Cystic fibrosis transmembrane conductance regulator-mRNA delivery: a novel alternative for cystic fibrosis gene therapy

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Abstract

Background Cystic fibrosis (CF) is the most frequent lethal genetic disease in the Caucasian population. CF is caused by a defective gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP- and ATP-dependent Cl⁻ channel and central regulatory protein in epithelia. CFTR influences the fluid composition of the mucus in the respiratory tract. The most common mutation inducing CF, ΔF508, impairs CFTR processing within the cell and thus prevents functional CFTR expression in the apical membrane. The present study aimed to investigate the functional restoration of CFTR in human CF airway epithelia after transfection with optimized wild-type (wt)CFTR-mRNA.

Methods We used primary cultured human nasal epithelial (HNE) cells and the human bronchial epithelial cell line CFBE₄₁o⁻ that stably expresses ΔF₅₀₈-CFTR and carried out transepithelial Ussing chamber measurements after transfection with optimized wtCFTR-mRNA. We confirmed the data obtained using immunofluorescence and protein biochemical approaches.

Results Transfection of the CFBE₄₁o⁻ cells with wtCFTR-mRNA restored cAMP-induced CFTR currents similar to the values seen in control cells (16HBE14o⁻). Using immunofluorescence approaches, we demonstrated that a considerable amount of CFTR is located at the apical surface in the CF cells after transfection. Western blot analyses of wtCFTR-mRNA transfected CFBE₄₁o⁻ cells confirmed these findings. Furthermore, we demonstrated physiological relevance by using primary cultured HNE cells and showed an almost two-fold increase in the cAMP-stimulated CFTR current after transfection.

Conclusions From these data, we conclude that CFTR-mRNA transfection could comprise a novel alternative for gene therapy to restore impaired CFTR function. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords cystic fibrosis; functional restoration; inherited disease; ion transport

Introduction

Cystic fibrosis (CF) is the most common life shortening autosomal inherited disorder, affecting one in 2500 newborns in the Caucasian population [1]. The disease is caused by mutations in a gene coding for a Cl⁻ channel, the
CF transmembrane conductance regulator (CFTR). Beside its function as a cAMP- and protein kinase A-dependent, ATP-gated ohmic 10-pS Cl\(^{-}\) channel, CFTR appears to be involved in the regulation of a whole orchestra of other ion channels and transport systems in epithelial cells [2]. It is suggested that CFTR exhibits regulatory influences on epithelial Na\(^{+}\) channels [3], K\(^{+}\) channels [4] and other Cl\(^{-}\) channels. It is commonly accepted that the many regulatory functions of CFTR and the failure of these lead to the impaired epithelial properties evoking the complex CF symptoms. Yet, the exact molecular mechanisms are far from being understood and some of the putative regulatory interactions are subject to vivid discussions. The combined ion transport defects in the airways lead to highly viscous mucus, which gives rise to bacterial infection and inflammation with several bacterial strains, such as Staphylococcus aureus and Pseudomonas aeruginosa [5]. Chronic lung disease is the major factor that contributes to mortality and morbidity in CF patients.

When, in 1989, the CFTR gene was cloned [6] researchers had high hopes of restoring CFTR function using gene therapy and, during the 1990s, there was widespread enthusiasm with respect to designing viral wild-type (wt) CFTR gene delivery for CF patients [7]. However, it became obvious that therapy approaches using adenoviruses and adeno-associated viruses as CFTR-DNA delivery vectors evoked unwanted immune responses and failed to correct CFTR symptoms in the airways of CF patients [8,9]. Therefore, further developments focused on nonviral gene therapy vectors such as cationic liposomes or cationic polymers. Yet, only a special plasmid complexed with GL67 ([cholest-5-en-3-ol (3b)3-[(3-aminopropyl)[4-[3-aminopropyl]amino] butyl] carbamate]) resulted in CFTR-mRNA expression in the mouse lung that lasted at least 56 days without eliciting an immune response [10]. Although not successful, all of these CFTR gene therapy approaches delivered valuable insights in the pitfalls of DNA gene delivery to CF epithelia [11]. It became clear that viral vectors activate the immune system quite rapidly and that repeated delivery increased the immune response. Recently, Davies et al. [12] demonstrated a single dose nonviral lipid-mediated CFTR gene therapy approach restoring CFTR expression and function that was also accompanied by a dose-dependent systemic inflammatory response. The second problem associated with nonviral delivery systems appeared to be the successful transport of the introduced DNA into the nucleus for further processing because nuclear pore complexes are not tailored to readily transfer DNA from the cytoplasm into the nucleus [13].

All of these problems of ‘conventional’ DNA gene delivery could be circumvented by using mRNA instead of DNA and delivery by newly developed nonviral vectors such as tailor-made polyethyleneglycols (PEGs) [14]. Indeed, mRNA-based gene delivery with PEGs has several advantages compared to DNA delivery: (i) viral vector induced immunogenicity is avoided; (ii) mRNA is efficient even when cells are no longer dividing; (iii) mRNA does not need to be transferred into the nucleus and is translated in the cytoplasm; (iv) the risk of insertional mutagenesis is omitted; (v) the mRNA construct is far smaller than DNA; and (vi) mRNA can be repeatedly applied. Compared to DNA transfection processes, much less is known about mRNA evoked immune responses. Especially, immunogenicity of in vitro transcribed RNA could elicit immune responses such as those described for cultured dendritic cells [15]. Furthermore, gene expression using unmodified mRNA decreases within 3 days, requiring repeated application in high frequencies [16]. Moreover, possible difficulties may also include mRNA stability and therefore the duration of the mRNA effect in the target cells, which could make frequent dosing necessary. Yet, future research on mRNA stabilization, optimization of delivery strategies and improved production procedures should overcome these drawbacks. In the present study, we present proof-of-concept for mRNA-based functional restoration of impaired CFTR functions in a human bronchial CF cell line stably expressing ΔF508-CFTR (CFBE410\(^{+}\)). We show that, after mRNA transfection, the human bronchial CF cells functionally behave very similar to healthy bronchial epithelia (16HBE140\(^{+}\)). The amount of functional CFTR molecules in the CF cells after mRNA transfection is even larger than in non-CF control cells. Furthermore, we verify these data using primary cultured human epithelial (HNE) cells and demonstrate their physiological relevance in a well differentiated and reliable cell culture system. From these data, we conclude that in vivo mRNA transfection methods could be promising for the treatment of CF. This new strategy to restore CFTR protein function by means of mRNA delivery was termed ‘transcript therapy’ [17].

Materials and methods

Constructs for in vitro transcription

Human CFTR was excised from pGZCUBI-CFTR (provided by S. H. Cheng; Genzyme Corporation, Framingham, MA, USA) and cloned into the PstI-AvaI sites of a T7 promoter containing pST1-2β-globin UTR-A (20, 70 or 120) construct, kindly provided by U. Sahin (Johannes Gutenberg University, Mainz, Germany). This vector has been described in detail [18] and used previously [17]. The constructs were cloned using standard molecular biology techniques.

mRNA preparation

For in vitro transcription (IVT) pST1-wt-CFTR with different poly(A) tail length (20, 70 and 120 adenines) were used.
The linearized plasmids were extracted with phenol/chloroform and precipitated with ethanol. The IVT reaction was carried out using the mMessage mMachine T7 Kit (#AM 1344; Ambion, Foster City, CA, USA), which includes cap analog [m7G(5’G)] in an ultra high-yield transcription reaction and RNase inhibitor that protects the synthesized mRNA from degradation by any contaminating ribonucleases. Next the reaction was purified using the RNeasy plus Mini Kit (Qiagen, Hilden, Germany) and subsequent ethanol/ammonium acetate precipitation to achieve good quality mRNA. The generated mRNA was stored at −20°C or −70°C in nuclease-free water. Under these conditions, no degradation of RNA is detectable after 1 year.

The concentration of mRNA was determined by absorbance measurement at 260 nm (Abs 260) in a micro-volume spectrophotometer (Nanodrop, Thermo Scientific, Wilmington, DE, USA). The integrity and size distribution of mRNA was analyzed via denaturing agarose gel electrophoresis and ethidium bromide staining (Figure S1).

**Oocyte preparation and injection of CFTR-mRNA**

Oocytes were taken from mature female *Xenopus laevis* (purchased from the African Xenopus Facility, Knysna, South Africa) and isolated as described previously [19]. Briefly, oocyte clusters were surgically removed from the ovaries of the frogs (anesthetized with tricain, 1.5 g/l) and stored in oocyte Ringer (ORi) containing: NaCl 90 mM, KCl 2 mM, Hepes 10 mM, CaCl2 1.8 mM (pH 7.6). Defolliculation of the oocytes was performed by incubation in collagenase solution (1 mg/ml; Serva, Mannheim, Germany) for 120 min followed by 10 min in Ca2+-free ORi to remove follicle cells. Healthy looking stage V-VI oocytes were selected and injected with 50 nl of total volume containing 3.3 ng of each CFTR-mRNA into the cytoplasm of the oocyte. Injected oocytes were stored up to 6 days in ORi at 16°C.

**Oocyte electrophysiology**

All measurements were performed under voltage-clamp conditions using the standard two-microelectrode technique with some modifications and additional equipment as described in detail elsewhere [19]. Briefly, individual oocytes were placed in a small plexiglas chamber (volume of 1 ml) and were continuously superfused with a flow rate of approximately 3.5 ml/min. Oocytes were impaled with conventional micro-electrodes filled with 1 M KCl (resistance: approximately 1 MΩ). Two Ag/AgCl pellets were used as bath electrodes. Electrodes were connected to an oocyte voltage-clamp amplifier (OC-725C; Warner Instruments, Hamden, CT, USA) and holding currents were digitally recorded. All measurements were performed with oocytes clamped to a holding potential of −60 mV. We used ORi (see below) as the initial bathing solutions in all experiments. Conventionally, a flow of negative charge (i.e. Cl−) from the oocyte cytosol to the outside is termed the inward current and is plotted downward in all graphs.

For impedance and capacitance measurements two Digital Signal Processing (DSP) boards (Model 310B; Dalano Spry, Rochester, NY, USA) were used. They were equipped with two high speed (300 kHz) analog to digital converters (14 bit) and two digital to analog converters (12 bit). The DSP boards were connected to the oocyte voltage clamp via an interface consisting of anti-aliasing filters, programmable gain amplifiers and digital control circuits that were controlled by the DSP boards. We used one DSP board to record membrane conductance (Gm) and clamp current (Im). Gm was measured by imposing a sine wave with a frequency of 0.25 Hz and an amplitude of 5 mV to the oocyte membrane. Current and voltage changes evoked by the sine wave were sampled with a frequency of 625 Hz. We used regression analysis to calculate Gm from the amplitudes of the current and voltage sine waves in a way that enabled the system to update Gm values every 15 s. The use of a low-pass filter with a cut-off of 3 Hz for the current and voltage signals enabled us to simultaneously record Gm and Im, whereas the high frequency membrane capacitance (Cm) was measured with the second board. Cm was measured with five sine waves with frequencies ranging from 73 to 293 Hz, yet only the three lowest frequencies (from 73 to 146 Hz) were used for data interpretation (for an explanation, see Results). The reported values illustrate records at f = 73 Hz.

**Cell culture (primary HNE cells)**

Primary cell culture of the HNE cells was performed as described previously [20]. The nasal epithelial cells were isolated by enzymatic digestion for 24–48 h and afterwards seeded on collagen coated (0.15 mg/ml collagen type I; Biochrom AG, Berlin, Germany) Transwell® permeable filters with a diameter of 6.5 mm (Costar #3470; Corning Inc., Lowell, MA, USA). The cells were cultured with serum-free F-12 Nutrient Mixture (Ham) (Invitrogen/Gibco, Karlsruhe, Germany) supplemented with the agents: insulin (2 μg/ml) (Invitrogen/Gibco), epidermal growth factor (13 ng/ml) (Sigma, Deisenhofen, Germany), endothelial cell growth supplement (7.5 μg/ml) (Becton Dickinson GmbH, Heidelberg, Germany), triiodothyronine (3 nM) (Sigma), hydrocortisone (100 nM) (Sigma), gentamycin (10 μg/ml) (Biochrom AG), penicillin/streptomycin (100 U/ml)
(Invitrogen/Gibco), L-glutamine (2 mM) (Invitrogen/Gibco) and transferrin (4 μg/ml) (Invitrogen/Gibco). Previously, it was shown that these supplements had no effect on the electrical parameters of the HNE cells [20]. The cell culturing procedure, the medium and its supplements were chosen so as to optimize the efficient isolation of human nasal epithelial cells. Fibroblast contamination is reduced by serum-free media and cell cluster formation is avoided by filtering through special cell strainers. Furthermore, we identified and validated epithelial cells using specific antibodies against vimentin and keratin (data not shown).

Cells were incubated in 95% air and 5% CO2 at 37°C. Seven to 9 days after seeding the cells on the membrane, we normally obtained confluent monolayers and proceeded with the Ussing chamber measurements.

Ethical statements

We obtained nasal specimens from patients undergoing nasal surgery. The samples were typically nasal polyps or nasal turbinates of patients suffering from chronic sinusitis. The study was approved by the committees for human studies of the University of Muenster (Ethik Kommission Muenster). Declaration of Helsinki protocols were followed and all patients provided their written informed consent. All animal work was approved by local authorities (review board institution: 'Veterinaer- und Lebensmittelueberwachungsamt Muenster in Westfalen', approval ID number: A72/2003-39.32.7.1).

Cell culture (bronchial epithelial cell lines)

16HBE14o− and CFBE41o− cells were obtained from Dr Dieter Gruenert (Department of Otolaryngology – Head and Neck Surgery, University of California, San Francisco, CA, USA). The cells were grown on T-25 cell culture flasks in Eagle’s Minimal Essential Medium with L-glutamine (MEM) in addition to 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 1% L-glutamine at 37°C in 5% CO2 and 95% air. MEM, penicillin (10 000 U/ml)/streptomycin (10 mg/ml) and FCS Gold were obtained from PAA Laboratories GmbH (Pasching, Austria). After reaching confluence, cells were split using trypsin/ethylenediaminetetraacetic acid (EDTA) solution (0.25%/0.02%; Biochrom AG) and then cultured on cell culture dishes (diameter 35 mm) for Western blot analyses, on glass coverslips for immunofluorescence staining and on permeable filter supports for transepithelial measurements. All cell culture supports were coated with the fibronectin/collagen/bovine serum albumin (BSA) coating solution before every experiment. The fibronectin/collagen/BSA coating solution was composed of LHC basal medium (Invitrogen/Gibco), human fibronectin (Biochrom AG), bovine collagen type I (VWR International, Darmstadt, Germany) and bovine serum albumin (Invitrogen).

Transfection efficiency

To test mRNA transfection efficiency in the epithelial cell lines and primary cultures, we used the pEGFP-C1 plasmid (Clontech/Takara Bio Europe, Saint-Germain-en-Laye, France) to introduce the reporter gene GFP. For control experiments, we carried out pEGFP-C1/GFP-small interfering (si)RNA co-transfection, H2O transfections and used mock transfected cells. To explore the transfection efficiency of 16HBE14o− and CFBE41o− cells using Lipofectamine 2000 transfection reagent (Invitrogen), 0.5 × 10⁵ cells were seeded per glass coverslip (diameter 12 mm) 3 days before transfection and were cultivated in MEM.

Primary cultured HNE cells were seeded also on glass coverslips 3 days before transfection and the transfected cells were subsequently cultivated in HNE culture medium. On the day of transfection, all cells were close to full confluence. For approximately 3 h before transfection, cells were cultivated in MEM or HNE culture medium without serum or antibiotics, respectively. Cells were transfected with 1 μg of pEGFP-C1 or co-transfected with 2.5 pmol/cm² of GFP-specific siRNA (siRNA to reagent ratio of 2:3), 2.5 pmol/cm² nonbinding siRNA (negative control = mock-transfected) or a respective amount of water. After 24 h of incubation, cells were fixed with glutaraldehyde and autofluorescence was quenched with sodium borohydride. Transfection efficiency was assessed 24 h after transfection and determined by analysis of the fluorescence intensities using a fluorescence microscope (LSM 510 META; Carl Zeiss, Oberkochen, Germany) Microscopic images were recorded using the AxioCamMRm and the LSM 510 4.2 SP1 software (Carl Zeiss).

Regulatory auto-exposure was used, even though the exposure time was adjusted manually for comparison of total fluorescence intensities. All images were recorded using a defined analysis area (1388 × 1040 pixels) and a locked exposure time of 100 ms to enable direct comparison. For compensation images, confluent regions of the cell layer were used for the analysis.

Transfection of the cells with wtCFTR-mRNA

For transfection with wtCFTR-mRNA CFBE41o− and 16HBE41o− cells were seeded at a density of 1 × 10⁵ cells/cm² per Costar filter (diameter 6.5 mm, #3470; Corning Inc.) or on cell culture dishes (diameter 35 mm)
and cultivated for 7 days. Medium was replaced every 48 h. Twenty-four hours before transfection, the culture medium was replaced by medium without serum and antibiotics. After 7 days, the cells were transfected with wtCFTR-mRNA (2.4 µg/cm² using Lipofectamine™ 2000 transfection reagent; Invitrogen). Experiments were performed 24 h after transfection.

The HNE cells were seeded on collagen coated Transwell® permeable filters with a diameter of 6.5 mm (Costar #3470; Corning Inc.) and cultivated for 7 days. Twenty-four hours before transfection, the culture medium was replaced by medium without antibiotics. Cells were also transfected with wtCFTR-mRNA (2.4 µg/cm²) using Lipofectamine™ 2000 in accordance with the manufacturer’s instructions for DNA transfections. Transfection complexes were removed after 4 h and replaced by normal culture medium. After 24 h of incubation, cells were measured in the Ussing chamber.

Transepithelial measurements

Transepithelial measurements were performed in modified Ussing chambers designed by Professor Willy Van Driessche (KU Leuven, Leuven, Belgium). We used Ag/AgCl electrodes, which were connected to the Ringer solution. The Vₑ was clamped to 0 mV with a low-noise voltage clamp. The transepithelial short-circuit current (Iₑsc) and conductance (Gₑ) were continuously and simultaneously recorded (ImpDsp 1.4; KU Leuven). All values were normalized to 1 cm². After adapting the cells to the Ringer solution (130 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 5 mM glucose, 10 mM Hepes; pH 7.3), an ‘activation cocktail’ consisting of the membrane permeable cAMP analogue 8-[4-chlorophenylthio (CTP)]-cAMP (100 mM; Biolog, Bremen, Germany) and IBMX (1 mM; AppliChem GmbH, Darmstadt, Germany) was applied to the basolateral side of the monolayer to activate CFTR. We inhibited this Cl⁻ current using the specific CFTR channel blocker CFTRinh172 (Tocris Biosience, Bristol, UK) (20 µM). Unless otherwise stated, all chemicals were obtained from Roth (Karlsruhe, Germany).

Western blot analysis

For Western blot experiments, the transfection reagent Lipofectamine™ 2000 was removed after 4 h and replaced by medium as recommended. Twenty-four hours after transfection, cells were trypsinized and, after centrifugation (500 g for 5 min), pellets were resuspended in 55 µl of lysis buffer (1 mM Tris, 15 mM NaCl, 0.2 mM EDTA, 2% Triton X-100) and 1% protease inhibitor cocktail (Sigma) and placed on ice for 30 min. The extracts were homogenized with a Sonifer transsonic cell disrupter (Branson, Danbury, CT, USA) and centrifuged at 4000 g for 30 min at 4°C to remove nonsoluble material. The concentration of the proteins in the supernatant was measured photometrically using the BCA test (Pierce, Rockford, IL, USA). For the detection of CFTR protein, 25 µg of total protein was separated via sodium dodecylsulfate polyacrylamide gele electrophoresis (SDS-PAGE) (6% acryl amide). A tank blot aperture (Mini Trans-Blot® Electrophoretic Transfer Cell; Bio-Rad, Muenchen, Germany) was used to transfer proteins to a polyvinylidene fluoride membrane at 25 V on ice overnight. Nonspecific binding sites were blocked for 2 h at room temperature with 5% nonfat dry milk in Tris-buffered saline/Tween (TBST: 10 mM Tris HCl, pH 7.4; 140 mM NaCl; 0.1% Tween 20). The CFTR was detected with a monoclonal mouse anti-human and murine CFTR primary antibody (#ABR-01129; Dianova, Hamburg, Germany), with a concentration of 1:1000 diluted in 5% nonfat dry milk/TBST for 1 h at room temperature. The specificity of this CFTR antibody was confirmed using CFTR specific siRNA (data not shown). After washing in TBST, the membrane was incubated for 1 h at room temperature with the affinity purified goat anti-mouse immunoglobulin (Ig)G conjugated with alkaline phosphatase (Dianova) diluted 1:10 000 in 5% nonfat dry milk/TBST. After washing the membrane in TBST again, detection was carried out with NBT (nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate). For densitometric evaluation of the intensity of the CFTR bands, we used ImageJ, version 1.41 (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). For control experiments, we prepared whole cell protein extracts from nontransfected CFBE410° cells and 16HBE14° cells. To assure comparable protein amount and expression, we routinely use anti-α-tubulin (Tubulin, alpha, DLN-09993; Dianova) for normalization of the Western blot data. This normalization is already included in the presented evaluation.

Immunofluorescence staining

We seeded 3.5 × 10⁵ CFBE410° cells and 5 × 10⁵ 16HBE14° cells, respectively, on four glass cover slips placed in a cell culture dish (diameter 35 mm). Twenty-four hours after transfection, the cells were fixed with 0.05% glutaraldehyde in Hepes buffer (10 mM Hepes, 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM, 5 mM glucose) for 10 min at 37°C. Nontransfected CFBE410° and 16HBE14° cells were prepared similarly. The following steps were carried out at room temperature. Cells on the cover slips were washed five times for 5 min with ice-cold phosphate-buffered saline (PBS: 140 mM NaCl, 2 mM KCl, 4 mM Na₂HPO₄, 1 mM KH₂PO₄, DOI: 10.1002/jgm

pH 7.4) and thereafter treated with 100 mM Glycine/PBS for 30 min. After another washing step (5 × 5 min with PBS), the cells were blocked with 10% (v/v) normal goat serum (NGS) for 30 min. After blocking, the cells were incubated for 1 h in a humidity chamber at a 1:500 concentration of mouse anti-CFTR antibody (Dianova) diluted in NGS. Before application of the secondary antibody, the cells were washed another five times for 5 min with PBS. The Quantum dot (Qdot)-labeled secondary antibody (Qdot®655 goat F(ab′)2 anti-mouse IgG conjugate (H + L); Invitrogen) was subsequently applied to the cells at a 1:200 concentration in NGS for an incubation time of 1 h in a humidity chamber. The cells were washed additionally five times for 5 min in PBS, another two times for 5 min in HEPES buffer and were then fixed once again in 0.05% glutaraldehyde in Hapes buffer for 10 min. After washing four more times for 5 min in Hapes buffer and five short times in deionised water, the cells on the cover slips were mounted in Dako fluorescent mounting medium (Dako, Glostrup, Denmark) on a microscope slide. Additionally, negative controls of CFBE41o and 16HBE14o cells were prepared that were treated with the secondary but not with the primary antibody.

Previously, we incubated cells with a primary anti-α-tubulin antibody (1:200) (Dianova) and Cy3-labelled anti-mouse secondary antibody (1:500) (Dianova) to ensure that the cells are not permeabilized during preparation. We detected α-tubulin in the cells by permeabilization with paraformaldehyde (0.37%) [21–23].

Fluorescence microscopy

Fluorescence microscopy was carried out with the inverted fluorescence microscope LSM 510 META (Zeiss, Jena, Germany) equipped with the alpha plan-fluar × 100/1.45 oil immersion objective. We used the filterset F31-118Q (AHF Analysetechnik AG, Tübingen, Germany) to visualize the Qdots. To display the autofluorescence of the cells as a result of the glutaraldehyde fixation, filterset 09 (Zeiss) was used. Cell surface areas of the cells were selected manually and Qdot-labeled CFTR molecules in the plasma membrane of the cells were detected by focusing on the upper surface of the cells.

The number of Qdot-labeled CFTR-molecules/μm² was counted for each cell manually using ImageJ, version 1.41. Nonspecific binding of Qdots was eliminated by subtracting the amount of Qdots counted in the negative controls (cells incubated only with the secondary antibody).

Analysis of fluorescence intensities

Analysis of total fluorescence intensities was performed using ImageJ, version 1.41. The plug-in for RGB (red, green, blue color model) measurement analyzes the intensity of each channel of an image and furthermore displays the average channel intensity [(R + G + B)/3]. The latter value was employed for analysis and was compared with that of other images. The average of nontransfected cells was set to 100% and the transfected average was normalized to that value to be expressed as a multiple of the nontransfected value.

Statistical analysis

Where applicable, data are expressed as the arithmetic mean ± SEM. In the case of oocyte experiments, n is the number of oocytes and N the number of female donor frogs. In the case of cell culture experiments, n gives the number of replications. Variances were checked using an analysis of variance F-test and, subsequently, the appropriate statistical method was used. Where applicable, statistical evaluations were compared with Student’s t-test. Differences were considered statistically significant when p ≤ 0.05 (*) or p ≤ 0.01 (**). All statistical tests were performed using Origin, version 7.0 (OriginLab Corporation, Northampton, MA, USA).

Results

Optimising CFTR-mRNA in X. laevis oocytes

Before we performed the CFTR-mRNA transfection of human epithelial cells, we set out to optimize the CFTR-mRNA construct. We designed CFTR-mRNA constructs with three different poly(A) tail lengths: 20, 70 and 120 adenines, respectively. These different constructs were injected into X. laevis oocytes and cAMP-stimulated changes in CFTR current (I(m)), conductance (G(m)) and capacitance (C(m)) were measured. The capacitance of a membrane is an optimal measure of the membrane surface area [24]. Figure 1 shows typical traces of these parameters. CFTR-expressing oocytes were clamped to −60 mV and a cAMP-cocktail was added. Shortly after cAMP-cocktail application, all three parameters increased considerably and simultaneously in oocytes injected with the 120 adenines mRNA-CFTR construct. The increase in capacitance shows that additional membrane material including CFTR molecules are transferred from intracellular pools and inserted into the oocyte membrane as described in detail previously [24]. Oocytes injected with the mRNA-poly(A) tail of 70 adenines showed similar but substantially lower activation by cAMP, whereas those oocytes expressing the mRNA with the 20 poly(A) tail
did not respond to cAMP at all. Non-injected control oocytes also showed no response to the cAMP-cocktail. Figures 2a to 2c summarize the results obtained with the different poly(A) tail lengths of the CFTR-mRNA. A poly(A) tail of 20 adenines is obviously too short for proper mRNA translation and protein expression, whereas the other constructs (70 and 120 adenines, respectively) evoke good CFTR expressions. All parameters (i.e. current, conductance and capacitance) are increased upon stimulation with cAMP. Obviously, the expression rate of the 120 poly(A) tail constructs was clearly higher than the 70 poly(A) constructs. Because the 120 adenines poly(A) tail had the highest impact on all parameters in the oocytes, we used this construct for all further transfection experiments with the human cell lines and primary cultured HNE cells.

Transfection efficiency

Transfection efficiency was determined for both cell lines (16HBE14o− and CFBE41o−) and the primary cultured HNE cells, respectively. We analyzed the cells 24 h after transfection. This analysis clearly demonstrated that samples transfected with GFP exhibited a markedly increased fluorescence as compared to controls after 24 h (data not shown). Measurement of total RGB intensities revealed a significant increase of fluorescence intensity after 24 h of approximately 223% in GFP transfected 16HBE14o− cells compared to controls (mock-transfected) (n = 5). In CFBE41o− cells, even higher transfection efficiencies could be demonstrated: here, the total fluorescence intensity in GFP transfected cells increased after 24 h by approximately 268% (n = 5). Fluorescence intensities of the primary HNE cells showed an increase of approximately 328% in GFP transfected cells compared to the controls (n = 5). Measurement of the different negative controls (co-transfection of pEGFP-C1 plasmid and GFP-specific siRNA and water transfected cells) revealed fluorescence intensities comparable to mock-transfected control cells in all performed experiments (n = 5). From these data, we assume high transfection efficiencies for the analyzed cell lines and the primary cultured cells.

Transepithelial measurements

We measured the transepithelial current (Isc) in CFBE41o− and the appropriate 16HBE14o− control cells. After application of the cAMP-cocktail, Isc increased in the control cells (Figure 3a). In CFBE41o− cells, this CFTR activation failed as a consequence of the improperly folded CFTR, thereby leading to missing integration into the cell membrane. Therefore, Isc shows no change after cAMP-cocktail application (Figure 3b).

However, when CFBE41o− cells are transfected with wtCFTR-mRNA, they behave in the same way as 16HBE14o− cells: cAMP activation of CFTR is restored (Figure 3c): short-circuit current is strongly increased, demonstrating functional expression of CFTR. The statistical evaluation of these experiments is indicated in Figure 3d.

Furthermore, the cAMP-cocktail evoked activation sensitive to the specific CFTR blocker CFTRinh172, which decreased CFTR current significantly (Figure 4). CFTRinh172 inhibited 54% of the CFTR current in 16HBE14o− cells and 95% in wtCFTR-mRNA transfected CFBE41o− cells.

The higher rate of inhibition in CFBE41o− cells may demonstrate that, in these cells, more CFTR molecules
Figure 2. Validation of different poly(A) tail lengths (20, 70 and 120 adenins, respectively) by heterologous expression in X. laevis oocytes. Shown are the cAMP-dependent CFTR current (a), conductance (b) and capacitance (c) displayed as the differences with and without cAMP activation. Non-injected controls as well as A20-CFTR-mRNA injected oocytes show no changes in the electrophysiological parameters after cAMP addition and therefore no activation of CFTR. By contrast, A120-CFTR-mRNA injected oocytes show a large cAMP response demonstrated by an increase of all three parameters. Therefore, the most efficient CFTR activation was reached by injection of CFTR-mRNA with a 120 poly(A) tail (*p < 0.05; **p < 0.01).

Figure 3. Time courses of typical transepithelial measurements. Shown is the short-circuit current ($I_{sc}$) in 16HBE14o− control cells (a), CFBE41o− cells (b) and wtCFTR-mRNA transfected CFBE41o− cells (c), respectively. After application of the cAMP-cocktail, $I_{sc}$ increased in the control cells as shown in (a). In a second step, we inhibited this Cl− current with the specific inhibitor CFTRinh172. In CFBE41o− cells, no activation and therefore no response to the CFTRinh172 can be observed (b). When CFBE41o− cells are transfected with wtCFTR-mRNA, they show an increase of $I_{sc}$ similar to 16HBE14o− cells, which could also be inhibited by the CFTRinh172. The cAMP activation of CFTR is restored, demonstrating functional expression of CFTR (c). (d) Statistical evaluation of the short-circuit current ($\Delta I_{sc}$). Shown is the activation of CFTR mediated current for 16HBE14o− control cells (2.66 ± 0.48), nontransfected CFBE41o− cells (0.66 ± 0.26) and wtCFTR-mRNA transfected CFBE41o− cells (2.63 ± 0.64) (*p < 0.05).
are expressed after mRNA transfection than in the control cells (16HBE14o\(^{-}\)).

To verify these functional data, we carried out Ussing chamber experiments using primary cultured HNE cells. Twenty-four hours after transfection with wtCFTR-mRNA, the short-circuit current is increased as a result of the cAMP application. The statistical evaluation revealed an almost two-fold increase in the transfected cells compared to the controls (Figure 5). Furthermore, this response could also be inhibited by the specific CFTR blocker CFTRinh172 and is strongly enlarged in the wtCFTR-mRNA transfected cells. Because we could not observe an obvious reduction by the inhibitor in the control cells (\(n = 33\)), we found a 37.37% inhibition of the cAMP activated CFTR current in the wtCFTR-mRNA transfected HNE cells (\(n = 17; **p < 0.01\)).

**Immunofluorescence measurements**

For further verification and validation of the restoring effect of wtCFTR-mRNA transfection in CFBE41o\(^{-}\) cells, we used immunofluorescence analysis to detect the amount of CFTR molecules in the apical membrane and nearby regions. CFBE41o\(^{-}\) cells were seeded on glass cover slips. Twenty-four hours after transfection of the CFBE41o\(^{-}\) cells, the cells were fixed and stained with an anti-CFTR primary antibody. A Qdot-labeled anti-mouse secondary antibody was used to localize the CFTR molecules in the membrane. Nontransfected CFBE41o\(^{-}\) and 16HBE14o\(^{-}\) cells were also prepared. Images of the stained cells are shown in Figure 7a. For statistical evaluation, the amount of Qdots/\(\mu^2\) of each cell was counted and compared with the normalized control cells (100%) (Figure 7b). Our data show that transfected CFBE41o\(^{-}\) cells express approximately 220% more CFTR molecules than the nontransfected CFBE41o\(^{-}\) cells. Moreover, the amount of CFTR molecules after transfection of the CFBE41o\(^{-}\) cells is even higher than in the 16HBE14o\(^{-}\) control cells (approximately 150%).

**Discussion**

CF is caused by mutations in the CFTR gene leading to impaired Cl\(^{-}\) channel function in diverse epithelia lining
CFTR-mRNA delivery for the treatment of CF

Figure 6. (a) Western blot analyses. Total membrane proteins (25 μg) from CFBE41ο− and 16HBE14ο− cells were isolated by using 2% Triton X-100 and separated on a 6% SDS-PAGE. To identify CFTR, we used a monoclonal anti-CFTR antibody that detects a CFTR band in the range of 170 kDa. Furthermore, we used an α-α-tubulin antibody that detects the protein in the range of 57 kDa for normalization. (a) Lane 1, 16HBE14ο−; lane 2, nontransfected CFBE41ο−; lane 3, transfected CFBE41ο−. (b) Statistical evaluation of the relative CFTR protein amount in transfected and nontransfected epithelial cells. Nontransfected CFBE41ο− cells exhibited approximately 50% less CFTR protein compared to the transfected CFBE41ο− cells. Relative protein expression of the 16HBE14ο− control protein was normalized to 1.0 (100%) (n = 8; **p < 0.01).

The ducts of, for example, the pancreas, sweat glands and large and small airways. To date, it is not known how the loss of Cl− channel function alone leads to the whole plethora of CF related abnormalities associated with impaired CFTR function, including Na+ hyperabsorption in the airways [25–27], dysfunction of several ion transport systems [2,28], modified regulation of exocytosis and endocytosis [29,30], and susceptibility to certain bacteria that colonize the airways eventually leading to their destruction [31,32]. With the cloning and identification of the CFTR gene in 1989 [6,33], researchers were enthusiastic that it would be possible to develop a gene therapy protocol to restore CFTR function. However, it turned out that the ambitious goal to cure the disease by delivery of intact CFTR-DNA constructs is more complicated than was initially thought. Early gene therapy trials showed that significant difficulties were associated with expressing foreign genes, especially in the airways of CF patients using conventional DNA delivery strategies.

Therefore, we set out to develop a strategy using mRNA instead of DNA for correction of CFTR function. The present study aimed to investigate the functional expression and insertion of wtCFTR in the apical plasma membrane of human CF airway epithelia after wtCFTR-mRNA transfection in vitro. The current opinion is that mRNA is rapidly degraded when handled outside strict sterile laboratory conditions. Although proper handling of mRNA is not trivial, special modifications can increase the stability of exogenous mRNA considerably [17].

In general, it was assumed that a poly(A) tail of approximately 70 adenines would guarantee maximal protein expression in X. laevis oocytes and other cells [34]. Holtkamp et al. [18] demonstrated that an unmasked poly(A) tail with a free 3′ end significantly enhanced the stability of mRNA. It has been shown and is further confirmed by our data that the pharmacokinetic properties and especially the translational efficacy of the mRNA can be drastically improved by insertion of a 120 bp long poly(A) tail. Encoding the 120 bp poly(A) tail in the template vector facilitates in vitro translation of mRNA and achieves better results than enzymatic polyadenylation [18].

Therefore, before the transfection experiments, we optimized the appropriate CFTR-mRNA. The aim was to generate a robust CFTR-mRNA molecule that is not too extensive in length because the CFTR-mRNA itself is already 4.5 kb in size and may interfere with the transfection procedure. Thus, we designed CFTR-mRNA constructs with three different poly(A) tail lengths: 20, 70 and 120 adenines, respectively, assuming that 20 adenines may be sufficient for a sufficient expression efficiency. We injected these constructs into X. laevis oocytes and measured cAMP-stimulated changes in CFTR current (Im), conductance (Gm) and capacitance (Cm). With these experiments, we could clearly demonstrate that the 120 adenine CFTR construct revealed the strongest increase in all three parameters after cAMP-cocktail application.

Referring to these data, we used this construct to transfect human bronchial epithelial cells, thereby building a bridge from the heterologous oocyte expression system to a human cell model. We carried out Ussing chamber experiments and measured the transepithelial current (Im) in CFBE41ο− cells and the appropriate control cells (16HBE14ο−). In these measurements, Im increased after application of the the cAMP-cocktail in 16HBE14ο− control cells and could be blocked by the specific CFTR inhibitor CFTRinh172. As expected, the nontransfected CFBE41ο− cells showed no response to cAMP. We used the wtCFTR-mRNA to transfect the CFBE41ο− cells and,
Figure 7. (a) Representative images of immunofluorescence CFTR detection. CFTR molecules were detected at the apical plasma membrane of CF bronchial epithelial cells (CFBE41o−), wtCFTR-mRNA transfected CF and control cells (16HBE14o−). CFBE41o− cells were transfected with wtCFTR-mRNA and fixed 24 h after transfection. Nontransfected CFBE41o− and control cells were fixed likewise. The specific monoclonal anti-CFTR antibody and a Qdot655-labeled secondary antibody were used to detect the CFTR molecules at the apical membrane of these cells. The cells were not permeabilized during the immunofluorescence staining process. The autofluorescence (AF) of the cells is a result of fixation with glutaraldehyde, which allows the identification of cell borders. The amount of CFTR molecules in wtCFTR-mRNA transfected cells is increased compared to the nontransfected CFBE41o− cells and respective controls. Arrows indicate representative Q dots in the different preparations. (b) Statistical evaluation of CFTR molecules. Quantification of CFTR at the apical surface of CFBE41o− cells, wtCFTR-mRNA transfected CFBE41o− cells and 16HBE14o− cells. Single CFTR molecules were detected with the combination of a specific monoclonal anti-CFTR antibody and a Qdot-labeled secondary antibody. The abundance of Qdot-labeled CFTR molecules/μm² at the apical surface was counted for each cell. The amount of Qdots in the non-CF cells (16HBE14o−) was normalized to 100%. Nonspecific binding of Qdots was eliminated from these results. The CFTR protein expression is increased in the transfected cells in contrast to the nontransfected cells (n = counted cells; **p < 0.01).
after 24 h, observed an increase in cAMP-stimulated $I_{sc}$ that was comparable to that of the control cells. Furthermore, the cAMP-induced current could also be blocked by CFTRinh172. These results demonstrate that mRNA transfection using the wtCFTR-mRNA restores Cl– current in CFTR deficient cells in a suitable manner. To transfer the obtained data into a relevant physiological context, we carried out transepithelial measurements using wtCFTR-mRNA transfected primary cultured HNE cells. We showed an almost two-fold increase in $I_{sc}$ after cAMP application. This increase reflects the expected response that we already received in the previously used cell lines. Accordingly, we verified the findings using protein biochemistry. First, we proved an increased amount of CFTR protein using Western blot procedures. Thus, we could show that, 24 h after wtCFTR-mRNA transfection, CFBE41o– cells expressed comparable amounts of CFTR as the wt control cells, whereas nontransfected CFBE41o– cells expressed approximately 50% less CFTR protein.

To demonstrate that the CFTR protein is integrated properly into the membrane, we performed immunofluorescence measurements that clearly confirmed CFTR expression in wtCFTR-mRNA transfected CFBE41o– cells 24 h later. The amount of CFTR was even higher than in 16HBE14o– control cells.

The availability of an efficient standardized and affordable nonviral technology, as a basis for transcript therapy of CF, could represent tremendous progress in the field of CF research. However, it could be envisaged that transcript therapy may not be limited to application for CF but may have an impact in many other indications.

Potentially, mRNA delivery to restore a defective protein has several profound advantages compared with DNA delivery (e.g. smaller size, no need to cross the nuclear membrane, no risk of mutagenesis) [35]. Because of its pharmaceutical properties, mRNA can be easily manufactured and purified yielding large doses of standardized specific mRNAs for the transfection procedures [15]. All of these advantages of the use of mRNA as an alternative for gene therapy are faced with one limitation: in vitro transcribed unmodified mRNA stimulates the mammalian innate immune system by activation of a class of pattern recognition receptors termed Toll-like receptors (TLRs). The 13 so far known mammalian TLRs termed TLR1 to TLR13 recognize conserved bacterial molecules referred to as pathogen-associated molecular patterns and orchestrate the initiation of strong unwanted immune responses via tumor necrosis factor-α. Exogenous mRNA appears to be a potent activator of TLR3, TLR7 and TLR8, leading to the stimulation of genes that are involved in inflammation [15]. However, this disadvantage can easily be circumvented by chemical modifications of the mRNA used as recently demonstrated by Kormann et al. [17]. In that study, 25% of uridine and cytidine was replaced by modified bases (2-thiouridine and 5-methyl-cytidine, respectively) in the mRNA, resulting in a substantial decreased activation of the innate immune response in vitro and in vivo. Further modifications of uridine, cytidine and guanosine nucleotides could additionally suppress the immunostimulatory effects of exogenous mRNA as measured by drastically decreased secretion of tumor necrosis factor alpha and interleukins [36].

Thus, chemical modification of mRNA appears to overcome the major limitation of a potential mRNA therapy by overcoming mRNA-associated immunogenicity. Additional chemical modifications could further increase the stability of the mRNA and prevent degradation of the mRNA molecules, such as by insertion of phosphorothioate cap analogs, as described recently [37].

Another problem that has emerged in numerous studies is the efficacy of delivering the respective transcript to the target tissues. Some nonviral delivery systems have been developed such as mRNA/cationic lipid lipoplexes [38] and nanoapatite particles [39] that protect the mRNA against degradation and yield effective delivery to the target cells.

In the case of CF, wtCFTR-mRNA could be delivered as an aerosol to Airways of CF patients [40]. Recently, Hasenpuch et al. [41] showed that magnetized aerosols comprising iron oxide nanoparticles resulted in improved gene delivery to the lungs of mice. This technique may serve to further improve delivery of the therapeutic mRNA to the Airways of CF patients.

Further investigations in controlled preclinical and clinical trials will be necessary to proof the concept of transcript therapy for the treatment of CF.

In conclusion, the results of the present study provide evidence that mRNA transfection of CF airway epithelial cells could be capable of restoring CFTR function defective in the Airways of CF patients. Our data yield the proof principle that mRNA delivery may serve as a novel concept for the treatment of CF. Further translational studies on the basis of our findings will be necessary to bring ‘transcript therapy’ to the patients.

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References


Supporting information

Additional supporting information may be found in the online version of this article at the publisher’s web-site.