21st Scientific Meeting

Innovative Therapies in CF

29th – 30th September 2022

Schloss Montabaur (Germany)

MUKOVISZIDOSE
Mukoviszidose Institut gGmbH

Organization

Scientific advisory board of the German Research Community for Cystic Fibrosis (FGM) & Mukoviszidose Institute gGmbH (MI)

Chairs

PD Dr. Frauke Stanke (Hannover)
Dr. Simon Gräber (Berlin)

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	Member of the Federal Executive Board Mukoviszidose e.V. Anna-Maria Dittrich (DE/Hannover)
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1:45-2:15 pm	**Chloride-transport mechanisms of SLC26A9** Jan-Philipp Machtens (DE/Jülich)
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	Exploring CFTR functions to identify novel therapeutic strategies John Hanrahan (CA/Montreal)

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11:30-11:45 am	**Correction of the drug-refractory CFTR mutation L227R by prime editing** *(Abstract Talk)* Mattijs Bulcaen (BE/Leuven)
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12:45-1:15 pm	**Phage-inspired antimicrobials** Zuzanna Drulis-Kawa (PL/Wrocław)
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1:30 pm	**Closing of the Meeting**

Session I: Alternative channels

Alternative chloride transporter SLC26A9 as a novel therapeutic target in cystic fibrosis

Anita Balazs

The solute carrier family 26, member 9 (SLC26A9) is an epithelial chloride transporter that is expressed in several organs affected in patients with cystic fibrosis (CF) including the lungs, the pancreas, and the intestine. In vitro studies suggest a multifaceted functional interaction between SLC26A9 and CFTR that may be implicated in normal transepithelial chloride secretion in health, as well as impaired chloride/fluid transport in CF. Genetic association studies and investigations utilizing SLC26A9-deficient mice identified SLC26A9 as a disease modifier and supported an important role of this alternative chloride transporter in the pathophysiology of several organ manifestations in CF. These data suggest that targeting SLC26A9 may be an attractive therapeutic strategy to restore epithelial chloride secretion in patients with CF, irrespective of their CFTR genotype. In addition, pharmacological activation of SLC26A9 may help to augment the effect of CFTR modulator therapies. Future development of compounds that may increase the expression, trafficking, and stability of SLC26A9 protein are needed to explore this alternative therapeutic target in CF and potentially in other muco-obstructive lung diseases.

Chloride-transport mechanisms of SLC26A9

Jan-Philipp Machtens

The epithelial anion transporter SLC26A9 is involved in various physiological functions in the gastrointestinal tract and the lungs, including airway surface hydration and mucus clearance, and has emerged as a possible disease modifier of cystic fibrosis. SLC26A9 mediates uncoupled Cl- transport across the cell membrane. However, the molecular mechanisms and the conformational changes underlying its molecular functions are not fully understood.

For example, it is unclear whether SLC26A9 mediates channel-like, conductive transport of Cl- ions as an ion channel or operates as an alternating-access transporter. Therefore, despite being a promising therapeutic target in cystic fibrosis, the lack of structural and mechanistic understanding of its molecular physiology impaired the development of specific pharmacological modulators of SLC26A9 so far.

Recently, cryo-electron microscopy structures in the so-called inward-facing conformation provided first insights into the molecular architecture of SLC26A9 at near-atomic resolution. These structures enabled us to study its physiological transport functions using atomistic molecular-dynamics (MD) simulations. Using a combination of extensive MD simulations, machine learning, and Markov state modeling, we explored the conformational landscape of SLC26A9 and observed hitherto unknown conformations, including an outward-facing and an open-channel conformation. Surprisingly, our simulations demonstrate that SLC26A9 can mediate Cl- transport using both slow alternating-access and fast channel-like mechanisms. However, whereas the former mechanism may represent an evolutionary relic of the SLC26 transporter family, our Computational

Electrophysiology simulations suggest that only channel-mediated transport exhibits the Cl- conductance and selectivity required to fulfill its physiological function. Combined with electrophysiological experiments, we aim for a validated understanding of Cl- transport by SLC26A9 and hope to guide rational drug design to support the treatment of cystic fibrosis.

Role of inflammatory stimuli in modulating ion transport in the airway epithelium

Luis Galietta

The properties of the airway surface fluid (ASF) are controlled by the activity of ion channels and transporters. In cystic fibrosis (CF), loss of CFTR chloride channel function causes airway surface dehydration and impairment of mucociliary clearance (MCC) that leads to bacterial infection and airway inflammation. Inflammation may alter the expression/function of CFTR and other ion transport proteins thus modifying ASF properties and further disrupting MCC. Our aim was to investigate the effects of a panel of cytokines to understand how inflammation is involved in CF lung disease.

We used cultured bronchial epithelia from CF and non-CF individuals differentiated under air-liquid interface condition. Gene expression was determined by bulk and single-cell RNAseq. Electrogenic ion transport was evaluated with short-circuit current recordings. The properties of the ASF were studied with the Fluorescence Recovery After Photobleaching (FRAP) technique.

We found that the treatment with IL-17 plus TNF-α, two cytokines with an important role in CF and other chronic obstructive respiratory diseases, induces a profound change of epithelial cell transcriptome with upregulation of several genes involved in ion transport, anti-bacterial defense, and neutrophil recruitment. In particular, the treatment modified the expression of the SLC26A4 anion exchanger and the ATP12A proton pump. The overall result of IL-17/TNF-α treatment was a marked dehydration and hyperviscosity of ASF as demonstrated by FRAP experiments. Interestingly, a pharmacological SLC26A4 inhibitor was able to improve the fluidity of ASF in CF epithelia thus indicating that chloride/bicarbonate exchange mediated by SLC26A4 is in some way involved in airway surface dehydration. These results suggest that SLC26A4 (a.k.a. pendrin) is a possible therapeutic target to ameliorate MCC in CF, particularly for patients carrying mutations insensitive to present CFTR modulators.

Activation and pharmacological mechanisms in the calcium-activated chloride channel TMEM16A

Andy K. M. Lam

The calcium-activated chloride channel TMEM16A is a ligand-gated anion channel that opens in response to an increase in intracellular calcium concentration. Its involvement in many epithelial processes has attracted considerable interest as it may provide an alternative chloride efflux pathway to bypass the CFTR defect in cystic fibrosis. To facilitate TMEM16A drug discovery, our group combines cryo-electron microscopy and electrophysiology to gain molecular insights into the mechanisms and pharmacology of this channel. We have previously determined

high-resolution structures of TMEM16A in resting and activated states, which, together with functional experiments, revealed the conformational and electrostatic dynamics that impact on both activation and ion conduction. In a more recent effort, we have identified a hydrophobic gate that prevents ion conduction in the closed state, the conformational changes resulting from its disruption, and the gating transitions involved during channel activation. We determined a structure of TMEM16A in complex with a potent channel blocker, which revealed an inhibitor binding pocket in the extracellular vestibule of the hourglass-shaped pore and an open-like conformation that involves a rearrangement of several pore-lining helices. Together, these studies provide a detailed molecular view into the activation and pharmacological mechanisms in TMEM16A, which will inform structure-based drug discovery for diseases such as cystic fibrosis.

A New Oligonucleotide Antisense Therapy for all Patients with Cystic Fibrosis

Christie Mitri

Introduction: Vertex modulators have demonstrated good clinical outcomes for patients with cystic fibrosis (CF) with specific CFTR gene mutations. However, there are still patients for whom those treatments are unsuitable and require alternative CFTR-independent strategies. Anoctamin 1 (TMEM16A or ANO1), a calcium-activated chloride channel expressed in the airways of patients with CF, could compensate for CFTR deficiency. In previous studies, we showed that the expression and activity of TMEM16A were decreased in CF bronchial epithelial cells due to microRNA-9 overexpression. To this end, we have developed an antisense oligonucleotide (ASO TMEM16A) that prevents microRNA-9 from binding to TMEM16A mRNA, thereby amplifying its expression and activity.
Here, we have investigated the effects of ASO TMEM16A in vitro and in vivo on CF deregulated parameters and prepared for preclinical studies by assessing the best administration route and the ASO TMEM16A's toxicity and specificity
Methods: The experiments are performed on cell lines and primary cells with different mutations. The CF mouse model is used to study the different administration routes, complete survival data, acute toxicity, and long-term effects.
Results: The first results show that ASO TMEM16A acts directly on the TMEM16a channel without inducing calcium mobilization and is very specific to the 3'UTR of TMEM16A mRNA. ASO TMEM16A does not induce inflammatory cytokines expression nor alters either intracellular calcium mobilization or cell proliferation. While studying different administration routes on mice, we showed that ASO TMEM16A is detectable 30 days after subcutaneous injection or intranasal instillation. CF mice that typically die of intestinal obstruction upon weaning showed a significant lifespan increase of up to 200 days after ASO TMEM16A treatment. Acute administration of 50 times the effective dose did not show behavioral changes in the mouse, nor macroscopic or pathological changes. In addition, all of the blood markers were found to be normal. In addition, our preliminary results show an improvement in male CF mice fertility by restoring vas deferens abnormalities, also described in CF male patients.

Conclusion: This strategy could apply to all patients with CF, regardless of their mutations. It can be combined with CFTR modulators or TMEM16a potentiators to obtain additive effects and target multiple fronts, pulmonary symptoms, gastrointestinal symptoms, and male fertility problems.

Evaluation Of TMEM16A As A Modifier Of The Cystic Fibrosis Lung Phenotype Utilising CF Patient-Specific Human Induced Pluripotent Stem Cells

Ulrich Martin

Introduction: Previous studies have suggested that the calcium-activated chloride channel TMEM16A may serve as a potential pharmacological target to treat the CF lung phenotype. CFTR and TMEM16A are main contributors to chloride secretion within the airway epithelium. Interestingly, it was observed that TMEM16A expression is enhanced in CF as well as under mucus-hypersecretion associated conditions. However, the precise physiological function of TMEM16A remains largely unknown. It was hypothesised that, in CF either the stimulation of TMEM16A could improve mucociliary clearance, or that its inhibition could prevent mucus accumulation. However, so far no complex in vitro model of CF respiratory epithelial cells is available to investigate both conditions in more detail. Therefore, the project aims to utilise human induced pluripotent stem cell (hiPSC) derived respiratory epithelium with either TMEM16A knockout ($T16^{KO}$) or TMEM16A(abc) ($T16abc^{OX}$) overexpression to elucidate TMEM16A function in CF.
Methods: Gene targeting by transfection of Cas9 protein, respective guide RNAs and donor sequences was performed to obtain both, T16KO [super] and $T16abc^{OX}$ in WT and CF- hiPSCs. The $T16^{KO}$ was achieved via a 9.3 kb sequence deletion. For the $T16abc^{OX}$ a transgenic cassette was integrated into the AAVS1 locus enabling for doxycycline-inducible overexpression of the TMEM16A(abc) isoform. Further, a multistep differentiation protocol was applied to differentiated hiPSCs into respiratory epithelia in air-liquid-interface (ALI) cultures. Functional characterisation of ALI cultures was performed via Ussing chamber recordings and measurement of the cilia beating frequency.
Results: The generation of homozygous $T16^{KO}$ and $T16abc^{OX}$ hiPSC lines was achieved utilising healthy donor-derived (WT), CF patient-specific (ΔF508 homozygous) (CF) and corresponding gene-corrected (Corr) CF-hiPSCs respectively. After generation, all subclones maintained a stable karyotype and pluripotency. Subsequent differentiation of WT, CF, CF-$T16^{KO}$ and CF-$T16abc^{OX}$ hiPSCs succeeded to generate respiratory epithelia showing expression of typical epithelial markers like TUBB4A, MUC5AC and TMEM16A. Initial studies are conducted on ALI cultures to analyse TMEM16A-dependent changes of the transepithelial ion transport as well as mucus properties.
Conclusion: It will be crucial to elucidate the physiological function of TMEM16A, to define the therapeutic intervention necessary for targeting TMEM16A in CF therapy. Here, our disease model represents a valuable and unique approach to study TMEM16A in ALI cultures derived from $T16^{KO}$ and $T16abc^{OX}$ hiPSCs.
We are thankful to the Mukoviszidose e.V. for financial support.

Session II: CFTR modulation

Molecular mechanisms of small molecule correctors and their ability to rescue rare mutations

Michael Schlierf

Cystic fibrosis (CF) is one of the most common inherited diseases in the world and is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR). To date, more than 2,000 mutations have been described in patients and the numbers are steadily increasing. The most common CFTR mutation is F508del, which causes severe misfolding and misfunction of CFTR. Several small molecules have been identified over the past decade and are now increasingly approved by the FDA for the treatment of CF caused by F508del. By contrast, other CFTR mutations, particularly those found within the transmembrane (TM) regions of CFTR which cause both misfolding and channel dysfunction, are often not in the focus of larger studies. While being carried by hundreds of patients, these mutations are often identified in less than 3 patients per mutation. As a result, the molecular effects of such rare mutations are poorly understood, and even less is known about the action mechanisms of small molecules, largely also because of the inherent difficulty of probing structural changes and drug effects in CFTR TM domains. To tackle this challenge, we recently developed an assay to probe structural and dynamic effects imposed on CFTR TM domains upon mutation and drug action. Based on this proof-of-concept study, we have established a screening assay to unravel structure–activity relationships of pharmacological CFTR modulators on CFTR TM segments. We have generated a library of transmembrane hairpins bear patient derived mutations and a library of small molecules and show which small molecules can rescue misfolding of which mutations. We anticipate these results to allow further studies in cell models, followed by animal models and potentially approval for patients with rare mutations.

Predicting active compound combinations in cystic fibrosis: a comprehensive systems biology approach

Liza Vinhoven

During the last years, different small-molecule therapeutics have been developed for clinical applications, which not only alleviate symptoms of people with Cystic Fibrosis but also amplify CFTR function directly. However, most of the therapeutics developed until now only target specific mutations or mutation classes, and are consequently not effective for all patients. The latest research efforts, therefore, focus on developing combination therapies to target multiple defects at once. For this purpose, high-throughput screens have been performed, where thousands of substances have been tested in different cell models. These result in a plethora of data and various candidate compounds, often with an unclear mode of action. To elucidate the mechanism of action for promising candidate substances, support the identification of drug targets and suggest compound combinations for testing in the wet-lab, we combined different bioinformatics approaches, such as systems biological modelling, molecular docking and ligand-based target identification.

In order to provide an overview over already tested compounds, we established the publicly available database CandActCFTR (https://candactcftr.ams.med.uni-goettingen.de), where substances from experimental publications are listed and categorized according to their interaction with CFTR. It becomes apparent that for about 70 % of the active compounds it is unknown whether they affect CFTR directly through physical interaction, or indirectly through its interactome. To address this question, we modelled the life-cycle of CFTR using a computational model and annotated it with information regarding interacting compounds. The model is written in the community standard Systems Biology Graphical Notation (SBGN) format and adheres to the Minimal Information Required In the Annotation of Models (MIRIAM) guidelines. It is composed of two datasets, a core map manually curated from small-scale experiments in human cells, and a coarse map including interactors identified in high-throughput efforts. Both data layers are divided into submaps focusing on different stages of the CFTR lifecycle and different processes the ion channel is involved in. At the moment, the manually curated core map includes 225 different molecular entities and 156 reactions from 221 experimental publications. The high-throughput data layer currently encompasses 1384 unique interactors from four publications. The CFTR Lifecycle Map is publicly available online on our server, based on the MINERVA (Molecular Interaction Network Visualization) platform (https://cf-map.uni-goettingen.de), which is the standard platform used for systems biology disease maps.
To identify drug targets and elucidate the mechanism of action of active compounds, we used publicly available interaction databases and mapped the compounds to potential targets in the CFTR interactome. This data, however, still leaves the mechanism of action for the majority of the active compounds unexplained. Therefore, in order to suggest possible modes of action for all active compounds in the database, we used two complementary in silico target identification approaches, namely target-based molecular docking and ligand-based similarity searches. We identified possible targets for all active compounds, which will help to understand which compound classes affect CFTR at which stage of its life cycle and which compounds can be combined to alleviate different defects in its biogenesis. Based on these results, we can now suggest compounds and compound combinations which will be tested in the wet-lab using different established assays at the sites of our cooperation partners.

Keynot Session

Exploring CFTR functions to identify novel therapeutic strategies

John W. Hanrahan

At least three general therapeutic approaches to treatment of the basic defect in CF lung disease have been pursued since the gene was identified. The first aims to deliver normal cftr or repair the mutation, and exciting developments in gene delivery and genome editing may lead to therapies in the next few years. The second approach is to intervene at the protein level by restoring the function of mutated CFTR. Modulators that partially rescue defects in CFTR folding, trafficking, and channel function are the standard of care although they do not help all patients. The third general approach is to correct the phenotype by alleviating downstream consequences of CFTR dysfunction, for example through targeting alternative channels, reducing inflammation, improving host defense and other mechanisms. This third approach requires an understanding of CFTR and its functions, which appear more complex than was previously thought. This presentation will briefly discuss these approaches and point out gaps in our understanding of CFTR and its role in pathophysiology that could impact therapy.

Formulations for gene transfer that are efficient, safe, and can be administered repeatedly to the lung are needed for gene therapy. Repeated delivery of DNA was achieved by the UK Respiratory Gene Therapy Consortium using a liposome formulation (GL67A) and it proved to be safe and stabilized lung function, however the clinical benefit was not sufficient to warrant further development. Although much attention has shifted back to lentiviral and adeno-associated viral vectors, advances in nanotechnology and materials science may improve non-viral delivery of DNA and chemically modified RNA sufficiently for use in CF. Polymeric nanoparticles (NPs) offer design flexibility and we have previously used NPs prepared from PLGA (poly(lactic-co-glycolic acid) / PEI (polyethyleneimine) to deliver CFTR DNA to the CFBE41o- airway cell line and rat lung in vivo. Recently we have begun studying branched star, polymeric nanocarriers that can be tuned for loading both nucleic acids and drugs and conjugated with antibodies to allow targeting to specific cell types in the highly heterogeneous airway epithelium.
Pharmacotherapies have been successful, but they provide variable benefit for different patients. This has generated much interest in precision medicine, for example testing multiple drugs on well differentiated primary nasal cells or iPSCs from a patient to identify the one that is most efficacious for that individual. We compared functional correction of F508del CFTR by lumacaftor in primary bronchial epithelial cell cultures from different 20 transplant patients and confirmed that cellular responses vary significantly between individuals, supporting the potential value of precision medicine. However, we also found a significant “design effect” that results from group sampling when drugs are evaluated in vitro using a small sample of cells taken from a large population. Fortunately, increasing the number of technical replicates can solve this statistical problem, enabling drug effects to be compared in vitro and used for decision making.

Bicarbonate secretion is defective in CF airways, which lowers the pH of airway surface liquid slightly and may compromise host defense. Using custom made, flexible ion-sensitive electrodes we recently discovered large pH oscillations on the airway surface during breathing. Airway surface liquid reached surprisingly alkaline values (pH ≈ 9!) during inhalation when the airway lumen was filled with room air having low PCO2. We also found that these pH oscillations are inherently antimicrobial and defective in CF but can be restored in vivo by acute bicarbonate supplementation. Clinical trials with inhaled bicarbonate are underway at two CF centres and further studies of airway surface liquid pH regulation may improve the design of such studies and interpretation of the results. For example, the ability of the airway surface to tolerate high luminal pH suggests more alkaline buffers could be useful. It will be interesting to find out if transient alkalinizations that occur in normal airways during the breathing cycle affect mucus rheology, immunity, and other aspects of airway physiology.

Abstract Session CFTR

Changes in intestinal CFTR protein expression in p.Phe508del homozygous and compound heterozygous patients with cystic fibrosis prior and during elexacaftor-tezacaftor-ivacaftor therapy

Anna-Maria Dittrich

Introduction: Defects in expression, maturation or function of the epithelial membrane glycoprotein CFTR are causative for the progressive disease cystic fibrosis (CF). Recently, molecular therapeutics that improve CFTR maturation and functional defects have been approved. We aimed to verify whether we could detect an improvement of CFTR expression and maturation by triple therapy with elexacaftor-tezacaftor-ivacaftor (ELX/TEZ/IVA).

Methods: Rectal suction biopsies of 22 p.Phe508del homozygous or compound heterozygous CF patients obtained pre- and during treatment with ELX/TEZ/IVA were analysed by CFTR western blot that was optimized to distinguish CFTR glycoisoforms.

Results: CFTR western immunoblot analysis revealed that – compared to baseline – the levels of CFTR protein increased by at least twofold in most samples upon treatment with ELX/TEZ/IVA compared to baseline ($p < 0.02$). However, polydispersity of the mutant CFTR protein was lower than that of the fully glycosylated wild type CFTR Golgi isoform, indicating an incompletely glycosylated p.Phe508el CFTR protein isoform in patients with CF both pre- and prost-treatment.

Conclusion: Treatment with ELX/TEZ/IVA increased protein expression by facilitating the posttranslational processing of mutant CFTR but apparently did not succeed in generating the polydisperse spectrum of N-linked oligosaccharides that is characteristic for the wild type CFTR band C glycoisoform.

Nasal and colon-derived primary cells theratyping for a personalized medicine approach in CF patients carrying ultra-rare genotypes

Claudio Sorio

Introduction: More than 30% of CF patients in Italy are not eligible for current CFTR modulators treatment. Most of these patients bear rare or ultra-rare mutations, making it difficult for them to be enrolled in clinical trials as well. Thus, new personalized approach to evaluate their responsiveness to CFTR modulators are needed. We used both rectal organoids (colonoids) and, when possible, primary nasal brushed cells (hNEC) derived from the same CF patients with rare mutations to evaluate their responsiveness to CFTR modulators.

Methods: We generated colonoids and (in selected cases) reprogrammed nasal epithelial cells and analyzed Cystic fibrosis transmembrane conductance regulator (CFTR) function (forskolin-induced swelling (FIS) assay and CFTR-mediated chloride secretion in Ussing chamber) and expression (western blotting). The effectiveness of CFTR modulator therapy in some patients was also reported. CFTR dependent sweat was tested by optical beta adrenergic test (1) and chloride sweat concentration was determined following the Gibson and Cooke method.

Results: FIS and short-circuit current (Isc) measurements indicated the presence of a residual CFTR function. FIS detected a strong increase by treatment with the

CFTR potentiator ivacaftor (VX-770) and the corrector tezacaftor (VX-661). In the case carrying the ultrarare CFTR variants W57G/A234D the addition of elexacaftor (VX-445) increased the swelling, also in the absence of forskolin treatment. CFTR-mediated chloride secretion assay of colonoid monolayers indicated a residual function of 7% of wild-type (WT) CFTR, a relative enhancement of the forskolin-induced, CFTR-specific current response to VX-661 of 20% of WT CFTR reaching 28% with VX-661/VX-445 and 32% with VX-661/VX-445/VX770 combination. Increased CFTR protein levels and function were detected in both intestinal organoids and nasal brushing cultures. Patients treatment with CFTR modulators showed decreased sweat chloride (i.e., meanly about 60 mmol/L as compared to baseline), improvement of ppFEV1 (i.e., >8%) and six-minute walk test, increased body mass index after the first 8 weeks of treatment with ivacaftor. Other rare genotypes (4382delA/R1066C, F508del/711+5G>A, Q39X/T465N, L227R/L227R) will be presented for discussion.

Conclusion: Clinical parameters we recoded confirms that the response predicted on both colon and nasal epithelial cells correlates well with in vivo therapeutic endpoints, confirming the efficacy of such models as a predictor of clinical effectiveness of novel therapies in patients bearing rare ultra-rare mutations for a personalized medicine approach. Full validation of these data require to increase the number of cases studied.

Supported by: Italian Cystic Fibrosis Research Foundation (grants# 7/2016,13/2018 and 9/2020; Lega Italiana Fibrosi Cistica, Associazione Veneta- Onlus; Cystic Fibrosis Foundation grant#ASSAEL08A0.

1) Optical Measurements of Sweat for in Vivo Quantification of CFTR Function in Individual Sweat Glands.Treggiari D, Kleinfelder K, Bertini M, Tridello G, Fedrigo A, Pintani E, Iansa P, Casiraghi A, Minghetti P, Cipolli M, Sorio C, Melotti P.J Cyst Fibros. 2021 Sep;20(5):824-827. doi: 10.1016/j.jcf.2021.03.003..

Session III: Gene therapy

siRNA mediated silencing of ENaC as a therapeutic strategy for cystic fibrosis

Stephen L. Hart

Loss of the cystic fibrosis transmembrane conductance regulator (CFTR) in cystic fibrosis (CF) leads to hyperabsorption of sodium and fluid from the airway due to upregulation of the epithelial sodium channel (ENaC). This leads to thickened mucus and depleted airway surface liquid (ASL) and impaired mucociliary clearance. ENaC regulation is, thus, a promising target for CF therapy. Our aim was to investigate whether silencing of ENaC could restore appropriate epithelial ion and fluid transport in the CF airway epithelium and so offer a route to a novel therapy for CF. We had developed siRNA nanoparticle formulations comprising epithelial targeting peptides and lipids, and compared their diffusion rates through mucus and found that anionic nanoparticles, as expected, displayed enhanced mucus mobility. Transfections to silence the major protein subunit, aENaC, were then performed in air– liquid interface (ALI) cultures of CF epithelial cells. SiRNA- mediated silencing of aENaC was examined by quantitative RT-PCR with silencing of 30% achieved in ALI cultures from a single dose. This was improved to approximately 50% by three repeat doses. Correction of the ion and fluid transport defects in the CF epithelium after silencing of ENaC was demonstrated by measurement of the transepithelial potential (Vt), short circuit current (Isc), airway surface liquid depth and ciliary beat frequency (CBF) with each parameter restored to normal levels. We then assessed efficiency of silencing of murine aENaC in normal mice, as well as the inflammatory response in lung tissue sections. A single dose of siRNA delivered by anionic or cationic lipid/peptide nanoparticles in mouse lung silenced ENaC by approximately 30%, which persisted for at least 7 days with anionic formulations displaying lower levels of inflammation. By extrapolation from our ALI culture data, this amount of silencing should prove sufficient to achieve a therapeutic effect for CF in vivo.

Development of novel self-amplifying RNA for long-term restoration and enhancement of CFTR function

Nathalie Francois

Objective: Cystic fibrosis (CF) is caused by a defect in the gene that encodes the cystic fibrosis transmembrane (CFTR) protein. The disease affects about 80,000 people worldwide but still lacks an efficient therapy for all mutation classes. CFTR supplementation therapy using a non-replicating mRNA encoding hCFTR already proved to be feasible. However, long-lasting protein supplementation with a non-replicating mRNA is difficult to achieve. Self-amplifying (sa) RNAs cause a much longer protein expression. Therefore, a sa-RNA encoding hCFTR formulated in an efficient delivery system, would provide a more beneficial therapy. Here, we present a novel sa-RNA technology to achieve long-lasting hCFTR expression.
Method: The coding sequence of the hCFTR was optimized and inserted into a synthetic saRNA that is based on the Venezuelan Equine Encephalitis Virus. The hCFTR encoding saRNA was evaluated for expression and functionality in CF bronchial epithelial cells (CFBE41o-) and lung carcinoma cells (A549). In parallel, saRNA transfection of CFBE41o- cells was compared to conventional mRNA

transfection of CFBE41o- cells. For this, in vitro expression of hCFTR was evaluated 24h after sa- and mRNA transfection in CFBE41o- cells by RT-qPCR, Western Blot, using CFTR specific 596 antibody. hCFTR specific ELISA assays are ongoing. IF-staining was performed for CFBE41o- cells grown in submerged culture and transfected with saRNA. In vitro hCFTR saRNA functionality was evaluated 24 and 48 hours after transfection of stably expressing halide-sensitive YFP CFBE41o- cells using the yellow fluorescent protein (YFP) halide functional assay.
Results: In both cell lines, RT-qPCR showed a higher presence of the hCFTR mRNA compared to controls after transfection of the cells with saRNA and mRNA. The functional glycosylated hCFTR protein (170 kDa band) was detected by Western Blot and was visually more present in saRNA transfected cells compared to cells transfected with conventional mRNA. A high presence of the protein was also demonstrated by IF staining. The YFP-based functional assay showed a 7-fold higher functionality of the CFTR protein in CFBE41o- cells transfected with saRNA.
Conclusion: Our study proved expression and functionality of hCFTR protein after in vitro transfection of a hCFTR encoding saRNA. A proof-of-concept study in vivo that studies the extent and duration of the CFTR correction is necessary and will be done using a CFTR knock-out mice model after finishing the ongoing optimization of the carrier for optimal delivery of the saRNA.

Correction of the drug-refractory CFTR mutation L227R by prime editing

Mattijs Bulcaen
Introduction: Gene therapy for cystic fibrosis (CF) has been pursued since the early 90's and focused on a gene addition approach, which adds a CFTR cDNA to restore CFTR function. Gene correction on the other hand, allows to restore mutations in patients' chromosomes, thereby preserving endogenous gene expression and regulation and possibly providing a permanent cure. 2021 and 2022 have excited the gene editing field with promising data from the first-ever in vivo human gene editing trials (TTR amyloidosis) as well as ex vivo gene editing data for sickle cell anemia, underscoring the potential for other inherited diseases, like CF. We hypothesized that prime editing (PE) could permanently correct drug-refractory CF-causing mutations, such as L227R (c.680 T>G).
Methods: To obtain optimal editing efficiencies, we screened over 20 guide combinations via transfection in HEK293T cells overexpressing mutant CFTR. After three days, genomic DNA was extracted and evaluated by Sanger sequencing. Gene editing efficiency was also verified at protein level to detect restored CFTR glycosylation by Western blot, plasma membrane (PM) localisation by confocal microscopy/flow cytometry and ion channel function by HS-YFP quenching. Additionally, we investigated the uses of engineered prime editing guides (epegRNAs) that contain a structural terminal motif capable of protecting the RNA against intracellular degradation. To validate our approach in a more relevant and translational model, we delivered the PE enzyme together with the required guides to patient-derived rectal organoids via lentiviral vectors (LV) and measured functional recovery via forskolin-induced swelling (FIS). In order to determine PE efficiency in this model, we developed an AI-based algorithm capable of recognising functionally corrected organoids by their swelling phenotype.

Results: The L227R-CFTR mutant presented with a severe processing defect, shown by the complete absence of PM CFTR. With an optimized PE approach however, we were able to achieve a genetic correction of 19 ± 2,6 (mean ± SEM) in HEK293T cells, which correlated with the numbers of corrected cells expressing PM-CFTR as well as with a rescue in ion channel function (HS-YFP quenching). LV delivery of PE components to intestinal organoids, resulted in a functional recovery of CFTR activity which can be attributed to correction of the endogenous CFTR gene. In preliminary experiments ~30% of organoids were corrected in the FIS assay. Both in HEK293T cells and in preliminary organoid experiments, the protected epegRNAs reached higher efficiencies compared to their respective (uncapped) pegRNA variants.
Conclusion: We have shown that the drug-refractory L227R processing mutation can be corrected by PE leading to a functional recovery of CFTR in cell lines and rectal organoids. Ongoing work focuses on increasing editing efficiency as well as on a detailed on/off-target analysis to fully uncover PE's potential to precisely and efficiently correct mutant *CFTR* alleles as a potential future cure for CF patients.
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Session IV: Anti-infective therapies

Harnessing and tailoring the unique properties of iPSC-derived macrophages for next generation immunotherapies

Mania Ackermann

Macrophages can be found in various tissues and play an important role in organ function by sensing and eradicating pathogens, regulating immune responses and contributing to tissue homeostasis and repair. Nowadays, increasing numbers of macrophage-based cell therapies are entering (pre-) clinical studies, e.g. for the treatment of lung or liver cirrhosis, chronic inflammations or cancer. We have also previously introduced a novel immunotherapy concept utilizing the adoptive transfer of macrophages to target pulmonary infections caused by different gram-positive and -negative pathogens. However, the diverse functions, activation stages and overall plasticity of macrophages, highlight the necessity to establish the robust production and tailored design of specific macrophage subsets allows further development of novel macrophage-based (immuno-) therapies. Here, we demonstrate the continuous production of standardized, highly pure CD45+CD11b+CD14+CD163+ iPSC-derived macrophages (iMonoMac) in scalable quantities using industry compatible bioreactors. To tailor the iMonoMac cell product further to the therapeutic application, we first enhanced the anti-microbial function of macrophages, by pre-loading of the cells with the antibiotics Gentamicin and Levofloxacin. Interestingly, Gentamicin remained intracellular in the iMonoMac for >24 hours, hinting to a direct role in the enhanced intracellular killing. In contrast, Levofloxacin was released from iMonoMac into the medium, suggesting a possibility to utilize preloaded iMonoMac as a carrier to deliver drugs to the site of infection in addition to the intrinsic antimicrobial potential of the cell. Next, we utilized polarization with different cytokines to imprint pro- or anti-inflammatory phenotypes in the iMonoMac, as demonstrated by changes in surface marker profile, cytokine secretion or gene expression. Of note, these M1- or M2-iMonoMac maintain key characteristics of their activation profile upon a secondary inflammatory stimulus. In summary, we here provide the tailored design of iMonoMac, which can be used for various cell-based immunotherapies and future applications in the field of regenerative medicine.

Phage-inspired antimicrobials

Zuzanna Drulis-Kawa

Phages are viruses that infect bacteria. The "predator-prey" interactions are recognized as a potentially effective way to treat infections. Phages, as well as phage-derived proteins, are intensively studied to become a future alternative or supportive antibacterials used alone or in combination with standard antibiotic regimens treatment.

Phages infect the host after successful receptor recognition and adsorption to the cell surface. The irreversible adherence must be preceded by the passage of diverse carbohydrate barriers such as capsule polysaccharides (CPS), O-polysaccharide chains of lipopolysaccharide (LPS) molecules, extracellular polysaccharides (EPS) forming biofilm matrix, and peptidoglycan (PG) layers. For that purpose, bacteriophages are equipped with virion-associated carbohydrate

degrading enzymes, termed polysaccharide depolymerases or lysins, that recognize, bind and degrade the polysaccharide compounds.
Unlike wide-spectrum antibiotics, phage therapy is characterized by a high selectivity. This narrow specificity allows for the selective killing of a given target pathogen, saving accompanying microflora. Bacteriophage therapy is currently being evaluated as a critical complement to traditional antibiotic treatment. However, the emergence of phage resistance is perceived as a major hurdle to the sustainable implementation of this antimicrobial strategy. My presentation refers to the application of phages and phage depolymerases as effective antibacterials against *Pseudomonas aeruginosa* and Klebsiella pneumoniae pathogens.

TMA analogues as anti-inflammatory agents in the treatment of Cystic Fibrosis

Ilaria Lampronti

Introduction: A series of new generation TMA (4,6,4'-trimethyl angelicin) analogues was projected and synthetized in order to ameliorate the anti-inflammatory activity, possibly maintaining also the CFTR correction effect known in the lead compound TMA, but with reduced or absent toxicity. Since the transcription factor NF-kB plays a critical role in IL-8 expression, the use of agents able to interfere with the NF-kB pathway represents an interesting therapeutic strategy.
Methods: Electrophoretic mobility shift assay (EMSA) analysis was performed to study the mechanism of action of different TMA analogues. The IL-8 expression was evaluated during TNF-alpha-mediated or *Pseudomonas aeruginosa*-mediated induction of pro inflammatory responses in CF (Cystic Fibrosis) bronchial epithelial cells treated with TMA derivatives in order to investigate their ability in reducing cytokine expression. We finally tested one selected TMA-derivative (the most active) in preclinical in vivo experiments. C57Bl/6NCr male mice (Charles River) were utilized for *P. aeruginosa* acute infection.
Results: Through preliminary EMSA experiments, we identified several TMA derivatives able to inhibit the NF-kB/DNA complex. The selected active molecules were then analyzed in order to evaluate the anti-inflammatory effect using both *P. aeruginosa* (PAO1) infection and TNF-alpha stimulus on CF cells. It was demonstrated that two TMA analogues were able to decrease the gene expression of IL-8. At the same time, these molecules were found to have no pro-apoptotic, mutagenic and phototoxic effects, facilitating our decision to test them also in vivo, in mouse models of acute *P. aeruginosa* lung infection. The anti-inflammatory effect of one molecule (GY971a mesylate salt) was confirmed in vivo: it was able to deeply decrease the neutrophils count in the mice model, without evident toxicity.
Conclusion: Starting from new synthetic TMA derivatives, studied to improve the chemical-pharmaceutical and pharmacological aspects, we were able to identify GY971a, a novel derivative with very interesting activities that we'd like to develop as possible drug for CF

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