFTR in detergent, purified full length CFTR reconstituted in liposomes and soluble domain of CFTR (ie NBD1). We isolated a large collection of binders and specifically, we identified three different families of nanobodies that bind distinct epitopes of NBD1 CFTR and recognize full-length CFTR in a cellular context.

Furthermore, all the nanobodies generated bind conformational epitopes of NBD1. Differential scanning calorimetry and fluorescence thermal shift assays demonstrated that both WT and dF508-NBD1 are thermally stabilized upon binding of different nanobodies. To our knowledge, this is

the first evidence for conformational binders to CFTR. We are currently investigating the functional potential of the different binders using various methods including binding in native vs. denaturing condition, flow cytometry in CFTR expressing cells and thermostabilization assays. Our approach aims at identifying binders with the necessary features of a CFTR-specific molecular chaperone/corrector, which could open new avenues to develop CFTR correctors.

This initial success demonstrates our ability to identify binders with the necessary features of a CFTR-specific molecular chaperone/corrector and will open new avenues to develop CFTR correctors with thermostabilizing properties.

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