CFTR MRNA COMPOSITIONS AND RELATED METHODS AND USES

RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Serial No. 61/783,663, filed March 14, 2013, the disclosure of which is hereby incorporated by reference.

BACKGROUND

[0002] The present invention relates to cystic fibrosis transmembrane regulator (CFTR) mRNA compositions, uses of same, and methods of making and using same.

[0003] Cystic fibrosis is an autosomal inherited disorder resulting from mutation of the CFTR gene, which encodes a chloride ion channel believed to be involved in regulation of multiple other ion channels and transport systems in epithelial cells. Loss of function of CFTR results in chronic lung disease, aberrant mucus production, and dramatically reduced life expectancy. See generally Rowe et al., New Engl. J. Med. 352, 1992-2001 (2005).

[0004] Despite cloning of the CFTR gene in 1989, effective therapy for replacing CFTR for the treatment of cystic fibrosis has yet to be developed. The literature has documented numerous difficulties encountered in attempting to induce expression of CFTR in the lung. For example, viral vectors comprising CFTR DNA triggered immune responses and CF symptoms persisted after administration. Conese et al., J. Cyst. Fibros. 10 Suppl 2, S114-28 (2011); Rosenecker et al., Curr. Opin. Mol. Ther. 8, 439-45 (2006). Non-viral delivery of DNA, including CFTR DNA, has also been reported to trigger immune responses. Alton et al., Lancet 353, 947-54 (1999); Rosenecker et al., J Gene Med. 5, 49-60 (2003). Furthermore, non-viral DNA vectors encounter the additional problem that the machinery of the nuclear pore complex does not ordinarily import DNA into the nucleus, where transcription would occur. Pearson, Nature 460, 164-69 (2009).

[0005] Another source of difficulties in inducing CFTR expression in the lung is the lung environment itself. Pulmonary surfactant has been reported to reduce transfection efficiency for cationic lipid transfer vehicles such as Lipofectamine (DOSPA:DOPE).

[0006] Ernst et al., J. Gene Med. 1, 331-40 (1999). Also, Rosenecker et al., 2003, supra, identified multiple inhibitory components present in the airway surface liquid which can interfere with either polymer-mediated or lipid-mediated transfection. Messenger RNA therapy has been proposed as a general approach for inducing expression of a therapeutic or replacement protein. The concept of introduction of messenger RNA (mRNA) as a means of protein production within a host has been reported previously (Yamamoto, A. et al. Eur. J. Pharm. 2009, 71, 484-489; Debus, H. et al. J. Control Rel. 2010, 148, 334-343). However, apparent lung-specific difficulties have been reported for mRNA delivery using certain lipoplexes formulations. For example, a comparison of *in vitro* and *in vivo* performance of lipoplexes carrying mRNA or DNA revealed that even though the mRNA composition gave higher expression in cultured cells, measureable expression was detected only with the DNA composition when administered intranasally to mouse lung. Andries et al., Mol. Pharmaceut. 9, 2136-45 (2012).

[0007] It should also be noted that CFTR is a relatively large gene relative to model or reporter genes such as firefly luciferase (FFL). Compare the lengths of the wild-type CFTR coding sequence (SEQ ID NO: 2) and the FFL coding sequence (SEQ ID NO: 7). The difference in length can impact stability under some circumstances, and therefore whether and how much protein expression any given dose of mRNA will produce. Furthermore, although *in vitro* synthesis of mRNA is generally preferable to synthesis by cells due to the absence of normal cellular mRNA and other cellular components which constitute undesirable contaminants, *in vitro* synthesis of mRNA with a long coding sequence, such as CFTR mRNA, is substantially more difficult to achieve than *in vitro* synthesis of mRNA with a relatively short coding sequence such as FFL.

[0008] PCT patent publication WO2007/024708 and US patent publications US2010/0203627 and US2011/0035819 discuss the therapeutic administration of CFTR mRNA but provide neither a demonstrated reduction to practice of production of functional CFTR in the lung following administration of CFTR mRNA or sufficient guidance for overcoming the difficulties associated with inducing CFTR expression in the lung using *in vitro*-transcribed CFTR mRNA. These include difficulties with achieving *in vitro* synthesis of the mRNA and difficulties specific to the interactions of mRNA compositions with lung-specific substances that investigators such as Andries et al., supra, have found to render mRNA compositions ineffective

for induction of expression even while corresponding DNA-based compositions did provide some level of expression.

[0009] Thus, there is a need for improved materials, formulations, production methods, and methods for delivery of CFTR mRNA for induction of CFTR expression, including in the mammalian lung, for the treatment of cystic fibrosis.

SUMMARY OF THE INVENTION

[0010] The present disclosure is based, in part, on the development of formulations of CFTR mRNA and non-naturally occurring CFTR mRNA and methods of administration thereof that can induce functional CFTR expression *in vivo*. The compositions, methods, and uses according to the disclosure can provide CFTR expression in the lung of a large mammal with a favorable safety profile suitable for effective treatment of cystic fibrosis.

[0010a(i)] In one aspect, there is provided a method of producing cystic fibrosis transmembrane conductance regulator (CFTR) protein in a lung of a mammal, the method comprising:

administering to the lung of the mammal by aerosolization a composition comprising an mRNA-loaded nanoparticle, wherein the mRNA is an in vitro transcribed mRNA and has a coding sequence at least 80% identical to SEQ ID NO: 3,

wherein the mRNA encodes a human CFTR protein comprising the amino acid sequence of SEQ ID NO:1.

[0010a(ii)] In one aspect, there is provided a pharmaceutical composition comprising an mRNA-loaded nanoparticle,

wherein the mRNA is an in vitro transcribed mRNA and has a coding sequence at least 80% identical to SEQ ID NO: 3, and

wherein the mRNA encodes a human cystic fibrosis transmembrane conductance regulator (CFTR) protein comprising the amino acid sequence of SEQ ID NO:1.

[0010a(iii)] In one aspect, there is provided the use of a composition comprising an mRNA-loaded nanoparticle, wherein the mRNA is an in vitro transcribed mRNA and has a coding sequence at least 80% identical to SEQ ID NO: 3, and

wherein the mRNA encodes a human cystic fibrosis transmembrane conductance regulator (CFTR) protein comprising the amino acid sequence of SEQ ID NO:1 in the preparation of a medicament for the treatment of cystic fibrosis.

[0010a(iv)] In one aspect, there is provided an in vitro transcribed mRNA comprising a coding

sequence at least 80% identical to SEQ ID NO: 3 for use in treating cystic fibrosis in a mammal.

[0010a] In one aspect, there is provided a use of an *in vitro* transcribed mRNA comprising a coding sequence at least 80% identical to SEQ ID NO: 3 in the manufacture of a medicament for treating cystic fibrosis in a mammal.

[0010b] In another aspect, there is provided a composition comprising an mRNA and a carrier, wherein the mRNA is an *in vitro* transcribed mRNA and has a coding sequence at least 80% identical to SEQ ID NO: 3.

[0010c] In one aspect, there is provided pharmaceutical composition comprising an mRNA-loaded nanoparticle,

wherein the mRNA is an *in vitro* transcribed mRNA and has a coding sequence at least 80% identical to SEQ ID NO: 3, and

wherein the mRNA encodes a human cystic fibrosis transmembrane conductance regulator (CFTR) protein comprising the amino acid sequence of SEQ ID NO:1, wherein the pharmaceutical composition is to be administered to the lung by nebulization or aerosolization.

[0010d] In one aspect, there is provided the use of a composition comprising an mRNA-loaded nanoparticle, wherein the mRNA is an *in vitro* transcribed mRNA and has a coding sequence at least 80% identical to SEQ ID NO: 3, and

wherein the mRNA encodes a human cystic fibrosis transmembrane conductance regulator (CFTR) protein comprising the amino acid sequence of SEQ ID NO:1 in the preparation of a medicament for the treatment of cystic fibrosis wherein the medicament is to be administered via nebulization or aerosolization.

[0010e] In one aspect, there is provided an *in vitro* transcribed mRNA comprising a coding sequence at least 80% identical to SEQ ID NO: 3 for use in treating cystic fibrosis in a mammal, wherein the mRNA is to be administered to the lung of the mammal by nebulization or aerosolization.

[0010f] In one aspect, there is provided an *in vitro* transcribed mRNA comprising a coding sequence at least 80% identical to SEQ ID NO: 3 for use in treating cystic fibrosis in a mammal, wherein the mRNA comprises a coding sequence which encodes a human cystic fibrosis transmembrane conductance regulator (CFTR) protein of SEQ ID NO:1, and wherein the mRNA is to be administered to the lung of the mammal by nebulization or aerosolization.

[0011] Thus, in one embodiment, the present disclosure provides a method of *in vivo* production of CFTR, in particular, in the lung of a subject (e.g., a mammal) in need of delivery by delivering an mRNA encoding a CFTR protein. In some embodiments, the mRNA encoding a CFTR protein is delivered directly to the lung of the subject. As used herein, a "CFTR protein" encompasses any full length, fragment or portion of a CFTR protein which can be used to substitute for naturally-occurring CFTR protein activity and/or reduce the intensity, severity, and/or frequency of one or more symptoms associated with Cystic fibrosis. For example, a suitable CFTR protein according to the present invention may have an amino acid sequence identical to the wild-type human CFTR protein (SEQ ID NO:1). In some embodiments, a suitable CFTR protein according to the present invention may have an amino acid sequence at least about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the wild-type human CFTR protein (SEQ ID NO:1).

[0012] In one embodiment, the disclosure provides a method of inducing CFTR expression in epithelial cells in a lung of a mammal, the method comprising contacting the epithelial cells in the lung of the mammal with a composition, wherein: the composition is a pharmaceutical composition comprising an *in vitro* transcribed mRNA;the *in vitro* transcribed mRNA comprises a coding sequence which encodes SEQ ID NO: 1. In another embodiment, the

in vitro transcribed mRNA comprises a coding sequence which encodes an amino acid sequence at least about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 1.

[0013] In one embodiment, the disclosure provides a method of inducing CFTR expression in a mammalian target cell, the method comprising contacting the mammalian target cell with a composition, the composition comprising an *in vitro* transcribed mRNA encoding the amino acid sequence of SEQ ID NO: 1. In another embodiment, the *in vitro* transcribed mRNA comprises a coding sequence which encodes an amino acid sequence at least about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 1.

[0014] In another embodiment, the disclosure provides a non-naturally occurring mRNA molecule comprising a coding sequence, a 5'-UTR, and a 3'-UTR, wherein the coding sequence encodes the amino acid sequence of SEQ ID NO: 1 and the coding sequence is at least 80% identical to SEQ ID NO: 3. In another embodiment, the coding sequence encodes the amino acid sequence at least about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 1 and/or the coding sequence is about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 3.

[0015] In another embodiment, the disclosure provides a non-naturally occurring mRNA molecule comprising a coding sequence, a 5'-UTR, and a 3'-UTR, wherein the coding sequence encodes the amino acid sequence of SEQ ID NO: 1 and the coding sequence comprises at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% of the non-wild-type bases listed in Table 1 at the positions of the coding sequence listed in Table 1 relative to the wild-type coding sequence of SEQ ID NO: 2.

[0016] In another embodiment, the disclosure provides a non-naturally occurring mRNA molecule comprising a coding sequence, a 5'-UTR, and a 3'-UTR, wherein the coding sequence encodes the amino acid sequence of SEQ ID NO: 1 and the coding sequence comprises at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% of the non-wild-type bases listed in Table 2 at the corresponding positions of the coding sequence listed in Table 2 relative to the wild-type coding sequence of SEQ ID NO: 2.

[0017] In some embodiments, the disclosure provides a non-naturally occurring mRNA molecule comprising a coding sequence for a signal peptide. In a particular embodiment, the disclosure provides a non-naturally occurring mRNA comprising a coding sequence for a growth hormone leader sequence. In certain embodiments, the disclosure provides a non-naturally occurring mRNA comprising a coding sequence of SEQ ID NO:18 or SEQ ID NO:19. In some embodiments, the invention provides a non-naturally occurring mRNA comprising a coding sequence at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% to SEQ ID NO:18 or SEQ ID NO:19.

[0018] In some embodiments, the disclosure provides a non-naturally occurring mRNA molecule comprising a sequence of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ UD NO:15, SEQ ID NO:16, or SEQ ID NO:17. In some embodiments, the disclosure provides a non-naturally occurring mRNA molecule comprising a sequence at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% to any of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ UD NO:15, SEQ ID NO:16, or SEQ ID NO:17.

[0019] In another embodiment, the disclosure provides a polynucleotide comprising a sequence complementary to the sequence of an mRNA according to the disclosure.

[0020] In another embodiment, the disclosure provides a composition comprising the polynucleotide according to the disclosure, an RNA polymerase, and nucleoside triphosphates.

[0021] In another embodiment, the disclosure provides a pharmaceutical composition comprising an mRNA according to the disclosure.

[0022] In another embodiment, the disclosure provides a nebulization or aerosolization apparatus loaded with a pharmaceutical composition according to the disclosure.

[0023] In another embodiment, the disclosure provides a cultured cell comprising an mRNA according to the disclosure and functional CFTR expressed from the mRNA.

[0024] In another embodiment, the disclosure provides a use of a pharmaceutical composition according to the disclosure for the induction of expression of functional CFTR.

[0025] In another embodiment, the disclosure provides a method of inducing CFTR expression in epithelial cells in a lung of a mammal, the method comprising contacting the

epithelial cells with a composition, wherein the composition is a pharmaceutical composition comprising an mRNA according to the disclosure.

[0026] In another embodiment, the disclosure provides a method of inducing CFTR expression in a mammalian target cell, the method comprising contacting the mammalian target cell with a composition, the composition comprising an mRNA according to the disclosure.

[0027] In another embodiment, the present disclosure provides a method of treating cystic fibrosis by administering to a subject in need of treatment an mRNA encoding a CFTR protein as described herein. In one embodiment, the mRNA is administered to the lung of the subject. In one embodiment, the mRNA is administered by inhalation, nebulization, intranasal administration or aerosolization. In various embodiments, administration of the mRNA results in expression of CFTR in the lung of the subject.

[0028] In a particular embodiment, the present disclosure provides a method of treating cystic fibrosis by administering to the lung of a subject in need of treatment an mRNA comprising a coding sequence which encodes SEQ ID NO:1. In some embodiments, the present disclosure provides a method of treating cystic fibrosis by administering to the lung of a subject in need of treatment an mRNA comprising a coding sequence which encodes an amino acid sequence at least about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the wild-type human CFTR protein (SEQ ID NO:1). In another particular embodiment, the present disclosure provides a method of treating cystic fibrosis by administering to the lung of a subject in need of treatment an mRNA comprising a coding sequence of SEQ ID NO:3. In some embodiments, the present disclosure provides a method of treating cystic fibrosis by administering to the lung of a subject in need of treatment an mRNA comprising a coding sequence at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO:3.

[0029] In yet another embodiment, the present disclosure provides methods for making an mRNA encoding a CFTR protein as described herein. In one embodiment, the disclosure provides a method of making CFTR mRNA *in vitro*, comprising contacting an isolated polynucleotide with an RNA polymerase in the presence of nucleoside triphosphates, wherein: the isolated polynucleotide and RNA polymerase are not contained within a cell; the isolated polynucleotide is a template for the RNA polymerase; the isolated polynucleotide comprises a

promoter operably linked to a template sequence; the template sequence comprises a coding sequence complement which is complementary to a sequence encoding SEQ ID NO: 1; and: (a) the template sequence comprises fewer cryptic promoters than the complement of SEQ ID NO: 2, (b) the template sequence comprises fewer direct and/or inverted repeats than SEQ ID NO: 2, (c) the template sequence comprises complements of fewer disfavored codons than SEQ ID NO: 2, or (d) the GC content of the coding sequence complement is lower than the GC content of SEQ ID NO: 2.

[0030] In another embodiment, the disclosure provides a method of making CFTR mRNA *in vitro*, comprising contacting an isolated polynucleotide according to the disclosure with an RNA polymerase in the presence of nucleoside triphosphates, wherein: the isolated polynucleotide and RNA polymerase are not contained within a cell; the isolated polynucleotide is a template for the RNA polymerase; and the isolated polynucleotide comprises a promoter operably linked to a template sequence, and the RNA polymerase synthesizes mRNA comprising a coding sequence encoding SEQ ID NO: 1.

[0031] In some embodiments of such uses and methods of treatment, the *in vitro* transcribed mRNA is a naturally occurring or wild-type mRNA encoding human CFTR (SEQ ID NO: 2) modified to include non-naturally occurring UTRs. In other embodiments, the *in vitro* transcribed mRNA is a non-naturally occurring mRNA as described above.

[0032] Additional objects and advantages of the disclosure will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention. The objects and advantages of the disclosure will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims.

[0033] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the disclosure, as claimed.

[0034] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several embodiments of the invention and together with the description, serve to explain the principles of the disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

[0035] The foregoing aspects and advantages of this invention may become apparent from the following detailed description with reference to the accompanying drawings.

[0036] **Figure 1A.** Detection of mature "C" band for human CFTR protein 24 hours after transfection with human CFTR mRNA. Successful protein production was observed for both unmodified and modified (SNIM) mRNA (comprising 25% of 2-thiouridine and 5-methylcytidine). Immunoprecipitation was performed using R&D Systems MAB25031 antibody and detection using Ab570.

[0037] **Figure 1B.** Western Blot analysis of CFTR KO mouse lungs 24 hour post-exposure of PEI/unmodified human CFTR mRNA nanoparticles. Mice were treated via nebulization (Pari Boy jet nebulizer) over the course of approximately one hour. Immunoprecipitation of human CFTR protein derived according to provided methods was performed. Mature "C" band is detected in all treated mice while unobserved in control mice.

[0038] **Figure 2.** Current-voltage plot of 8-Br-cAMP evoked currents of treated (4 ug hCFTR mRNA) and untreated HEK293T cells. A large current is induced within the hCFTR mRNA transfected cells as compared to the untreated cells. Treated cells that were exposed to a specific CFTR protein inhibitor, CFTRinh-172, show a marked reduction (~89%) in Cl- ion current flow.

[0039] **Figure 3.** Histogram plots of 8-Br-cAMP evoked currents of treated (4 ug hCFTR mRNA) and untreated HEK293T cells upon application of a +80mV membrane potential. A large current is induced within the hCFTR mRNA transfected cells as compared to the untreated cells. Treated cells that were exposed to a specific CFTR protein inhibitor, CFTRinh-172, show a marked reduction (~89%) in Cl- ion current flow.

[0040] **Figure 4.** Current-voltage plot comparing profiles of HEK 293 cells of native, forskolin and GlyH-101 exposure. No significant changes in current were observed in any scenario.

[0041] **Figure 5.** Current-voltage plot of forskolin-evoked currents of treated (4 ug hCFTR mRNA) and untreated HEK293 cells. A large current is induced within the hCFTR

mRNA transfected cells as compared to the untreated cells. Treated cells that were exposed to a specific CFTR protein inhibitor, GlyH-101, show a marked reduction (~95%) in Cl- ion current flow as demonstrated in the step plot (+100 mV) on the right side of the graph.

[0042] **Figure 6.** *In situ* hybridization of human CFTR mRNA in untreated (PBS) (left) and treated (right) CFTR KO mouse lungs. Mice were exposed to 30 ug of encapsulated unmodified hCFTR mRNA in PEI nanoparticles via intratracheal administration. Substantial positive staining is observed throughout both lungs at 24 hours post-administration.

[0043] **Figure 7.** *In situ* hybridization of human CFTR mRNA treated CFTR KO mouse lungs at different magnification views (up to 20x magnification). Mice were exposed to 30 ug of encapsulated unmodified hCFTR mRNA in PEI nanoparticles via intratracheal administration.

[0044] **Figure 8.** High magnification (40x) representative lung section demonstrating *in situ* hybridization of human CFTR mRNA treated (right) CFTR KO mouse lungs. Human CFTR mRNA was detected in the apical cytoplasm of target bronchial epithelial cells 24 hours post administration. Mice were exposed to 30 ug of encapsulated unmodified hCFTR mRNA in PEI nanoparticles via intratracheal administration.

[0045] **Figure 9.** Comparison of *in situ* hybridization staining of human CFTR mRNA treated CFTR KO mouse lungs at six hours (left) and 24 hours (right) post-administration. Mice were exposed to 30 ug of encapsulated unmodified hCFTR mRNA in PEI nanoparticles via intratracheal administration. Intense positive intracellular staining is observed within six hours throughout both lungs within bronchial and alveolar regions while substantial positive staining is still observed at 24 hours post-administration.

[0046] **Figure 10.** *In situ* hybridization of human CFTR mRNA in untreated (PBS) (top) and treated (bottom) CFTR KO mouse lungs. Mice were exposed to 15 ug of encapsulated unmodified hCFTR mRNA in C12-200 lipid nanoparticles via intratracheal administration. Substantial positive staining is observed throughout both lungs at 6 hours post-administration.

[0047] **Figure 11.** High magnification (40x) representative lung sections demonstrating *in situ* hybridization of human CFTR mRNA treated CFTR KO mouse lungs. Human CFTR mRNA was detected in the apical cytoplasm of target bronchial epithelial (left) as well as intracellular alveolar regions (right) six hours post administration. Mice were exposed to 15 ug

of encapsulated unmodified hCFTR mRNA in C12-200 lipid nanoparticles via intratracheal administration.

[0048] **Figure 12.** Screening of different cell lines for hCFTR expression. Immunoblot of CHO and COS-7 (A) and BHK and PKC (B) cells transfected with hCFTR coding constructs. Protein lysates were prepared 24 hrs post transfection and screened using MA1-935 as primary antibody. Arrow indicates putative CFTR. See the discussion of MA1-935 specificity in Example 6.

[0049] **Figure 13.** Cross reactivity of different anti-human CFTR antibodies. (A) – Mouse anti-human CFTR MA1-935 (Chemicon): (B) – Mouse anti-human CFTR AB570 (Cystic Fibrosis Foundation): (C) – Mouse anti-human CFTR AB596 (Cystic Fibrosis Foundation): (D) Rabbit anti-human CFTR G449 (Rockefeller University). Arrow indicates CFTR.

[0050] **Figure 14.** Immunoprecipitation of human CFTR using three different antibodies (R29, R66/17 and R66/16) followed by immunodetection using AB596. Lane 1: T84 cells (positive control), Lane 2: untreated pig lung tissue (300 mg), Lane 3: treated pig lung tissue (697 mg), Lane 4: treated pig lung tissue (163 mg).

[0051] **Figure 15.** Immunoprecipitation and Western blotting of a mouse at 24 hrs post IT spray application of 20 μg hCFTR SNIM RNA/10 μg FFL SNIM RNA each in the HGT5001 Formulation of Example 6. T84 cells served a positive control showing the mature glycosylated C-band and the mannosylated B-band of hCFTR. "supernatant" remaining cellular extract fraction without immunoprecipitated fraction. "IP" immunoprecipitated fraction.

[0052] **Figure 16.** Immunoprecipitation of hCFTR from T84 cells using MAB25031 followed by immunodetection using AB570 (A) and MAB1660 (B).

[0053] **Figure 17.** Immunoprecipitation of CFTR from NIH3T3 cells at 72 hrs post transfection with different constructs.

[0054] **Figure 18.** Immunoprecipitation of CFTR from NIH3T3 cells at 72 hrs post transfection with different constructs using 500 ug protein and MAB1660 (left and center panels) and increased amount of total protein (8 mg) using MAB25031 (right panel).

[0055] **Figure 19.** Immunoprecipitation of hCFTR using MAB25031 and subsequent immunodetection using AB570 from pig lung samples post hCFTR SNIM RNA delivery in the

PEI Formulation of Example 6. Lane 1: sample from luciferase-negative left caudal lobe of pig #2, Lane 2: sample from luciferase-positive lung regions of pig #1.

[0056] **Figure 20.** Nebulisation was performed on anesthetized and ventilated pigs (left). The nebulizer was connected in-line to the ventilation system (right, see white arrow).

[0057] **Figure 21.** Luciferase expression measured in homogenates of pig tissue specimens from different lung regions after aerosol administration of 1 mg FFL SNIM RNA in the PEI Formulation of Example 6 with the EFlow mesh nebulizer. Lung specimens were ex vivo cultured overnight before luciferase measurements (pg luciferase/mg lung tissue).

[0058] **Figure 22.** BLI of luciferase expression in representative pig tissue specimens from different lung regions after aerosol administration of 1 mg FFL SNIM RNA in the PEI Formulation of Example 6. Lung specimens were ex vivo cultured overnight before measurements.

[0059] **Figure 23.** BLI imaging of luciferase expression in representative pig tissue specimens from different lung regions after aerosol administration of 1 mg FFL SNIM RNA in the PEI Formulation of Example 6 using a PARI BOY jet nebulizer. Lung specimens were ex vivo cultured overnight before measurements.

[0060] **Figure 24.** BLI of luciferase expression in representative pig tissue specimens from different lung regions after aerosol administration of each 1 mg FFL SNIM RNA and hCFTR mRNA in the PEI Formulation of Example 6 using an Aeroneb mesh nebulizer. Lung specimens were ex vivo cultured overnight before measurements.

[0061] **Figure 25.** BLI of luciferase expression in representative pig tissue specimens from different lung regions after aerosol administration of 1 mg FFL SNIM RNA in "SHIRE Formulation #3" (HGT5001:DOPE:Chol:DMGPEG2K (50:25:20:5) (mol ratio) using an Aeroneb mesh nebulizer. Lung specimens were ex vivo cultured overnight before measurements.

[0062] **Figure 26.** BLI of luciferase expression in pig tissue specimens from different lung regions from one untreated control pig. The other untreated control pig showed the same result (data not shown).

[0063] **Figure 27.** BLI of luciferase expression in lung specimens of once-treated pigs #3 and #6. Aerosol administration of each 1 mg FFL SNIM RNA and hCFTR SNIM RNA in the PEI Formulation of Example 6 was performed using an Aeroneb mesh nebulizer. Slices of the entire pig lung are shown. Upper three rows: pig #3, lower three rows: pig #6.

[0064] **Figure 28.** BLI of luciferase expression in lung specimens of twice-treated pigs #4 and #8. Aerosol administration of each 1 mg FFL SNIM RNA and hCFTR SNIM RNA in the PEI Formulation of Example 6 was performed using an Aeroneb mesh nebulizer. Slices of the entire pig lung are shown. Upper three rows: pig #4, lower three rows: pig #8.

[0065] **Figure 29.** BLI of luciferase expression in lung specimens of three times-treated pigs #1 and #2. Aerosol administration of each 1 mg FFL SNIM RNA and hCFTR-mRNA SNIM RNA in the PEI Formulation of Example 6 were performed using an Aeroneb mesh nebulizer. Slices of the entire pig lung are shown. Upper three rows: pig #1, lower three rows: pig #2.

[0066] **Figure 30.** Luciferase IHC on lung tissue of three times treated pig #1. Aerosol administration of each 1 mg FFL SNIM RNA and hCFTR SNIM RNA in the PEI Formulation of Example 6 was performed using an Aeroneb mesh nebulizer. Luciferase expression appeared in reddish-pink colour (Anti-Luciferase pAb 1:300, G7451, Promega, Refine AP-Kit, chromogen: New fuchsine).

[0067] **Figure 31.** Highly BLI-positive lung tissue of threefold treated pig #1 was subjected to hCFTR IP/WB. Lane 1:T84 cells (positive control), Lane 2: untreated pig lung tissue (300 mg), Lane 3: treated pig lung tissue (697 mg), Lane 4: treated pig lung tissue (163 mg). Mature complex-glycosylated hCFTR appeared as the disperse so-called C-band. Mannose-rich hCFTR appeared as the more dense so-called B-band. hCFTR expression was observed in T84 cells and pig lung tissue of hCFTR SNIM RNA treated pig #1, whereas no hCFTR expression was observed in untreated pigs.

[0068] **Figure 32.** Immunoprecipitation of hCFTR using MAB25031 and subsequent immunodetection using AB570 from pig lung samples post hCFTR SNIM RNA delivery in the PEI Formulation of Example 6. Lane 1: sample from luciferase-negative left caudal lobe of pig #2, Lane 2: sample from luciferase-positive lung regions of pig #1.

[0069] **Figure 33 A&B.** *In vitro* transfection of HEK 293T cells with C-terminal His_{10} tagged (CO-CFTR-C-His_{10}) and non-tagged (CO-CFTR) codon optimized human CFTR SNIM RNA. Following transfection, whole cell lysate was collected and analyzed for human CFTR expression by Western blot using (A) anti-CFTR antibody #217 and (B) anti-His antibody 1187. Transfected samples were compared to non-transfection HEK 293T control lysate (Lane 3).

[0070] **Figure 33 C.** *In vitro* transfection of HEK 293T cells with SNIM RNA encoding codon optimized human CFTR with a growth hormone leader sequence and a (GH-CO-CFTR) or SNIM RNA encoding a C-terminal His_{10} tagged codon optimized human CFTR (CO-CFTR-C-His_{10}). Following transfection, whole cell lysate was collected and analyzed for human CFTR expression by Western blot using anti-CFTR antibody #217. Transfected samples were compared to non-transfection HEK 293T control lysate (Lane 3).

[0071] **Figure 34.** *In vivo* transfection of CFTR knockout mice with C-terminal His_{10} tagged codon optimized human CFTR SNIM RNA encapsulated within either a lipid (cKK-E12) or polymeric (PEI) nanoparticle formulation. Following nebulized delivery of each respective mRNA formulation, Right and Left lung tissue lysate was collected and analyzed for CFTR expression by Western blot using anti-His antibody 1187. Control CFTR knockout lung tissue and CFTR-His_{10} HEK293 lysate was used as a negative and positive controls respectively.

[0072] **Figure 35.** Bioluminescent detection of FFL expression in porcine lung samples collected following nebulization with water for injection.

[0073] **Figure 36.** Bioluminescent detection of FFL expression in porcine lung samples collected following nebulization with 1 mg FFL SNIM RNA + 1 mg CO-CFTR SNIM RNA in a branched 25 kDa PEI formulation.

[0074] **Figure 37.** Bioluminescent detection of FFL expression in porcine lung samples collected following nebulization with 1 mg FFL SNIM RNA + 5 mg CO-CFTR SNIM RNA in a branched 25 kDa PEI formulation.

[0075] **Figure 38.** Bioluminescent detection of FFL expression in porcine lung samples collected following nebulization with 1 mg FFL SNIM RNA + 10 mg CO-CFTR SNIM RNA in a branched 25 kDa PEI formulation.

[0076] **Figure 39.** Relative quantification of CFTR expression in different chorots. Band intensities were normalized to 150kDa band in the protein ladder.

[0077] **Figure 40.** Representative example of a "CFTR-positive" bronchi with at least one epithelial cell detected within the epithelial cell layer and displaying a clear membrane localized CFTR signal via CFTR immunohistochemical staining using an anti-CFTR antibody.

[0078] **Figure 41.** Immunohistochemical staining of CFTR in porcine lung following aerosol delivery of control (WFI) or 5 mg CO-CFTR SNIM RNA.

[0079] **Figure 42.** Represents a "low" CFTR expression level, assayed in porcine lung by immunohistochemical staining with anti-CFTR following aerosol delivery of 5 mg CO-CFTR SNIM RNA.

[0080] **Figure 43.** Represents a "medium" CFTR expression level, assayed in porcine lung by immunohistochemical staining with anti-CFTR following aerosol delivery of 5 mg CO-CFTR SNIM RNA.

[0081] **Figure 44.** Represents a "high" CFTR expression level, assayed in porcine lung by immunohistochemical staining with anti-CFTR following aerosol delivery of 5 mg CO-CFTR SNIM RNA.

[0082] **Figure 45.** Immunohistochemical staining of CFTR in porcine lung following aerosol delivery of control (WFI) or 10 mg CO-CFTR SNIM RNA.

[0083] **Figure 46.** Quantification of relative numbers of CFTR-positive bronchi / bronchioles per animal. Analysis of each cohort (WFI; and 1mg, 5mg, 10mg human CFTR SNIM RNA) 24 hours post aerosol administration. CFTR expression normalized to signal intensity for 150 kDa protein standard. (WFI=9.4±5.6%, 1MG=15.2±6.6%, 5MG=25.4±14.1%, 10MG=20.9±3.7%; WFI vs 5MG p=0.0281, WFI vs 10MG p=0.0174)

[0084] **Figure 47.** Illustrates multiplex nucleic acid *in situ* detection of (A) ubiquitin C and (B) *dap* B in porcine lung, post aerosol delivery of water for injection by nebulizer.

[0085] **Figure 48.** Illustrates multiplex nucleic acid *in situ* detection of (A) ubiquitin C and (B) *dap* B in porcine lung, post aerosol delivery of 1 mg FFL SNIM RNA + 10 mg CO-CFTR SNIM RNA in a branched 25 kDa PEI formulation.

[0086] **Figure 49.** Illustrates multiplex nucleic acid *in situ* detection of (A) right cranialis and (B) left cranialis in porcine, post aerosol delivery of water for injection by nebulizer.

[0087] **Figure 50.** Illustrates multiplex nucleic acid *in situ* detection of (A) right cranialis and (B) left cranialis in porcine, post aerosol delivery of 1 mg FFL SNIM RNA + 1 mg CO-CFTR SNIM RNA in a branched 25 kDa PEI formulation.

[0088] **Figure 51.** Illustrates multiplex nucleic acid *in situ* detection of (A) right cranialis and (B) left cranialis in porcine, post aerosol delivery of 1 mg FFL SNIM RNA + 5 mg CO-CFTR SNIM RNA in a branched 25 kDa PEI formulation.

[0089] **Figure 52.** Illustrates multiplex nucleic acid *in situ* detection of (A) right cranialis and (B) left cranialis in porcine, post aerosol delivery of 1 mg FFL SNIM RNA + 10 mg CO-CFTR SNIM RNA in a branched 25 kDa PEI formulation.

[0090] **Figure 53.** Illustrates positive detection of active firefly luciferase (FFL) protein in a treated pig lung via luminescence upon exposure to FFL/CO-CFTR-C-His10 mRNA encapsulated cKK-E12 lipid nanoparticles. Pigs were treated with 1 mg FFL + 9 mg CO-CFTR-C-His10 mRNA encapsulated lipid nanoparticles via nebulization using a Pari jet nebulizer and sacrificed 24 hours post-treatment. FFL luminescence was visualized using an IVIS bioluminometer.

[0091] **Figure 54.** Illustrates exemplary results of hCFTR expression in HEK cells transfected using neubilized complexes given to pigs 10, 11 and 12 (1 mg dose).

[0092] **Figure 55.** Illustrates exemplary results of hCFTR expression in HEK cells transfected using neubilized complexes given to pigs 13, 14 and 15 (5 mg dose) and in HEK cells transfected using neubilized complexes given to pigs 19, 20 and 21 (10 mg dose).

[0093] **Figure 56.** Illustrates exemplary results of hCFTR expression in HEK cells transfected using neubilized complexes given to pig 16 (5 mg dose), 22 (10 mg dose) and 67 (1 mg dose).

[0094] **Figure 57.** Illustrates exemplary results of hCFTR expression in HEK cells transfected using neubilized complexes given to pigs 17, 18 (5 mg dose), 23, 24 (10 mg dose) and 68, 69 (1 mg dose).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0095] As used herein, the term "polynucleotide" is generally used to refer to a nucleic acid (e.g., DNA or RNA). The terms polynucleotide, nucleic acid, DNA, RNA, and mRNA include such molecules that are comprised of: standard or unmodified residues; nonstandard or modified residues; and mixtures of standard and nonstandard residues.

[0096] As used herein, the term "mRNA" is used to refer to modified and unmodified RNA including both a coding region and a noncoding region.

[0097] As used herein, the phrase "coding region" of an mRNA generally refers to that portion that when translated results in the production of an expression product, such as a polypeptide, protein, or enzyme.

[0098] A "nonstandard nucleobase" is a base moiety other than the natural bases adenine (A), cytosine (C), guanine (G), thymine (T), or uracil (U). The nonstandard nucleobase is an analog of a specific nucleobase (A, C, G, T, or U) when its base pairing properties in a nucleic acid double helix and locus of incorporation by DNA or RNA polymerases in a nucleic acid double helix (including a local RNA-DNA helix such as that formed during transcription of a DNA template by RNA polymerase) are most similar to one of the five previously listed nucleobases, with the exception that analogs of T will generally also be analogs of U and vice versa. For purposes of determining percentage identity of a first sequence relative to a second sequence, an analog of a base is not a mismatch to the natural base; for example, pseudouridine matches uridine, 5-methylcytidine matches cytidine, etc.

[0099] The term "nonstandard" used in conjunction with terms including but not limited to "nucleoside", "base", "nucleotide", or "residue" is to be interpreted in the same manner as if it were used in conjunction with "nucleobase."

[0100] "GC content" is the fraction or percentage of total nucleobase residues in a nucleic acid sequence that are guanine residues, cytosine residues, or analogs thereof. For

example, a 100 nt sequence that contains exactly 30 cytosines, exactly 30 guanines, exactly one cytosine analog, and exactly one guanine analog has a GC richness of 62%.

[0101] As used herein, a "disfavored codon" refers to a codon which is translated less efficiently or rapidly by mammalian cells than another codon for the same amino acid residue. Disfavored codons generally include codons with an A or U in the 3rd or "wobble" position of the codon. For a discussion of disfavored codons, see, e.g., U.S. Patent Publication 2009/0069256 A1.

[0102] A "non-naturally occurring mRNA molecule" is an mRNA that is not produced through normal transcription and splicing processes of wild-type cells. An mRNA may qualify as non-naturally occurring by virtue of its sequence (e.g., a series of codons and/or one or more UTRs that do not present in any naturally-occurring CFTR mRNA) and/or because it includes nonstandard nucleotide residues. A non-naturally occurring mRNA molecule may be *in vitro* synthesized.

[0103] In each of Tables 1 and 2 below, the NWT column indicates the non-wild-type base at the position (Pos.) in the CFTR coding sequence (see, e.g., SEQ ID NO: 3), and the WT column indicates the wild-type base at the same position (see, e.g., SEQ ID NO: 2 or the RefSeq entry for human CFTR (accession no. NM_000492.3, Feb. 10, 2013, version, available from GenBank; note that the sequence of NM_000492.3 contains noncoding sequence such that the coding sequence occurs at position 133 to 4575, such that, for example, position 7 in the tables below corresponds to position 139 of the NM_000492.3 sequence).

Non-naturally occurring CFTR mRNA

[0104] In addition to providing methods of producing functional CFTR *in vivo* using naturally occurring or wild-type CFTR mRNA (and compositions comprising that mRNA), the invention also provides non-naturally occurring CFTR mRNA that encodes CFTR protein (e.g., SEQ ID NO:1). In some embodiments, the non-naturally occurring CFTR mRNA is purified or isolated.

[0105] In other embodiments, the non-naturally occurring CFTR mRNA is present in a cell. In some embodiments, the cell comprising the non-naturally occurring CFTR mRNA did not synthesize the non-naturally occurring CFTR mRNA and/or does not comprise DNA

complementary to the non-naturally occurring CFTR mRNA and/or a functional CFTR gene; the cell may optionally comprise an inactive CFTR gene, such as a CFTR gene with a nonsense, missense, frameshift, insertion, or deletion mutation that renders the expression product of the gene nonfunctional. In some embodiments, the cell comprising the non-naturally occurring CFTR mRNA further comprises functional CFTR protein translated from the non-naturally occurring CFTR mRNA. The cell may be, e.g., a lung epithelial cell, a liver cell, or a kidney cell. In some embodiments, the cell is in a cell culture.

CFTR Coding Sequence

[0106] In some embodiments, CFTR mRNA according to the invention comprises a coding sequence with fewer complements of cryptic promoters than SEQ ID NO: 2 (i.e., the coding sequence of wild-type human CFTR), fewer direct and/or inverted repeats than SEQ ID NO: 2, fewer disfavored codons than SEQ ID NO: 2, and/or the GC content of the coding sequence is lower than the GC content of SEQ ID NO: 2.

[0107] Cryptic promoters, direct and/or inverted repeats and/or disfavored codons of a sequence may be recgnized by one skilled in the art using routine methods. For example, the direct and/or inverted repeat content of a sequence can be determined by sequence analysis (Liu et al., *Journal of Theoretical Biology* (2014) 344: 19-30). The cryptic promoter content of a sequence can also be determined by sequence analysis, e.g., presence of Shine-Dalgarno sequences within construct or the like.

[0108] In some embodiments, CFTR mRNA according to the invention is *in vitro*-transcribed, i.e., the mRNA was synthesized in an artificial setting not within a biological cell (e.g., a cell free *in vitro* transcription system). Generally, *in vitro* transcription involves providing a DNA template comprising a promoter and a sequence complementary to the desired mRNA (which may be circular or linear), an RNA polymerase, and nucleoside triphosphates in suitable reaction conditions (salts, buffers, and temperature). RNase inhibitors, reducing agents, and/or pyrophosphatase may be present in the reaction mixture. In some embodiments, the RNA polymerase is T7 RNA polymerase.

[0109] In some embodiments, the CFTR mRNA according to the invention comprises a coding sequence comprising at least 50%, at least 55%, at least 60%, at least 65%, at least 70%,

at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% of the non-wild-type bases listed in Table 1 at the positions of the coding sequence listed in Table 1 relative to the wild-type coding sequence of SEQ ID NO: 2.

Table 1. Non-wild-type bases that can be used in the coding sequence of mRNA encoding CFTR.

Pos.	NWT	WT
7	c	a
12	c	g
15	g	u
18	c	g
30	u	c
33	c	u
36	g	c
45	c	u
48	c	u
52	u	a
53	c	g
54	a	c
60	u	c
61	c	a
63	g	a
66	u	a
69	c	u
70	c	u
72	u	g
75	a	g
78	g	a
81	g	a
84	u	c
85	c	a
87	g	a
91	a	c
93	g	c
96	u	g
99	g	a
105	u	a
111	c	a

Pos.	NWT	WT
117	g	a
123	c	u
126	g	u
129	a	u
135	g	u
138	g	u
141	u	c
144	c	u
147	c	a
150	g	u
153	g	a
156	g	a
157	c	u
159	c	g
163	c	a
165	g	a
174	c	u
175	c	a
177	c	a
180	a	g
183	c	g
186	g	u
189	u	a
198	c	u
201	g	u
204	g	a
210	c	u
213	c	u
216	a	c
219	g	u
220	a	c

Pos.	NWT	WT
222	a	g
223	a	c
225	g	a
228	c	u
231	c	u
238	c	a
240	g	a
243	c	u
252	c	u
255	u	a
261	c	u
264	g	a
268	c	u
270	c	a
276	g	a
282	a	c
291	c	a
294	a	g
297	c	u
300	g	c
303	g	a
304	u	c
309	u	a
310	c	a
312	c	a
315	u	c
318	c	a
321	c	u
324	g	c
327	c	u
333	c	g

Pos.	NWT	WT
342	a	g
345	a	g
351	g	c
352	a	u
353	g	c
354	c	u
363	c	u
366	c	u
369	c	a
372	g	c
375	c	a
378	a	c
379	c	u
381	g	a
384	u	c
385	u	c
387	g	u
390	u	c
393	c	u
396	c	u
399	c	g
402	a	g
408	u	g
409	u	c
411	g	c
412	u	c
414	g	a
417	u	c
423	a	c
426	c	u
429	c	u
435	c	u
444	c	u
447	u	a
457	c	a
462	c	a
474	c	u
480	c	u
483	c	u
486	a	g

Pos.	NWT	WT
492	a	u
493	c	u
495	g	a
498	a	g
501	c	g
504	g	a
505	u	a
506	c	g
507	g	c
510	g	u
513	g	u
514	u	c
516	g	a
522	g	a
525	u	a
526	u	a
527	c	g
528	c	u
531	c	u
534	u	a
537	g	a
538	u	c
540	g	u
543	g	u
544	u	a
545	c	g
546	c	u
549	g	c
553	a	u
554	g	c
555	u	c
558	u	c
564	c	g
573	c	u
579	g	a
585	g	u
588	g	a
589	c	u
609	u	c
612	c	u

Pos.	NWT	WT
615	g	u
624	c	g
627	c	a
630	u	c
631	u	c
633	g	c
639	c	g
642	u	a
645	u	c
652	c	u
657	g	a
663	a	g
672	u	c
678	c	a
681	g	u
684	a	u
687	u	c
693	u	a
696	g	c
697	u	c
699	g	u
702	a	c
703	u	c
705	g	u
720	u	a
724	c	a
726	g	a
741	u	c
742	c	a
744	c	a
747	c	u
756	g	u
759	u	g
762	a	g
766	u	a
767	c	g
768	g	u
777	c	u
780	c	g
783	c	u

Pos.	NWT	WT
786	u	c
789	g	a
798	c	u
804	c	u
810	g	a
813	g	u
816	c	u
819	a	g
822	c	a
825	u	c
840	u	a
846	g	a
849	g	a
862	c	u
864	c	a
865	c	a
867	c	a
873	u	a
876	g	a
888	c	u
891	c	g
897	g	a
900	g	c
906	c	g
907	c	a
909	g	a
912	u	c
919	u	a
920	c	g
921	g	c
927	g	c
936	u	c
939	c	a
948	c	u
951	u	g
954	c	g
958	c	u
960	c	a
963	g	u
966	u	g

Pos.	NWT	WT
967	u	c
969	g	u
972	u	c
978	c	a
979	u	c
981	g	a
984	u	c
987	g	a
990	g	a
993	u	c
1002	c	g
1005	g	a
1008	u	a
1017	g	c
1020	u	c
1023	g	a
1035	a	u
1036	u	c
1047	a	g
1050	g	c
1053	a	u
1065	g	c
1071	c	u
1074	g	a
1077	g	a
1092	g	u
1101	g	a
1104	c	a
1113	c	a
1116	a	g
1119	c	u
1125	g	a
1137	g	a
1140	c	u
1146	c	a
1147	c	u
1152	g	a
1155	c	u
1158	u	c
1159	c	u

Pos.	NWT	WT
1161	u	a
1164	u	g
1170	g	a
1173	g	a
1179	a	g
1191	g	a
1194	g	a
1197	u	c
1200	u	c
1206	a	g
1212	u	a
1218	a	g
1222	c	u
1224	g	a
1239	g	a
1242	g	a
1245	u	c
1248	c	u
1254	c	u
1255	c	a
1257	c	a
1260	g	a
1263	c	u
1266	a	u
1272	g	u
1275	c	u
1278	u	c
1279	u	a
1280	c	g
1284	g	c
1287	u	c
1291	u	a
1292	c	g
1293	g	u
1296	c	u
1302	c	a
1305	g	u
1308	c	u
1311	a	u
1314	a	u

Pos.	NWT	WT
1317	c	u
1320	g	c
1321	u	c
1326	g	a
1329	c	u
1332	c	u
1344	u	a
1347	g	a
1350	g	a
1357	c	u
1359	u	g
1360	c	u
1362	c	g
1368	a	u
1371	g	u
1375	a	u
1376	g	c
1383	u	a
1386	g	a
1389	a	c
1392	a	g
1396	a	u
1397	g	c
1398	c	a
1401	c	u
1402	u	c
1404	g	a
1419	g	a
1422	g	a
1425	u	g
1431	c	u
1432	a	u
1433	g	c
1434	c	a
1440	g	u
1443	g	a
1449	a	g
1453	u	a
1454	c	g
1455	c	u

Pos.	NWT	WT
1458	g	a
1459	c	a
1461	u	a
1464	c	u
1474	a	u
1475	g	c
1476	c	u
1485	a	c
1491	c	u
1497	c	u
1500	a	c
1512	g	a
1515	c	u
1521	u	c
1524	c	u
1527	a	u
1530	a	u
1542	g	a
1545	c	u
1546	c	a
1555	u	a
1556	c	g
1557	g	c
1563	u	c
1566	g	a
1569	g	a
1575	g	a
1576	u	c
1578	g	a
1590	u	c
1593	u	c
1599	c	u
1602	c	a
1608	g	a
1611	u	c
1614	c	u
1617	c	a
1620	c	u
1621	u	c
1623	g	u

Pos.	NWT	WT
1632	g	u
1635	u	a
1638	u	c
1642	u	c
1645	u	a
1646	c	g
1647	g	u
1653	g	u
1656	g	a
1662	g	a
1663	c	a
1665	g	a
1668	c	u
1669	a	u
1670	g	c
1671	c	u
1672	c	u
1674	c	a
1677	g	a
1683	g	a
1698	a	u
1708	c	u
1710	g	a
1711	c	u
1713	u	a
1716	u	c
1719	a	u
1722	g	u
1734	c	a
1737	c	u
1740	a	u
1743	g	a
1758	c	a
1761	c	u
1764	g	a
1765	u	a
1766	c	g
1767	g	c
1770	c	u
1773	g	c

Pos.	NWT	WT
1782	u	g
1791	u	c
1794	g	a
1797	g	u
1800	a	g
1803	c	u
1804	c	u
1809	g	c
1812	a	u
1815	a	u
1827	c	u
1828	c	u
1830	u	a
1836	g	a
1839	g	u
1845	g	a
1848	c	a
1849	c	u
1851	g	a
1854	c	u
1855	c	u
1857	c	g
1860	c	u
1866	a	u
1867	u	a
1868	c	g
1869	g	c
1870	u	a
1871	c	g
1875	c	u
1881	c	u
1884	c	g
1887	u	a
1890	c	u
1896	g	a
1897	u	c
1899	g	c
1905	c	u
1906	u	c
1908	g	a

Pos.	NWT	WT
1914	g	a
1920	c	u
1921	u	a
1922	c	g
1923	a	c
1924	a	u
1925	g	c
1926	c	a
1938	g	a
1944	c	u
1947	a	u
1956	g	a
1959	c	u
1962	c	u
1965	g	a
1969	c	a
1971	g	a
1972	c	a
1974	g	a
1977	c	u
1980	g	a
1984	u	c
1986	g	a
1989	g	u
1992	a	g
1995	g	c
1996	c	u
1998	g	a
2004	a	u
2010	g	a
2011	c	u
2013	u	a
2016	g	a
2019	u	a
2025	c	u
2028	g	u
2031	a	c
2034	g	c
2040	c	a
2043	g	a

Pos.	NWT	WT
2049	g	a
2052	g	a
2055	g	a
2058	g	u
2064	g	a
2070	a	u
2076	a	g
2082	u	g
2085	g	a
2091	a	g
2097	c	u
2098	a	u
2099	g	c
2103	c	u
2104	u	c
2106	g	c
2112	u	a
2115	u	c
2121	a	u
2124	u	a
2127	c	a
2130	g	a
2133	c	u
2136	a	c
2139	c	u
2142	c	g
2145	g	a
2148	a	g
2154	a	c
2155	c	u
2157	g	a
2160	g	a
2169	a	c
2172	u	c
2184	g	u
2187	c	u
2190	a	g
2193	c	u
2194	c	u
2196	g	a

Pos.	NWT	WT
2200	c	a
2202	c	a
2208	u	g
2209	a	u
2210	g	c
2212	c	u
2214	c	a
2217	g	a
2220	g	a
2226	a	u
2232	a	g
2235	g	a
2241	c	g
2244	u	a
2247	u	g
2250	c	u
2253	g	c
2256	u	c
2257	u	a
2258	c	g
2259	g	c
2265	u	c
2266	u	a
2267	c	g
2268	a	c
2271	c	u
2274	a	c
2277	u	c
2280	a	g
2289	g	a
2290	a	c
2292	g	a
2293	c	a
2295	a	g
2301	a	g
2304	c	u
2307	g	c
2310	c	g
2316	c	g
2322	g	a

Pos.	NWT	WT
2325	u	c
2328	g	a
2331	a	u
2337	g	a
2340	g	u
2343	a	g
2355	c	a
2358	a	g
2361	g	a
2364	g	a
2367	c	a
2370	a	c
2373	g	a
2374	a	c
2388	u	g
2391	a	c
2394	c	u
2400	g	a
2403	u	c
2415	c	g
2418	c	u
2421	c	a
2424	c	u
2425	a	u
2426	g	c
2427	c	a
2428	c	a
2430	u	a
2434	c	u
2436	u	a
2439	g	u
2448	c	u
2451	a	c
2452	c	u
2454	u	g
2457	g	a
2460	c	a
2463	c	u
2472	c	u
2475	u	c

Pos.	NWT	WT
2484	u	c
2487	g	a
2490	a	g
2496	u	c
2499	c	u
2508	c	u
2514	a	g
2515	u	a
2516	c	g
2517	a	c
2520	c	a
2526	g	a
2532	a	u
2535	g	a
2548	u	c
2550	g	u
2553	u	a
2556	c	u
2559	c	u
2562	g	u
2565	g	c
2572	u	a
2573	c	g
2577	g	a
2583	c	u
2586	c	g
2589	c	a
2592	c	u
2598	u	c
2599	c	u
2601	c	a
2604	g	a
2607	c	u
2613	c	g
2616	u	a
2622	c	g
2625	a	u
2628	g	u
2631	a	u
2632	c	u

Pos.	NWT	WT
2634	u	g
2637	g	u
2640	c	g
2643	c	g
2649	g	c
2655	u	a
2658	u	c
2661	g	u
2664	c	u
2665	u	c
2667	g	u
2679	c	g
2683	u	a
2684	c	g
2688	a	u
2691	c	u
2692	u	a
2693	c	g
2694	a	u
2700	c	u
2703	u	c
2704	u	a
2705	c	g
2712	c	a
2724	u	c
2725	u	a
2726	c	g
2727	u	c
2730	a	c
2733	c	u
2742	c	u
2754	c	u
2760	a	g
2766	g	a
2776	c	u
2781	c	u
2784	g	u
2790	u	a
2797	c	a
2802	a	u

Pos.	NWT	WT
2805	c	a
2811	c	g
2814	u	g
2817	c	u
2820	g	u
2823	u	a
2829	u	a
2832	c	g
2835	c	g
2838	g	a
2842	c	u
2844	c	a
2850	u	c
2853	g	a
2857	c	u
2859	u	a
2863	a	u
2864	g	c
2865	c	u
2868	a	u
2871	g	u
2874	g	a
2877	u	a
2880	c	u
2886	c	a
2890	u	c
2892	g	c
2895	u	c
2899	c	u
2901	c	g
2904	g	a
2907	g	a
2910	a	u
2913	u	g
2917	u	c
2919	g	u
2923	c	a
2925	c	a
2931	a	c
2940	u	a

Pos.	NWT	WT
2964	c	u
2967	g	u
2970	g	c
2973	c	a
2976	c	u
2994	g	a
2995	c	u
2997	g	a
3000	c	u
3009	g	a
3015	u	a
3021	a	u
3027	u	a
3030	c	u
3031	c	u
3033	c	a
3036	g	a
3039	u	c
3045	u	c
3051	c	u
3054	g	a
3057	c	a
3060	u	g
3063	g	a
3069	c	a
3075	g	u
3081	c	u
3085	c	u
3088	c	a
3090	g	a
3093	c	a
3100	u	c
3102	g	c
3105	g	a
3108	g	c
3117	g	a
3120	u	c
3123	g	a
3132	g	a
3141	g	c

Pos.	NWT	WT
3145	u	a
3146	c	g
3147	g	u
3150	u	a
3153	c	u
3156	u	c
3159	g	u
3168	g	u
3171	c	a
3174	u	c
3177	g	a
3180	g	a
3184	u	c
3186	g	a
3192	g	a
3193	u	c
3195	g	u
3198	c	u
3204	u	c
3207	c	a
3208	a	c
3216	c	u
3228	a	u
3243	g	u
3250	c	u
3252	c	a
3258	g	u
3261	a	c
3264	u	c
3270	u	c
3276	u	c
3277	u	c
3280	a	u
3281	g	c
3282	u	a
3285	c	a
3288	c	g
3291	a	c
3297	u	c
3300	g	a

Pos.	NWT	WT
3304	c	a
3306	c	a
3309	u	a
3312	g	a
3324	g	c
3333	u	c
3336	c	u
3339	g	u
3342	g	u
3345	u	c
3348	u	c
3351	c	u
3357	c	u
3360	g	a
3363	c	a
3366	g	a
3372	g	a
3375	c	a
3378	g	a
3382	c	a
3384	g	a
3387	c	u
3402	a	u
3403	c	u
3405	c	a
3414	c	u
3417	u	c
3423	c	u
3426	u	a
3438	a	u
3441	g	a
3445	a	u
3446	g	c
3448	u	a
3449	c	g
3450	g	c
3453	u	a
3466	c	u
3472	a	c
3474	g	a

Pos.	NWT	WT
3477	c	u
3480	u	g
3481	u	a
3482	c	g
3483	g	c
3484	a	c
3486	g	a
3501	c	u
3510	g	a
3513	g	a
3516	g	a
3519	a	u
3522	g	a
3525	c	u
3528	a	c
3531	a	g
3532	a	u
3533	g	c
3534	u	a
3537	g	c
3543	c	a
3546	u	c
3555	g	c
3559	u	c
3561	g	c
3562	a	u
3563	g	c
3564	u	g
3567	g	a
3570	a	u
3576	c	u
3579	c	u
3585	c	u
3586	a	u
3587	g	c
3588	u	a
3600	g	a
3615	u	c
3616	a	u
3617	g	c

Pos.	NWT	WT
3618	c	a
3624	u	c
3627	g	a
3633	c	u
3636	g	c
3639	g	a
3642	c	u
3645	g	c
3648	g	a
3660	c	a
3663	g	a
3666	a	u
3669	g	a
3672	c	u
3675	a	c
3678	c	a
3679	c	u
3681	u	a
3684	a	g
3690	c	u
3693	g	c
3697	a	u
3698	g	c
3699	c	a
3702	u	a
3705	c	u
3708	c	u
3711	u	c
3715	c	a
3717	u	g
3723	g	c
3724	u	c
3726	g	c
3727	c	u
3729	c	g
3732	g	a
3735	g	a
3738	c	u
3741	g	a
3747	a	g

Pos.	NWT	WT
3750	a	g
3751	u	a
3752	c	g
3753	g	u
3756	g	u
3760	c	u
3762	g	a
3765	g	a
3768	c	u
3771	c	u
3780	u	a
3786	u	c
3789	a	u
3792	g	a
3795	u	a
3798	g	a
3810	c	u
3813	c	u
3816	u	g
3819	g	u
3826	a	u
3827	g	c
3828	c	a
3831	c	a
3834	c	u
3840	g	a
3847	c	a
3855	g	c
3864	a	g
3867	c	a
3870	c	a
3873	a	g
3876	g	a
3879	c	a
3885	c	u
3889	a	u
3890	g	c
3891	c	u
3897	c	a
3900	c	u

Pos.	NWT	WT
3901	c	a
3906	g	a
3909	u	c
3910	c	u
3912	c	g
3918	u	c
3931	u	a
3932	c	g
3933	a	u
3942	g	a
3945	u	a
3954	c	u
3957	g	a
3960	c	u
3969	c	g
3972	u	c
3973	c	a
3975	g	a
3976	a	u
3977	g	c
3981	a	g
3984	c	a
3987	g	a
3996	g	u
3999	a	g
4002	a	g
4005	c	u
4020	a	g
4029	a	c
4032	c	u
4038	g	a
4039	u	a
4040	c	g
4041	g	c
4047	g	c
4057	c	u
4059	c	g
4066	c	u
4071	g	u
4072	c	a

Pos.	NWT	WT
4077	c	u
4080	c	u
4084	u	a
4085	c	g
4089	a	g
4095	a	g
4098	u	c
4099	c	u
4101	u	g
4104	c	g
4105	u	c
4107	g	u
4116	u	c
4117	u	a
4118	c	g
4119	g	u
4122	c	u
4126	c	u
4131	c	u
4134	g	a
4140	g	a
4143	u	c
4146	g	a
4149	c	a
4152	c	u
4158	g	a
4161	a	u
4164	u	a
4167	g	a
4170	g	a
4173	g	a
4179	c	u

Pos.	NWT	WT
4182	c	u
4188	g	a
4191	g	a
4203	g	a
4206	u	c
4207	c	a
4209	u	g
4212	c	a
4215	g	a
4218	c	a
4224	c	g
4233	g	a
4240	c	u
4242	u	g
4248	c	a
4257	u	c
4260	g	a
4263	c	g
4266	c	g
4275	c	u
4287	g	a
4293	u	g
4296	u	c
4302	a	g
4303	u	a
4304	c	g
4305	a	c
4306	u	c
4308	g	c
4317	g	a
4320	g	c
4323	u	c

Pos.	NWT	WT
4324	u	a
4325	c	g
4326	a	c
4329	a	c
4335	u	c
4344	a	g
4347	u	c
4353	a	c
4357	a	c
4359	a	g
4362	u	c
4365	g	a
4366	u	a
4367	c	g
4368	g	c
4380	c	u
4383	a	g
4386	g	c
4392	c	u
4395	g	u
4398	c	u
4399	u	c
4407	a	g
4413	u	a
4422	a	g
4425	u	g
4431	c	u
4434	g	a
4435	c	a
4437	u	g
4443	a	g

[0110] In some embodiments, the CFTR mRNA according to the invention comprises a coding sequence comprising at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% of the non-wild-type bases listed in Table 2 at the corresponding positions of the coding sequence listed in Table 2 relative to the wild-type coding sequence of SEQ ID NO: 2.

Table 2. Subset of non-wild-type bases that can be used in the coding sequence of mRNA encoding CFTR.

Pos.	NWT	WT
7	c	a
15	g	u
18	c	g
33	c	u
45	c	u
54	a	c
60	u	c
61	c	a
63	g	a
66	u	a
72	u	g
81	g	a
84	u	c
85	c	a
87	g	a
93	g	c
96	u	g
126	g	u
129	a	u
135	g	u
138	g	u
141	u	c
147	c	a
150	g	u
159	c	g
163	c	a
165	g	a
175	c	a
177	c	a
180	a	g
183	c	g
186	g	u
189	u	a
201	g	u
213	c	u

Pos.	NWT	WT
216	a	c
225	g	a
238	c	a
240	g	a
252	c	u
255	u	a
270	c	a
282	a	c
291	c	a
294	a	g
304	u	c
309	u	a
310	c	a
312	c	a
315	u	c
318	c	a
324	g	c
327	c	u
333	c	g
342	a	g
345	a	g
351	g	c
369	c	a
372	g	c
375	c	a
378	a	c
384	u	c
385	u	c
390	u	c
396	c	u
399	c	g
409	u	c
412	u	c
417	u	c
423	a	c

Pos.	NWT	WT
429	c	u
435	c	u
444	c	u
447	u	a
457	c	a
462	c	a
492	a	u
498	a	g
501	c	g
504	g	a
507	g	c
510	g	u
514	u	c
525	u	a
526	u	a
527	c	g
531	c	u
534	u	a
538	u	c
544	u	a
545	c	g
555	u	c
558	u	c
564	c	g
573	c	u
588	g	a
615	g	u
624	c	g
631	u	c
642	u	a
645	u	c
663	a	g
672	u	c
684	a	u
697	u	c

Pos.	NWT	WT
702	a	c
703	u	c
720	u	a
724	c	a
726	g	a
741	u	c
742	c	a
744	c	a
756	g	u
759	u	g
762	a	g
768	g	u
777	c	u
780	c	g
786	u	c
789	g	a
798	c	u
813	g	u
816	c	u
819	a	g
825	u	c
840	u	a
864	c	a
865	c	a
867	c	a
873	u	a
891	c	g
897	g	a
900	g	c
906	c	g
907	c	a
909	g	a
912	u	c
921	g	c
927	g	c
939	c	a
948	c	u
951	u	g
954	c	g
960	c	a

Pos.	NWT	WT
963	g	u
966	u	g
967	u	c
972	u	c
979	u	c
984	u	c
990	g	a
993	u	c
1002	c	g
1008	u	a
1017	g	c
1020	u	c
1023	G	a
1035	a	u
1036	u	c
1047	a	g
1053	a	u
1065	g	c
1071	c	u
1092	g	u
1101	g	a
1116	a	g
1158	u	c
1161	u	a
1164	u	g
1170	g	a
1179	a	g
1194	g	a
1197	u	c
1200	u	c
1206	a	g
1212	u	a
1218	a	g
1245	u	c
1255	c	a
1257	c	a
1266	a	u
1275	c	u
1278	u	c
1279	u	a

Pos.	NWT	WT
1280	c	g
1293	g	u
1308	c	u
1311	a	u
1314	a	u
1317	c	u
1321	u	c
1350	g	a
1362	c	g
1368	a	u
1371	g	u
1383	u	a
1386	g	a
1389	a	c
1392	a	g
1401	c	u
1402	u	c
1425	u	g
1440	g	u
1449	A	g
1455	C	u
1458	G	a
1459	C	a
1461	U	a
1485	A	c
1497	C	u
1500	a	c
1521	u	c
1524	c	u
1527	a	u
1530	a	u
1557	g	c
1563	u	c
1569	g	a
1576	u	c
1590	u	c
1593	u	c
1599	c	u
1602	c	a
1611	u	c

Pos.	NWT	WT
1617	c	a
1620	c	u
1621	u	c
1635	u	a
1638	u	c
1642	u	c
1647	g	u
1653	g	u
1662	g	a
1674	c	a
1677	g	a
1683	g	a
1698	a	u
1713	u	a
1716	u	c
1719	a	u
1722	g	u
1734	c	a
1737	c	u
1740	a	u
1761	c	u
1765	u	a
1766	c	g
1767	g	c
1770	c	u
1782	u	g
1791	u	c
1797	g	u
1812	a	u
1815	a	u
1830	u	a
1839	g	u
1857	c	g
1860	c	u
1866	a	u
1869	g	c
1870	u	a
1871	c	g
1881	c	u
1887	u	a

Pos.	NWT	WT
1897	u	c
1906	u	c
1914	g	a
1923	a	c
1938	g	a
1947	a	u
1962	c	u
1965	g	a
1969	c	a
1971	g	a
1972	c	a
1974	g	a
1980	g	a
1984	u	c
1989	g	u
1992	a	g
1995	g	c
2010	g	a
2013	u	a
2019	u	a
2028	g	u
2031	a	c
2034	g	c
2040	c	a
2058	g	u
2076	a	g
2082	u	g
2104	u	c
2112	u	a
2115	u	c
2121	a	u
2124	u	a
2127	c	a
2136	a	c
2139	c	u
2142	c	g
2154	a	c
2169	a	c
2184	g	u
2187	c	u

Pos.	NWT	WT
2190	a	g
2200	c	a
2202	c	a
2208	u	g
2214	c	a
2220	g	a
2226	a	u
2232	a	g
2244	u	a
2247	u	g
2253	g	c
2256	u	c
2259	g	c
2265	u	c
2266	u	a
2267	c	g
2268	a	c
2271	c	u
2274	a	c
2277	u	c
2280	a	g
2289	g	a
2301	a	g
2304	c	u
2310	c	g
2316	c	g
2322	g	a
2325	u	c
2328	g	a
2331	a	u
2340	g	u
2343	a	g
2355	c	a
2358	a	g
2361	g	a
2364	g	a
2370	a	c
2373	g	a
2388	u	g
2391	a	c

Pos.	NWT	WT
2400	g	a
2403	u	c
2415	c	g
2428	c	a
2430	u	a
2436	u	a
2439	g	u
2448	c	u
2451	a	c
2454	u	g
2463	c	u
2484	u	c
2490	a	g
2514	a	g
2515	u	a
2516	c	g
2517	a	c
2526	g	a
2532	a	u
2535	g	a
2548	u	c
2553	u	a
2562	g	u
2572	u	a
2573	c	g
2583	c	u
2586	c	g
2589	c	a
2598	u	c
2601	c	a
2613	c	g
2622	c	g
2625	a	u
2628	g	u
2631	a	u
2634	u	g
2640	c	g
2643	c	g
2655	u	a
2658	u	c

Pos.	NWT	WT
2661	g	u
2665	u	c
2683	u	a
2684	c	g
2688	a	u
2694	a	u
2703	u	c
2704	u	a
2705	c	g
2712	c	a
2724	u	c
2725	u	a
2726	c	g
2727	u	c
2730	a	c
2742	c	u
2760	a	g
2781	c	u
2784	g	u
2802	a	u
2805	c	a
2811	c	g
2814	u	g
2820	g	u
2823	u	a
2829	u	a
2832	c	g
2844	c	a
2850	u	c
2859	u	a
2865	c	u
2868	a	u
2877	u	a
2890	u	c
2895	u	c
2901	c	g
2907	g	a
2910	a	u
2913	u	g
2917	u	c

Pos.	NWT	WT
2923	c	a
2925	c	a
2931	a	c
2970	g	c
2976	c	u
3000	c	u
3021	a	u
3030	c	u
3033	c	a
3039	u	c
3045	u	c
3051	c	u
3054	g	a
3060	u	g
3063	g	a
3069	c	a
3075	g	u
3088	c	a
3090	g	a
3108	g	c
3120	u	c
3141	g	c
3145	u	a
3146	c	g
3147	g	u
3150	u	a
3156	u	c
3159	g	u
3174	u	c
3184	u	c
3192	g	a
3193	u	c
3198	c	u
3228	a	u
3243	g	u
3252	c	a
3258	g	u
3261	a	c
3264	u	c
3270	u	c

Pos.	NWT	WT
3276	u	c
3277	u	c
3282	u	a
3288	c	g
3297	u	c
3304	c	a
3306	c	a
3336	c	u
3339	g	u
3345	u	c
3348	u	c
3366	g	a
3375	c	a
3382	c	a
3387	c	u
3402	a	u
3405	c	a
3417	u	c
3426	u	a
3438	a	u
3448	u	a
3449	c	g
3450	g	c
3474	g	a
3477	c	u
3480	u	g
3481	u	a
3482	c	g
3483	g	c
3486	g	a
3501	c	u
3510	g	a
3513	g	a
3519	a	u
3528	a	c
3531	a	g
3534	u	a
3537	g	c
3546	u	c
3555	g	c

Pos.	NWT	WT
3559	u	c
3564	u	g
3570	a	u
3579	c	u
3588	u	a
3615	u	c
3624	u	c
3633	c	u
3648	g	a
3660	c	a
3666	a	u
3669	g	a
3672	c	u
3675	a	c
3681	u	a
3684	a	g
3693	g	c
3702	u	a
3711	u	c
3717	u	g
3723	g	c
3724	u	c
3729	c	g
3732	g	a
3741	g	a
3747	a	g
3750	a	g
3751	u	a
3752	c	g
3753	g	u
3756	g	u
3765	g	a
3786	u	c
3795	u	a
3810	c	u
3813	c	u
3816	u	g
3819	g	u
3847	c	a
3855	g	c

Pos.	NWT	WT
3864	a	g
3873	a	g
3879	c	a
3889	a	u
3890	g	c
3891	c	u
3901	c	a
3912	c	g
3918	u	c
3933	a	u
3945	u	a
3954	c	u
3957	g	a
3960	c	u
3972	u	c
3981	a	g
3984	c	a
3996	g	u
3999	a	g
4002	a	g
4005	c	u
4020	a	g
4029	a	c
4032	c	u
4039	u	a
4040	c	g
4041	g	c
4047	g	c
4059	c	g
4071	g	u
4072	c	a
4077	c	u
4080	c	u
4084	u	a
4085	c	g
4089	a	g
4095	a	g
4098	u	c
4104	c	g
4105	u	c

Pos.	NWT	WT
4116	u	c
4119	g	u
4134	g	a
4140	g	a
4143	u	c
4158	g	a
4161	a	u
4164	u	a
4173	g	a
4179	c	u
4188	g	a
4206	u	c
4207	c	a
4209	u	g
4212	c	a
4224	c	g
4242	u	g
4248	c	a

Pos.	NWT	WT
4257	u	c
4263	c	g
4266	c	g
4293	u	g
4303	u	a
4304	c	g
4305	a	c
4306	u	c
4320	g	c
4323	u	c
4324	u	a
4325	c	g
4326	a	c
4329	a	c
4335	u	c
4344	a	g
4347	u	c
4353	a	c

Pos.	NWT	WT
4362	u	c
4365	g	a
4366	u	a
4367	c	g
4368	g	c
4383	a	g
4386	g	c
4392	c	u
4395	g	u
4399	u	c
4407	a	g
4413	u	a
4422	a	g
4425	u	g
4434	g	a
4435	c	a
4437	u	g

[0111] In some embodiments, the present invention comprises a non-naturally occurring CFTR mRNA comprising a coding sequence of SEQ ID NO: 3. Additional exemplary non-naturally occurring CFTR mRNA coding sequences are described in the Brief Description of Sequences section, such as, for example, SEQ ID NOs:9, 10, 11, 12, 13, 14, 15, 16, or 17. In some embodiments, the present invention provides a CFTR mRNA comprising a coding sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to any of SEQ ID NO: 3, 9, 10, 11, 12, 13, 14, 15, 16, or 17. In some embodiments, a non-naturally occurring CFTR mRNA comprises a 5'UTR, 3'UTR, a signal peptide coding sequence or a cap or tail structure as described below.

[0112] The above-described CFTR mRNAs comprising coding sequence which differs from wild-type CFTR coding sequence can provide advantages with respect to efficacy and ease of preparation. For example, *in vitro* transcription reactions using a polynucleotide comprising template sequence complementary to the CFTR coding sequence can give greater RNA yield; a polynucleotide comprising said template sequence can be more stable (i.e., less prone to mutation) during growth in a host cell, reducing the amount of purification needed to generate template usable in a reaction; and the *in vivo* translation of an mRNA comprising the coding sequence can be higher.

Signal Peptide Sequence

[0113] In some embodiments, an mRNA encoding a CFTR protein incorporates a nucleotide sequence encoding a signal peptide. As used herein, the term "signal peptide" refers to a peptide present at a newly synthesized protein that can target the protein towards the secretory pathway. In some embodiments, the signal peptide is cleaved after translocation into the endoplasmic reticulum following translation of the mRNA. Signal peptide is also referred to as signal sequence, leader sequence or leader peptide. Typically, a signal peptide is a short (e.g., 5-30, 5-25, 5-20, 5-15, or 5-10 amino acids long) peptide. A signal peptide may be present at the N-terminus of a newly synthesized protein. Without wishing to be bound by any particular

theory, the incorporation of a signal peptide encoding sequence on a CFTR encoding mRNA may facilitate the secretion and/or production of the CFTR protein *in vivo*.

[0114] A suitable signal peptide for the present invention can be a heterogeneous sequence derived from various eukaryotic and prokaryotic proteins, in particular secreted proteins. In some embodiments, a suitable signal peptide is a leucine-rich sequence. See Yamamoto Y *et al.* (1989), *Biochemistry*, 28:2728-2732, which is incorporated herein by reference. A suitable signal peptide may be derived from a human growth hormone (hGH), serum albumin preproprotein, Ig kappa light chain precursor, Azurocidin preproprotein, cystatin-S precursor, trypsinogen 2 precursor, potassium channel blocker, alpha conotoxin lp1.3, alpha conotoxin, alfa-galactosidase, cellulose, aspartic proteinase nepenthesin-1, acid chitinase, K28 prepro-toxin, killer toxin zygocin precursor, and Cholera toxin. Exemplary signal peptide sequences are described in Kober, et al., *Biotechnol. Bioeng.*, 110: 1164-73, 2012, which is incorporated herein by reference.

[0115] In some embodiments, a CFTR encoding mRNA may incorporate a sequence encoding a signal peptide derived from human growth hormone (hGH), or a fragment thereof. A non-limiting nucleotide sequence encoding a hGH signal peptide is show below.

5' human growth hormone (hGH) sequence (SEQ ID NO:18):

AUGGCCACUGGAUCAAGAACCUCACUGCUGCUCGCUUUUGGACUGCUUUGCCUGC
CCUGGUUGCAAGAAGGAUCGGCUUUCCCGACCAUCCCACUCUCC

Alternative 5' human growth hormone (hGH) sequence (SEQ ID NO:19):

AUGGCAACUGGAUCAAGAACCUCCCUCCUGCUCGCAUUCGGCCUGCUCUGUCUCC
CAUGGCUCCAAGAAGGAAGCGCGUUCCCCACUAUCCCCCUCUCG

[0116] In some embodiments, an mRNA according to the present invention may incorporate a signal peptide encoding sequence having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more identity to SEQ ID NO:18 or SEQ ID NO:19.

5'-UTR, 3'-UTR, Poly-A Tail, Cap, and Nonstandard Nucleotide Residues

[0117] In some embodiments, the mRNA comprises a sequence in its 5'-UTR which is identical to SEQ ID NO: 4 or is at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 4.

[0118] In some embodiments, the mRNA comprises a sequence in its 3'-UTR which is identical to SEQ ID NO: 5 or is at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 5.

[0119] In some embodiments, the mRNA comprises a poly-A tail. In some embodiments, the poly-A tail has a length of at least 70, 100, 120, 150, 200, 250, 300, 400, or 500 residues. In some embodiments, the poly-A tail has a length ranging from 70 to 100, 100 to 120, 120 to 150, 150 to 200, or 200 to 300, 300 to 400, or 400 to 500 residues. Poly A tails can be added using a variety of art-recognized techniques. For example, long poly A tails can be added to synthetic or *in vitro* transcribed RNA using poly A polymerase (Yokoe, el al. Nature Biotechnology. 1996; 14: 1252-1256). A transcription vector can also encode long poly A tails. In addition, poly A tails can be added by transcription directly from PCR products. Poly A may also be ligated to the 3' end of a sense RNA with RNA ligase (see, e.g., Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1991 edition)). In some embodiments, a poly-U or poly-C tail may be used instead or in addition to a poly-A tail. For example, CFTR encoding mRNAs may include a 3' poly(C) tail structure. A suitable poly-C tail on the 3' terminus of mRNA typically include about 10 to 200 cytosine nucleotides (e.g., about 10 to 150 cytosine nucleotides, about 10 to 100 cytosine nucleotides, about 20 to 70 cytosine nucleotides, about 20 to 60 cytosine nucleotides, or about 10 to 40 cytosine nucleotides). The poly-C tail may be added to the poly-A tail or may substitute the poly-A tail.

[0120] In some embodiments, the mRNA comprises a 5'-cap, for example, a cap1 structure. For mRNA capping enzymes and procedures, see, e.g., Fechter, P.; Brownlee, G.G. "Recognition of mRNA cap structures by viral and cellular proteins" *J. Gen. Virology* 2005, *86*, 1239-1249; European patent publication 2 010 659 A2; U.S. Patent No. 6,312,926. A 5' cap is typically added as follows: first, an RNA terminal phosphatase removes one of the terminal phosphate groups from the 5' nucleotide, leaving two terminal phosphates; guanosine triphosphate (GTP) is then added to the terminal phosphates via a guanylyl transferase, producing a 5'5'5 triphosphate linkage; and the 7-nitrogen of guanine is then methylated by a methyltransferase. Examples of cap structures include, but are not limited to, m7G(5')ppp (5'(A,G(5')ppp(5')A and G(5')ppp(5')G.

[0121] In some embodiments, the mRNA comprises one or more nonstandard nucleotide residues. The nonstandard nucleotide residues may include, e.g., 5-methyl-cytidine ("5mC"), pseudouridine ("ψU"), and/or 2-thio-uridine ("2sU"). See, e.g., U.S. Patent No. 8,278,036 or WO2011012316 for a discussion of such residues and their incorporation into mRNA. In some embodiments, mRNA may be SNIM RNA. As used herein, SNIM RNA is an acronym of Stabilized Non-Immunogenic Messenger RNA, designating messenger RNAs produced by *in vitro* transcription (IVT) including certain percentages of modified nucleotides in the IVT reaction as described in PCT Publication WO 2011/012316. SNIM RNA used in the Examples disclosed herein was produced by IVT in which 25% of U residues were 2-thio-uridine and 25% of C residues were 5-methylcytidine. The presence of nonstandard nucleotide residues may render an mRNA more stable and/or less immunogenic than a control mRNA with the same sequence but containing only standard residues. In further embodiments, the mRNA may comprise one or more nonstandard nucleotide residues chosen from isocytosine, pseudoisocytosine, 5-bromouracil, 5-propynyluracil, 6-aminopurine, 2-aminopurine, inosine, diaminopurine and 2-chloro-6-aminopurine cytosine, as well as combinations of these modifications and other nucleobase modifications. Certain embodiments may further include additional modifications to the furanose ring or nucleobase. Additional modifications may

include, for example, sugar modifications or substitutions (e.g., one or more of a 2′-O-alkyl modification, a locked nucleic acid (LNA)). In some embodiments, the RNAs may be complexed or hybridized with additional polynucleotides and/or peptide polynucleotides (PNA). In embodiments where the sugar modification is a 2′-O-alkyl modification, such modification may include, but are not limited to a 2′-deoxy-2′-fluoro modification, a 2′-O-methyl modification, a 2′-O-methoxyethyl modification and a 2′-deoxy modification. In certain embodiments, any of these modifications may be present in 0-100% of the nucleotides—for example, more than 0%, 1%, 10%, 25%, 50%, 75%, 85%, 90%, 95%, or 100% of the constituent nucleotides individually or in combination.

Compositions comprising CFTR mRNA

[0122] In certain embodiments, the mRNA molecules of the invention may be administered as naked or unpackaged mRNA. In some embodiments, the administration of the mRNA in the compositions of the invention may be facilitated by inclusion of a suitable carrier. In certain embodiments, the carrier is selected based upon its ability to facilitate the transfection of a target cell with one or more mRNAs.

[0123] As used herein, the term "carrier" includes any of the standard pharmaceutical carriers, vehicles, diluents, excipients and the like which are generally intended for use in connection with the administration of biologically active agents, including mRNA.

[0124] In certain embodiments, the carriers employed in the compositions of the invention may comprise a liposomal vesicle, or other means to facilitate the transfer of a mRNA to target cells and/or tissues. Suitable carriers include, but are not limited to, polymer based carriers, such as polyethyleneimine (PEI) and multi-domain-block polymers, lipid nanoparticles and liposomes, nanoliposomes, ceramide-containing nanoliposomes, proteoliposomes, both natural and synthetically-derived exosomes, natural, synthetic and semi-synthetic lamellar bodies, nanoparticulates, calcium phosphor-silicate nanoparticulates, calcium phosphate

nanoparticulates, silicon dioxide nanoparticulates, nanocrystalline particulates, semiconductor nanoparticulates, dry powders, poly(D-arginine), nanodendrimers, starch-based delivery systems, micelles, emulsions, sol-gels, niosomes, plasmids, viruses, calcium phosphate nucleotides, aptamers, peptides, peptide conjugates, small-molecule targeted conjugates, and other vectorial tags. Also contemplated is the use of bionanocapsules and other viral capsid proteins assemblies as a suitable carrier. (Hum. Gene Ther. 2008 Sep;19(9):887-95).

[0125] In some embodiments, the carrier comprises an organic cation, such as a cationic lipid or a cationic organic polymer. If present, the cationic lipid may be a component of liposomal vesicles encapsulating the mRNA.

[0126] In certain embodiments of the invention, the carrier is formulated using a polymer as a carrier, alone or in combination with other carriers. Suitable polymers may include, for example, polyacrylates, polyalkycyanoacrylates, polylactide, polylactide-polyglycolide copolymers, polycaprolactones, dextran, albumin, gelatin, alginate, collagen, chitosan, cyclodextrins, protamine, PEGylated protamine, PLL, PEGylated PLL and polyethylenimine (PEI). When PEI is present, it may be branched PEI of a molecular weight ranging from 10 to 40 kDa, e.g., 25 kDa branched PEI (Sigma #408727). Additional exemplary polymers suitable for the present invention include those described in PCT Publication WO2013182683, the contents of which is hereby incorporated by reference.

[0127] The use of liposomal carriers to facilitate the delivery of polynucleotides to target cells is contemplated by the present invention. Liposomes (e.g., liposomal lipid nanoparticles) are generally useful in a variety of applications in research, industry, and medicine, particularly for their use as carriers of diagnostic or therapeutic compounds *in vivo* (Lasic, *Trends Biotechnol.*, 16: 307-321, 1998; Drummond et al., *Pharmacol. Rev.*, 51: 691-743, 1999) and are usually characterized as microscopic vesicles having an interior aqua space sequestered from an outer medium by a membrane of one or more bilayers. Bilayer membranes of liposomes are typically formed by amphiphilic molecules, such as lipids of synthetic or natural origin that comprise spatially separated hydrophilic and hydrophobic domains (Lasic, *Trends Biotechnol.*,

16: 307-321, 1998). Bilayer membranes of the liposomes can also be formed by amphiphilic polymers and surfactants (e.g., polymerosomes, niosomes, etc.).

In certain embodiments, the mRNA is complexed with lipid nanoparticles to facilitate delivery to the target cell. In certain embodiments, the compositions of the invention may be combined with a multi-component lipid mixture employing one or more cationic lipids, additional lipids such as non-cationic lipids (also referred to as helper lipids), cholesterol-based lipids, and/or PEGylated lipids for mRNA encapsulation.

Cationic Lipids

[0128] In some embodiments, a suitable lipid nanoparticle contains a cationic lipid. As used herein, the phrase "cationic lipid" refers to any of a number of lipid species that have a net positive charge at a selected pH, such as physiological pH. Some cationic lipids, in particular, those known as titratable or pH-titratable cationic lipids are particularly effective in delivering mRNA. Several cationic (e.g., titratable) lipids have been described in the literature, many of which are commercially available. Particularly suitable cationic lipids for use in the compositions and methods of the invention include those described in international patent publications WO 2010/053572 (and particularly, C12-200 described at paragraph [00225]) and WO 2012/170930, both of which are incorporated herein by reference. In some emodiments, the cationic lipid cKK-E12 is used (disclosed in WO 2013/063468), the teachings of which are incorporated herein by reference in their entirety. In some embodiments, the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride or "DOTMA" is used. (Feigner et al. (*Proc. Nat'l Acad. Sci.* 84, 7413 (1987); U.S. Pat. No. 4,897,355). DOTMA can be formulated alone or can be combined with the neutral lipid, dioleoylphosphatidyl-ethanolamine or "DOPE" or other cationic or non-cationic lipids into a liposomal transfer vehicle or a lipid nanoparticle, and such liposomes can be used to enhance the delivery of nucleic acids into target cells. Other suitable cationic lipids include, for example, 5-carboxyspermylglycinedioctadecylamide or "DOGS," 2,3-dioleyloxy-N-[2(spermine-

carboxamido)ethyl]-N,N-dimethyl-l-propanaminium or "DOSPA" (Behr et al. *Proc. Nat.'l Acad. Sci.* 86, 6982 (1989); U.S. Pat. No. 5,171,678; U.S. Pat. No. 5,334,761), l,2-Dioleoyl-3-Dimethylammonium-Propane or "DODAP", 1,2-Dioleoyl-3-Trimethylammonium-Propane or "DOTAP". Contemplated cationic lipids also include 1,2-distearyloxy-N,N-dimethyl-3-aminopropane or "DSDMA", 1,2-dioleyloxy-N,N-dimethyl-3-aminopropane or "DODMA", 1 ,2-dilinoleyloxy-N,N-dimethyl-3-aminopropane or "DLinDMA", l,2-dilinolenyloxy-N,N-dimethyl-3-aminopropane or "DLenDMA", N-dioleyl-N,N-dimethylammonium chloride or "DODAC", N,N-distearyl-N,N-dimethylamrnonium bromide or "DDAB", N-(l,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide or "DMRIE", 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-l-(ci s,cis-9,12-octadecadienoxy)propane or "CLinDMA", 2-[5'-(cholest-5-en-3-beta-oxy)-3'-oxapentoxy)-3-dimethy l-l-(cis,cis-9', l-2'-octadecadienoxy)propane or "CpLinDMA", N,N-dimethyl-3,4-dioleyloxybenzylamine or "DMOBA", 1 ,2-N,N'-dioleylcarbamyl-3-dimethylaminopropane or "DOcarbDAP", 2,3-Dilinolcoyloxy-N,N-dimethylpropylamine or "DLinDAP", l,2-N,N'-Dilinoleylcarbamyl-3-dimethylaminopropane or "DLincarbDAP", 1 ,2-Dilinoleoylcarbamyl-3-dimethylaminopropane or "DLinCDAP", 2,2-dilinoleyl-4-dimethylaminomethyl-[l,3]-dioxolane or "DLin- -DMA", 2,2-dilinoleyl-4-dimethylaminoethyl-[l,3]-dioxolane or "DLin-K-XTC2-DMA", and 2-(2,2-di((9Z,12Z)-octadeca-9,l 2-dien- 1-yl)-l ,3-dioxolan-4-yl)-N,N-dimethylethanamine (DLin-KC2-DMA)) (See, WO 2010/042877; Semple et al., Nature Biotech. 28: 172-176 (2010)), or mixtures thereof. (Heyes, J., et al., *J Controlled Release* 107: 276-287 (2005); Morrissey, DV., et al., *Nat. Biotechnol.* 23(8): 1003-1007 (2005); PCT Publication WO2005/121348A1).

[0129] In certain embodiments, the compositions and methods of the invention employ a lipid nanoparticles comprising an ionizable cationic lipid described in U.S. provisional patent application 61/617,468, filed March 29, 2013 (incorporated herein by reference), such as, e.g, (15Z, 18Z)-N,N-dimethyl-6-(9Z, 12Z)-octadeca-9, 12-dien-l -yl)tetracosa- 15,18-dien- 1 -amine (HGT5000), (15Z, 18Z)-N,N-dimethyl-6-((9Z, 12Z)-octadeca-9, 12-dien- 1 -yl)tetracosa-4,15,18-trien-l -amine (HGT5001), and (15Z,18Z)-N,N-dimethyl-6-((9Z, 12Z)-octadeca-9, 12-dien- 1 -yl)tetracosa-5, 15 , 18-trien- 1 -amine (HGT5002).

[0130] In some embodiments, one or more of the cationic lipids present in such a composition comprise at least one of an imidazole, dialkylamino, or guanidinium moiety. In a preferred embodiment, one or more of the cationic lipids does not comprise a quaternary amine.

Non-cationic/Helper Lipids

[0131] In some embodiments, a suitable lipid nanoparticle contains one or more non-cationic ("helper") lipids. As used herein, the phrase "non-cationic lipid" refers to any neutral, zwitterionic or anionic lipid. As used herein, the phrase "anionic lipid" refers to any of a number of lipid species that carry a net negative charge at a selected pH, such as physiological pH. In some embodiments, a non-cationic lipid is a neutral lipid, i.e., a lipid that does not carry a net charge in the conditions under which the composition is formulated and/or administered. Non-cationic lipids include, but are not limited to, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl-ethanolamine (DSPE), 16-O-monomethyl PE, 16-O-dimethyl PE, 18-1-trans PE, 1-stearoyl-2-oleoyl-phosphatidyethanolamine (SOPE), or a mixture thereof.

Cholesterol-based Lipids

[0132] In some embodiments, a suitable lipid nanoparticle comprises one or more cholesterol-based lipids. For example, suitable cholesterol-based cationic lipids include, for example, cholesterol, PEGylated cholesterol, DC-Choi (N,N-dimethyl-N-ethylcarboxamidocholesterol), 1,4-bis(3-N-oleylamino-propyl)piperazine (Gao, et al. *Biochem.*

Biophys. Res. Comm. 179, 280 (1991); Wolf et al. *BioTechniques* 23, 139 (1997); U.S. Pat. No. 5,744,335), or ICE.

PEGylated Lipids

[0133] In some embodiments, a suitable lipid nanoparticle comprises one or more PEGylated lipids. For example, the use of polyethylene glycol (PEG)-modified phospholipids and derivatized lipids such as derivatized ceramides (PEG-CER), including N-Octanoyl-Sphingosine-l-[Succinyl(Methoxy Polyethylene Glycol)-2000] (C8 PEG-2000 ceramide) is contemplated by the present invention in combination with one or more of the cationic and, in some embodiments, other lipids. In some embodiments, suitable PEGylated lipids comprise PEG-ceramides having shorter acyl chains (e.g., C_{14} or C_{18}). In some embodiments, the PEGylated lipid DSPE-PEG-Maleimide-Lectin may be used. Other contemplated PEG-modified lipids include, but are not limited to, a polyethylene glycol chain of up to 5 kDa in length covalently attached to a lipid with alkyl chain(s) of C_6-C_{20} length. Without wishing to be bound by a particular theory, it is contemplated that the addition of PEGylated lipids may prevent complex aggregation and increase circulation lifetime to facilitate the delivery of the lipsome encapsulated mRNA to the target cell.

[0134] In certain embodiments, the composition comprises one of the following combinations of lipids:

C12-200, DOPE, cholesterol, DMG-PEG2K;

DODAP, DOPE, cholesterol, DMG-PEG2K;

HGT5000, DOPE, cholesterol, DMG-PEG2K;

HGT5001, DOPE, cholesterol, DMG-PEG2K;

XTC, DSPC, cholesterol, PEG-DMG;

MC3, DSPC, cholesterol, PEG-DMG;

ALNY-100, DSPC, cholesterol, PEG-DSG;

cKK-E12, DOPE, Chol, PEGDMG2K.

[0135] In some embodiments, lipid:mRNA ratios can be 5:1 (mg:mg), 6:1, 7:1, 8:1, 9:1, 10:1 and greater up to 30:1 (mg:mg) or more. N/P ratios can be in the range of 1.1:1 up to 10:1 or higher. Example lipid ratios are 40:30:20:10, 55:20:20:5, 50:25:20:5 (cationic lipid:helper lipid:chol:PEG lipid).

[0136] In some embodiments, the pharmaceutical compositions according to the invention do not comprise a mucolytic agent (e.g., N-acetylcysteine, erdosteine, bromheksin, carbocysteine, guiafenesin, or iodinated glycerol).

Apparatuses loaded with a pharmaceutical composition

[0137] In some embodiments, a pharmaceutical composition according to the invention, such as a cationic lipid-based or PEI-based composition comprising a non-naturally occurring CFTR mRNA, is provided within an apparatus for administration to the respiratory system of a subject. The apparatus can be, e.g., an instillation, aerosolization, or nebulization apparatus. Suitable apparatuses include, for example, a PARI Boy jet nebulizer, Aeroneb® Lab nebulizer, MicroSprayer®, or EFlow mesh nebulizer. Alternatively, dry powder inhalers or aerosolization apparatuses such as portable inhalers may be used.

Uses and Methods

mRNA for Uses and Methods According to the Invention

[0138] Among other things, the present invention provides methods for *in vivo* production of a CFTR protein, in particular, in a lung of a mammal. In some embodiments, the invention provides methods of inducing CFTR expression in epithelial cells in a lung of a

mammal, comprising contacting the epithelial cells with a pharmaceutical composition comprising an *in vitro* transcribed mRNA, wherein the *in vitro* transcribed mRNA comprises a coding sequence encoding SEQ ID NO: 1 (the amino acid sequence of wild-type human CFTR). The invention also provides uses of pharmaceutical compositions comprising an *in vitro* transcribed mRNA, wherein the *in vitro* transcribed mRNA comprises a coding sequence encoding SEQ ID NO: 1, for the induction of CFTR expression in epithelial cells in a lung of a mammal.

[0139] The invention further provides methods of inducing CFTR expression in a mammalian target cell, the method comprising contacting the mammalian target cell with a composition, the composition comprising an *in vitro* transcribed mRNA encoding the amino acid sequence of SEQ ID NO: 1. The invention further provides a use of composition, the composition comprising an *in vitro* transcribed mRNA encoding the amino acid sequence of SEQ ID NO: 1, for the induction of CFTR expression in a mammalian target cell.

[0140] In some embodiments of such uses and methods of treatment, the *in vitro* transcribed mRNA is a naturally occurring or wild-type mRNA encoding human CFTR (SEQ ID NO: 2). In other embodiments, the *in vitro* transcribed mRNA is a non-naturally occurring mRNA as described above.

[0141] In certain embodiments, the *in vitro* transcribed mRNA comprises a coding sequence encoding SEQ ID NO: 1 which is at least 65%, 70%, 75%, 80%, 85%, 88%, 90%, 92% 95%, or 100% identical to SEQ ID NO: 2 (wild-type human CFTR mRNA coding sequence).

[0142] mRNA comprising a coding sequence encoding SEQ ID NO: 1 which is at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 2 may have greater cryptic promoter, direct and inverted repeat, and/or GC content than the mRNA discussed above. It was observed that vectors comprising SEQ ID NO: 2 frequently underwent insertion/deletion/rearrangement mutations in host cells under typical growth conditions, resulting in a heterogeneous population of vectors that could not be used directly for *in vitro* transcription. It was found that growing host cells under conditions such as lower temperature,

subdued light and/or low copy cells such as CopyCutter® reduced, but did not eliminate, the occurrence of mutation. Accordingly, it can be advisable for *in vitro* transcription reactions of mRNA comprising a coding sequence at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 2 to use a template obtained by growing the vector as described above, harvesting and linearizing the vector, and purifying the desired species for use in the transcription reaction. The purification step can be, e.g., size exclusion chromatography or weak anion exchange.

[0143] The *in vitro* transcribed mRNA for uses and methods according to the invention can comprise a 5'-UTR, 3'UTR, poly-A, poly-U and/or poly-C tail, cap, and/or nonstandard nucleotide residues, as discussed in the section above concerning such features.

Pharmaceutical Compositions for Uses and Methods

[0144] Pharmaceutical compositions for use according to the invention may comprise mRNA for uses and methods according to the invention as discussed in the preceding section and additional ingredients as discussed in the section above regarding compositions comprising CFTR mRNA. Thus, use and/or administration of pharmaceutical compositions comprising any of the carriers discussed above is contemplated.

[0145] In some preferred embodiments, pharmaceutical compositions comprise PEI, such as branched PEI having a molecular weight ranging from 10-40 kDa, for example, 25 kDa.

[0146] In other preferred embodiments, pharmaceutical compositions comprise a cationic lipid, a pegylated lipid, and an additional lipid (such as a neutral lipid). The cationic lipid, pegylated lipid, and/or additional lipid may be chosen from those listed in the section above regarding compositions comprising CFTR mRNA.

Routes of administration for induction of expression in lung

[0147] In some embodiments of methods and uses for induction of CFTR expression in a lung of a mammal, a pharmaceutical composition as described above is administered by a route chosen from intratracheal instillation, nebulization, and aerosolization. The apparatus for administering the composition can be chosen from the apparatuses listed in the section above regarding apparatuses loaded with a pharmaceutical composition.

[0148] In preferred embodiments, the composition is administered via nebulization or aerosolization. Some lipid formulations may have a tendency to aggregate when nebulization is attempted but it is generally possible to solve aggregation issues by adjusting the formulation, e.g., by substituting the cationic lipid.

Treatment of cystic fibrosis

[0149] Among other things, the present invention can be used for treating cystic fibrosis. In some embodiments, the present invention provides a method of treating cystic fibrosis by administering to a subject in need of treatment an mRNA encoding a CFTR protein as described herein or a pharmaceutical composition containing the mRNA. The mRNA or a pharmaceutical composition containing the mRNA may be administered directly to the lung of the subject. Various administration routes for pulmonary delivery may be used. In some embodiments, an mRNA or a composition containing an mRNA described herein is administered by inhalation, nebulization or aerosolization. In various embodiments, administration of the mRNA results in expression of CFTR in the lung of the subject (e.g., epithelial cells of the lung).

[0150] In a particular embodiment, the present invention provides a method of treating cystic fibrosis by administering to the lung of a subject in need of treatment an mRNA comprising a coding sequence which encodes SEQ ID NO:1. In certain embodiments, the present invention provides a method of treating cystic fibrosis by administering to the lung of a subject in need of treatment an mRNA comprising a coding sequence which encodes an amino

acid sequence at least about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO:1. In another particular embodiment, the present invention provides a method of treating cystic fibrosis by administering to the lung of a subject in need of treatment an mRNA comprising a coding sequence of SEQ ID NO: 3. In other embodiments, the present invention provides a method of treating cystic fibrosis by administering to the lung of a subject in need of treatment an mRNA comprising a coding sequence at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical to SEQ ID NO: 3. Additional exemplary non-naturally occurring CFTR mRNAs that can be used for treating cystic fibrosis are described in the Brief Description of Sequences section, such as, for example, SEQ ID NOs:9, 10, 11, 12, 13, 14, 15, 16, or 17. In some embodiments, non-naturally occurring CFTR mRNAs that can be used for treating cystic fibrosis comprises a coding sequence at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to any of SEQ ID NO: 3, 9, 10, 11, 12, 13, 14, 15, 16, or 17.

EXAMPLES

[0151] The following specific examples are to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. Without further elaboration, it is believed that one skilled in the art can, based on the description herein, utilize the present invention to its fullest extent.

[0152] Unless otherwise indicated, CFTR mRNA and SNIM RNA used in the Examples disclosed herein comprised a 5' UTR with the sequence of SEQ ID NO: 4, a coding sequence (CDS) with the sequence of SEQ ID NO: 3, and a 3' UTR with the sequence of SEQ ID NO: 5. FFL mRNA and SNIM RNA used in the Examples disclosed herein comprised a 5' UTR, CDS, and 3' UTR with the sequences of SEQ ID NOS: 6, 7, and 8, respectively.

Example 1: *In Vitro* Synthesized mRNA Encoding CFTR

[0153] *Messenger RNA Synthesis.* Human cystic fibrosis transmembrane conductance regulator (CFTR) mRNA and firefly luciferase (FFL) mRNA were synthesized by *in vitro* transcription from a plasmid DNA template encoding the gene, which was followed by the addition of a 5' cap structure (Cap 1) (Fechter, P.; Brownlee, G.G. "Recognition of mRNA cap structures by viral and cellular proteins" *J. Gen. Virology* **2005**, *86*, 1239-1249) and a 3' poly(A) tail of approximately 200 nucleotides in length as determined by gel electrophoresis. 5' and 3' untranslated regions were present in each mRNA product.

[0154] Exemplary non-naturally occurring CFTR mRNAs include SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ UD NO:15, SEQ ID NO:16, or SEQ ID NO:17described in the Brief Description of Sequences section.

Example 2: CFTR Expression and Activity in HEK cells

[0155] This example demonstrates that fully functional CFTR protein is expressed from synthetic human CFTR mRNA delivered to cells.

[0156] *Cells and CFTR transfection.* Human embryonic kidney HEK293T cells were grown in DMEM (Invitrogen Cat # 11965-092) supplemented with 10% fetal bovine serum, 2 mM L-Glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The day before transfection, cells were plated on 6-well plates at 50-60% confluence and incubated under normal tissue culture conditions (36°C in a humidified atmosphere of 5% CO2, 95% air). 60 μl Lipofectamine 2000 (Invitrogen Cat # 11668019) was diluted in 900 μl OptiMem reduced serum media (Invitrogen Cat # 31985-062) and gently vortexed. 24 μg CFTR mRNA (4 μg per plate) was diluted in 900 μl OptiMem media. The mRNA was immediately added to the diluted Lipofectamine and incubated at room temperature for 30 minutes. The plating media was gently aspirated from the HEK293T cells and replaced with 1 ml OptiMem Reduced Serum Medium. 300 μl of mRNA/Lipofectamine complex was added to each well and the cells allowed to rest

under normal tissue culture conditions for 24 hrs before being re-plated by mechanical detachment on poly-L-Lysine coated glass cover slips (BD Biosciences, BD Biocoat) so that the cells could be easily transferred to a recording chamber for electrophysiological recording. Cells were incubated under standard tissue culture conditions for a minimum of a further 24 hours and were used within 48 hours of final plating.

[0157] *Electrophysiological Recording*. Whole-cell patch-clamp recordings were conducted at room temperature using an Axopatch 200B amplifier with 5-8 MΩ electrodes. Data were digitized (50 kHz) and filtered (5 kHz) appropriately. Series resistance was compensated (70-80%) to minimize voltage errors. Voltage-clamp recordings were performed with pipette solution of the following composition: 140 mM NMDG-Cl; 5 mM EGTA; 1 mM MgCl2; 10 mM HEPES; pH 7.2; 310 mOsm/l. The bathing solution contained: 140 mM NaCl, 3 mM KCl, 2 mM MgCl2, 2 mM CaCl2, and 10 mM HEPES; pH 7.3, adjusted to 315 mOsm/l with D-glucose. Voltage clamp recordings commenced 3-5 minutes after establishing whole-cell configuration.

[0158] Cells were voltage-clamped at a holding potential of either -60 mV or 0 mV and a series of positive and negative voltage steps (either -80 mV to +80 mV or -100 to +100 mV in 20 mV increments) injected into the recorded HEK293T cells to evoke CFTR-induced whole-cell chloride (Cl-) currents. The membrane permeable analogue of cAMP, 8-Br-cAMP (500 μM, Sigma Aldrich) was applied for 4 mins to recorded cells to facilitate CFTR currents. The 'gold-standard' CFTR blocker, CFTRinh-172 (10 μM, Sigma) was applied at the end of each recording to block the CFTR induced Cl- current. Control recordings were performed in non-transfected HEK293T cells.

[0159] *Test Compounds*. Test compounds were applied using a DAD-16VC fast perfusion system (ALA Scientific Instruments, USA) with the ejection pipette placed approximately 200 μm from the recorded cell. 8-Br-cAMP was made as a 500 mM stock concentration in ddH20. CFTRinh-172 was made as a 10 mM stock in DMSO. All compounds were stored at -20°C and were rapidly defrosted and diluted to the desired final concentration immediately prior to use.

[0160] *Analysis*. All analysis was conducted using Clampfit (MDS Analytical Technologies) and Excel (Microsoft) software. All values are maximum evoked-peak current amplitude. Statistical differences in the data were evaluated by Student's t-test, paired or un-paired as appropriate and considered significant at $P < 0.05$.

[0161] *In Vitro Human CFTR Protein Production.* The production of human CFTR protein via hCFTR mRNA was accomplished via transfection of human CFTR mRNA in HEK293T cells described herein. Treated and untreated cells were harvested and subjected to immunoprecipitation methods 24 hours post-transfection. Detection of human CFTR protein via Western blot analysis demonstrates that the fully complex glycosylated CFTR protein (designated as "C" band) was produced from the synthetic messenger RNA (Figure 1A).

[0162] *In Vitro Human CFTR Protein Activity.* To determine the activity of the synthetic human CFTR mRNA-derived CFTR protein produced after transfection, whole cell patch clamp assays were performed in both HEK 293 and HEK 293T cells. Treated cells as well as control cells (untreated and mock transfected) were subjected to activator (8-Br-cAMP, forskolin) and inhibitor (CFTRinh-172, GlyH-101) substrates to help determine changes in current flow (chloride ion transport).

[0163] HEK293T cells were transfected with 4 ug of hCFTR mRNA and analyzed 24 hours post transfection. Whole cell clamp assays were conducted to measure current flow, as represented by chloride ion transport upon application of a set voltage. A plot of current vs voltage as a result of a voltage ramp of -80 mV to +80 mV (depicted in Figure 2) demonstrates substantial differences in current when comparing untreated versus hCFTR mRNA-treated cells. This increase in current after exposure to 8-Br-cAMP, a known activator of CFTR protein, is suggestive that human CFTR protein is present in these cells. Upon treatment of these previously transfected cells with a known specific CFTR inhibitor, CFTRinh-172, the respective current drops back down to near control levels (~89% decrease). Such a decrease after exposure of this inhibitor strongly supports the presence of human CFTR protein. These results in sum demonstrate that synthetic hCFTR mRNA can produce active human CFTR protein.

[0164] Separately, CFTR whole cell activity assays were performed using an automated system (IonWorks) within HEK293 cells. As described above, treated cells as well as control cells (untreated and mock transfected) were subjected to activator and inhibitor substrates to help determine changes in current flow (chloride ion transport). In these studies, forskolin was employed as the CFTR protein activator and a portion of the hCFTR mRNA-transfected cells were further exposed to a different specific CFTR inhibitor, GlyH-101. GlyH-101 is believed to act as a CFTR pore blocker which acts upon the extracellular membrane side of the protein. Notably, this action of mechanism is different from that of CFTRinh-172, which is reported to function from the intracellular side of the CFTR protein.

[0165] Figure 4 represents a current-voltage plot of the parental HEK293 cell line treated with forskolin as well as GlyH-101. No significant change in current was observed, suggesting that these specific CFTR activators/inhibitors have no effect on the endogenous proteins present in the cell line.

[0166] A plot of current vs. voltage as a result of a voltage ramp of -100 mV to +100 mV (depicted in Figure 5) demonstrates substantial differences in current when comparing untreated HEK293 cells versus hCFTR mRNA-treated cells. This increase in current after exposure to forskolin a known activator of CFTR protein, is indicative that human CFTR protein is present in these cells. Upon treatment of these previously transfected cells with a different known specific CFTR inhibitor, GlyH-101, the respective current drops back down to near control levels (~95% decrease). Such a decrease after exposure of this inhibitor strongly supports the presence of human CFTR protein.

[0167] In total, these inhibition data which are a result of two distinct mechanisms strongly support the identity of a fully functional CFTR protein derived from the synthetic human CFTR messenger RNA.

Example 3: *In Vivo* Expression of CFTR

[0168] This example demonstrates that CFTR protein is effectively expressed *in vivo* from a CFTR encoding mRNA delivered through pulmonary administration.

[0169] *Formulation Protocol 1.* Aliquots of 50 mg/mL ethanolic solutions of C12-200, DOPE, Chol and DMG-PEG2K were mixed and diluted with ethanol to 3 mL final volume. Separately, an aqueous buffered solution (10 mM citrate/150 mM NaCl, pH 4.5) of CFTR mRNA was prepared from a 1 mg/mL stock. The lipid solution was injected rapidly into the aqueous mRNA solution and shaken to yield a final suspension in 20% ethanol. The resulting nanoparticle suspension was filtered, diafiltrated with 1x PBS (pH 7.4), followed by water, concentrated and stored at 2-8°C. Final concentration = 1.09 mg/mL CFTR mRNA (encapsulated). Z_{ave} = 80.2 nm ($Dv_{(50)}$ = 55.5 nm; $Dv_{(90)}$ = 99.6 nm).

[0170] *Formulation Protocol 2.* Aliquots of a 2.0 mg/mL aqueous solution PEI (branched, 25 kDa) were mixed with aqueous solution of CFTR mRNA (1.0 mg/mL). The resulting complexed mixture was pipetted up and down several times and put aside for 20 minutes prior to injection. Final concentration = 0.60 mg/mL CFTR mRNA (encapsulated). Z_{ave} = 75.9 nm ($Dv_{(50)}$ = 57.3 nm; $Dv_{(90)}$ = 92.1 nm).

[0171]

Analysis of FFL and CFTR protein produced via intratracheal administered mRNA-loaded nanoparticles. All studies were performed using either female BALB/C mice or CFTR KO mice. FFL samples were introduced via either direct instillation (MicroSprayer®) or nebulization (PARI Boy or Aeroneb) respective dose of encapsulated FFL mRNA. CFTR mRNA was introduced using a PARI Boy jet nebulizer. Mice were sacrificed and perfused with saline after allowing time for expression.

[0172] *Intratracheal Administration of FFL mRNA.* FFL test materials were administered by a single intratracheal aerosol administration via a Microsprayer™ (50 µL/animal) while

animals are anesthetized with intraperitoneal injection of a mixture of ketamine 50-100 mg/kg and xylazine 5-15 mg/kg.

[0173] *Nebulization (Aerosol) Administration of FFL mRNA.* FFL test materials were administered by a single aerosol inhalation via Aeroneb® Lab nebulizer (nominal dose volume of up to 8 mL/group). The test material was delivered to a box containing the whole group of animals (n=4) and connected to oxygen flow and scavenger system.

[0174] *Administration of CFTR mRNA.* CFTR mRNA was prepared in the manner described in Example 6 below. Four CFTR knockout mice were placed in an aerosol chamber box and exposed to 2 mg total codon optimized unmodified human CFTR mRNA (comprising the coding sequence of SEQ ID NO: 3) via nebulization (Pari Boy jet nebulizer) over the course of approximately one hour. Mice were sacrificed 24 hours post-exposure.

[0175] *Euthanasia.* Animals were euthanized by CO_2 asphyxiation at representative times post-dose administration (± 5%) followed by thoracotomy and exsanguinations. Whole blood (maximal obtainable volume) was collected via cardiac puncture and discarded.

[0176] *Perfusion.* Following exsanguination, all animals underwent cardiac perfusion with saline. In brief, whole body intracardiac perfusion was performed by inserting 23/21 gauge needle attached to 10 mL syringe containing saline set into the lumen of the left ventricle for perfusion. The right atrium was incised to provide a drainage outlet for perfusate. Gentle and steady pressure was applied to the plunger to perfuse the animal after the needle had been positioned in the heart. Adequate flow of the flushing solution was ensured when the exiting perfusate flows clear (free of visible blood) indicating that the flushing solution has saturated the body and the procedure was complete.

[0177] *Tissue Collection.* Following perfusion, all animals had the liver and lungs (right and left) harvested. Select groups were subjected to approximately one half of the liver and both (right and left) lungs snap frozen in liquid nitrogen and stored separately at nominally -70°C. Select groups were subjected to approximately half of the liver placed in one histology cassette

per animal. Additionally, the lungs were inflated with 10% NBF through a cannula that was inserted into the trachea. The trachea was tied off with a ligature and the lungs (right and left) and trachea were placed intact in one histology cassette per animal. All histology cassettes were stored ambient in 10% NBF for 24 hours and transferred to 70% ethanol.

[0178] *Expression of FFL in FFL-treated mice.* Upon analysis of the tissue samples, FFL expression was detected in FFL-treated mice (data not shown).

[0179] *Expression of CFTR in CFTR knockout mice.* CFTR expression was detected by immunoprecipitation-Western blot analysis of CFTR mRNA-treated mouse lungs. Mature "C" band was detected in left and right lungs of all treated mice while unobserved in control mice (Figure 1B). Antibodies used were MAB25031 (R&D Systems) for immunoprecipitation and SAB4501942 (Sigma) for detection via Western blot analysis.

[0180] The results shown here indicate that a CFTR protein can be successfully expressed *in vivo* based on lung delivery of mRNA. Furthermore, the fact that CFTR mRNA has been successfully delivered to the lung of CFTR knock out mice and resulted in effective protein production in the lung indicates that CFTR mRNA based *in vivo* protein production may be used to treat the CFTR protein deficiency.

Example 4: Lung Delivery of CFTR mRNA Using Polymeric Nanoparticles

[0181] The delivery of human CFTR messenger RNA to the lungs of a mouse can be accomplished via either direct inhillation as well as nebulization. Using *in situ* hybridization methods, one can successful detect human CFTR mRNA after intratracheal administration of human CFTR mRNA-loaded nanoparticles to mice. Administration may be accomplished employing lipid-based nanoparticles (eg. C12-200) as well as polymeric nanoparticles (eg. Polyethyleneimine, PEI).

[0182] *Administration of CFTR mRNA Using Polymeric Nanocarriers.* CFTR KO mice were treated with polyethyleneimine (PEI)-based CFTR mRNA loaded nanoparticles via

intratracheal administration (30 ug encapsulated mRNA). The treated mice were sacrificed six hours and twenty-four hours post-administration and the lungs were harvested and fixed in 10% neutral buffered formalin (NBF). *In situ* hybridization was employed for detection of the exogenous human CFTR mRNA (Figure 6). Substantial staining was observed 24 hours post-administration with widespread distribution in both mouse lungs of the treated CFTR KO mice while no staining was observed for PBS-treated control mice.

[0183] Analysis of the treated lungs at higher magnifications (up to 20x magnification) revealed extensive positive intracellular staining throughout the bronchial and alveolar regions of both lungs (Figure 7). Upon further magnification (40x), positive staining within the cytoplasm of target apical bronchial epithelial cells was observed (Figure 8). Thus, one can conclude that the messenger RNA API was successfully delivered to the target apical bronchial epithelial cells. Further, while substantial staining can be observed at 6 hours post-administration, significant positive detection of hCFTR mRNA was still observed after 24 hours (Figure 9).

[0184] Substantial positive intracellular staining was observed throughout both lungs within bronchial and alveolar regions at 24 hours post-administration.

Example 5: Lung Delivery of CFTR mRNA Using Lipid-Based Nanoparticles

[0185] *Administration of CFTR mRNA Using Lipid-Based Nanocarriers.* As mentioned above, successful lung delivery of human CFTR mRNA can be accomplished via lipid nanoparticle based delivery vehicles. Disclosed here are examples of hCFTR mRNA-loaded cationic lipid nanoparticles utilizing C12-200 as the cationic lipid component.

[0186] Successful detection of human CFTR mRNA within the lungs of CFTR KO mice was achieved via in situ hybridization. Knockout mice were treated with 15 ug of hCFTR mRNA encapsulated in C12-200-based lipid nanoparticles and sacrificed 6 hours post-administration. Positive detection of hCFTR mRNA was observed throughout the bronchial and alveolar regions of both lungs when compared to PBS-treated control mice (Figure 10).

[0187] Upon further magnification (40x), positive detection of human CFTR mRNA was observed within the apical cytoplasm of bronchial epithelial cells and well as intracellular terminal alveolar regions (Figure 11).

[0188] In total, successful delivery of synthetic human CFTR messenger RNA can be achieved utilizing both polymeric (PEI) and lipid nanoparticle-based (C12-200) delivery systems. These systems afforded intracellular accumulation of the drug substance within the target cells of the mice. Further, substantial amounts of hCFTR mRNA were present in these target cells 24 hours post-administration.

Example 6: Validation of Human CFTR Expression Using Specific Antibody

[0189] *Antibody validation for human CFTR protein detection in mouse, pig, and cultured cells.* Experiments were performed to identify an antibody which is specific for the hCFTR protein, which does not cross-react with the mouse and swine analogue and which is available in sufficient supply for future experiments. Briefly, testing of various anti-hCFTR antibodies from academic and commercial sources led to identification of a combination of anti-hCFTR antibodies which were capable of detecting human CFTR protein after immunoprecipitation and Western blotting (IP/WB) without cross-reactivity for either murine or porcine CFTR. Thus, suitable anti-hCFTR antibodies for detection of hCFTR protein without cross-reactivity for either murine or porcine CFTR were identified based on IP/WB results.

[0190] Cells were transfected with hCFTR mRNA and protein lysates were prepared using ProteoExtract Transmembrane Kit (Merck) at 24 hrs post transfection and transmembrane fraction was screened by Western blotting for hCFTR using mouse anti-human CFTR antibody (MA1-935). Lysates from 16HBE cells were used as positive control. Figure 12A presents the data from CHO and COS-7 cells.

[0191] Baby Hamster Kidney cells (BHK), described as CFTR-negative in the literature, were transfected similar to CHO and COS-7 cells and protein lysates screened by Western blot.

In contrast to the previously published reports, a clear positive signal for CFTR could be observed using the mouse monoclonal anti-CFTR antibody (Figure 12B). To test the specificity of antibody used in Western blot analysis, Pig Kidney Cells from CFTR-knockout pig (PKC), kindly provided by Prof. Eckhardt Wolf (Ludwig Maximilians University, Munich), were used in transfection experiments and protein lysates screened for CFTR expression. As was evident in Figure 12B, no signal for CFTR could be detected in PKC cells. However, transfection did not result in any detectable hCFTR expression either. Using luciferase as a control for transfection, PKC cells were found to express luciferase several fold less efficient when compared to CHO or COS-7 cells. As no significant difference in the intensity of hCFTR band could be detected in any of the screened cell lines post transfection, extensive screening for other hCFTR antibodies with higher sensitivities and specificity towards hCFTR was performed.

[0192] *Antibody Screening via Western Blots*. Protein lysates were prepared from human bronchial epithelial cell line (BEAS-2B), human embryonic kidney cell line (HEK), mouse lungs and pig lungs using ProteoExtract Transmembrane Kit (Merck) and transmembrane fraction used for immunoblotting using different primary antibodies (MA1-935 from Thermo Scientific Pierce Antibodies, Rockford, IL, USA, AB596 from the Cystic Fibrosis Consortium, University of Pennsylvania, PA, USA, and AB570 from the Cystic Fibrosis Consortium, University of Pennsylvania, PA, USA). The data are summarized as Figure 13.

[0193] Whereas MA1-935 detected CFTR in all the three species, AB596 detects human and murine CFTR but not porcine and antibody G449 detects only human CFTR specifically. With AB570, it was not clear if the slightly low molecular weight bands observed with murine and porcine samples are indeed CFTR or non-specific products. In subsequent experiments (data not shown), it was found that MA1-935 recognizes a band which is not CFTR. Therefore, in general, MA1-935 results were considered as confirming results generated using other antibodies, but experiments in which the only anti-CFTR antibody used was MA1-935 were not considered conclusive.

[0194] *Immunoprecipitation of hCFTR (IP-hCFTR) from Tissue samples.* Given that all the screened antibodies produced several non-specific bands and none of them produced the characteristic banding pattern of hCFTR (C-band representing the fully glycosylated protein and B-band representing the core mannosylated form), immunoprecipitation (IP) of hCFTR and subsequent detection by Western blot was established to increase the sensitivity and specificity of detection thereby increasing the signal to noise ratio.

[0195] Initial IP experiments were performed in collaboration with Prof. Burkhard Tümmler (Medizinsche Hochschule Hannover) using protocols and antibodies published by van Barneveld et al. 2012, Immunochemical analysis of Mutant CFTR in Lung explants, *Cell Physiol. Biochem.* 30, 587-595 (2012)). Human colon carcinoma cells (T84) which overexpress hCFTR were used as positive controls for IP experiments.

[0196] Immunoprecipitation of hCFTR using three different antibodies (R29, R66/17 and R66/16) followed by immunodetection with AB596 resulted in specific detection of hCFTR in protein lysates from lungs of pigs treated with an aerosol of hCFTR SNIM RNA as described in Example 8 below (Figure 14).

[0197] *HGT5001 Formulation.* Aerosol experiments using hCFTR SNIM RNA in a formulation of HGT5001:DOPE;Chol;PEGDMG2K (relative amounts 50:25:20:5 (mg:mg:mg:mg)) ("HGT5001 Formulation") were performed in mice and protein lysates from the isolated lungs at 24 hrs post mRNA delivery were also analysed by IP using the same antibodies and conditions as for the pig lysates. However, no characteristic mature CFTR banding pattern could be detected for mouse samples (Figure 15).

[0198] *Immunoprecipitation of hCFTR (IP-hCFTR) from in vitro-transfected cells.* Initial IP results using tissue material from pigs provided the evidence for the technical feasibility of hCFTR detection post transcript delivery *in vivo*. However, as none of the antibodies used in immunoprecipitating CFTR (R29, R66/17 and R66/16) are commercially available, other commercially available antibodies were screened for their efficacy in IP reactions. Two antibodies from R&D systems (MAB25031 and MAB1660) were tested.

[0199] Protein lysates were prepared from T84 cells and 500 µg of total protein was used in the IP reaction using different concentrations of MAB25031 antibody. The amount of hCFTR protein immunoprecipitated was then detected by immunobloting using AB570 (Cystic Fibrosis Foundation). AB596 under these conditions resulted in much higher background and so was not tested further. As revealed in Figure 16A, there was no further increase in the amount of CFTR protein precipitated when the concentration of IP antibody was increased from 2 µg/ml to 4 µg/ml. Both the fully glycosylated and only core glycosylated forms (C- and B-band, respectively) were detected. The same immunoprecipitates were also screened using MAB1660 as primary antibody in western blot. With this antibody however, only band-C was visible (Figure 16B).

[0200] After the successful detection of endogenous hCFTR from T84 immunoprecipitates using MAB25031 antibody, experiments were performed in NIH3T3 cells with the aim to detect hCFTR protein post transfection. NIH3T3 cells were transfected with hCFTR SNIM RNA. Protein lysates were prepared at 72 hrs post transfection and protein amounts quantified using BCA method. Human CFTR protein was immunoprecipitated from 500 µg of total protein lysate using MAB25031 antibody at 2 µg/ml followed by immunoblotting using AB570 (Figure 17). However, no CFTR could be detected. Cells transfected with LacZ encoding mRNA were analysed as control samples for the effect of transfection per se on amount of CFTR protein.

[0201] Increasing the amount of total protein used in immunoprecipitation from 500 µg to 8 mg did not result in any detectable hCFTR protein post immunodetection with AB570. Another hCFTR specific antibody, MAB1660 (R&D Systems), was also screened for immunoprecipitation (Figure 18). However, this antibody does not precipitate CFTR as effectively as MAB25031. Therefore all future immunoprecipitations were performed with MAB25031.

[0202] Lack of hCFTR detection in mRNA transfected samples may not necessarily mean lack of functionality of the tested mRNAs as kinetic experiments using luciferase as

marker gene have shown that maximum expression with mRNA is observed at 24 hrs post transfection. Lack of hCFTR detection is rather due to insufficient hCFTR concentration in the tested samples or lack of specificity of the applied antibodies.

[0203] *PEI Formulation.* The established conditions were tested for their feasibility to detect hCFTR after hCFTR SNIM RNA in delivery to pigs (see Example 7) of a nanoparticle formulation with 25 kDa branched PEI ("PEI Formulation") prepared as follows.. The required amount of SNIM RNA was diluted just before application in water for injection (Braun, Melsungen) to a total volume of 4 ml and added quickly to 4 ml of an aqueous solution of branched PEI 25 kDa using a pipette at an N/P ratio of 10. The solution was mixed by pipetting up and down ten times and nebulized as two separate 4.0 ml fractions one after another to the pig lungs using the indicated nebulizer. One sample from the luciferase expressing lung areas from pig #1 and another from the caudal lobe of pig #2, where no luciferase activity could be detected, thus indicating lack of mRNA delivery and/or expression, were selected as positive and negative controls. Protein lysates prepared from these samples were immunoprecipitated using MAB25031 (R&D Systems) and hCFTR protein detected using AB570. As shown in Figure 19, luciferase expression correlated with the expression of hCFTR mRNA. Sample from the left caudal lobe from pig #2 where no luciferase activity was detectable, was also negative for hCFTR (lane 1) whereas hCFTR could be detected in samples from pig #1 which were positive for luciferase (lane 2).

Example 7: Aerosol Delivery of mRNA

[0204] *Establishment of encapsulated mRNA aerosol delivery to the lungs of pigs.* Aerosol administration of firefly luciferase (FFL) SNIM RNA to the pig lungs was established by a stepwise experimental procedure. In a first step FFL SNIM RNA formulations were nebulized to anaesthetized pigs during controlled ventilation. In a second step lungs were excised immediately after aerosol administration was completed and lung specimens were

incubated in cell culture medium overnight before ex vivo luciferase measurement was performed on lung specimens by BLI.

[0205] Pigs of the German Landrace were obtained from Technical University Munich, Weihenstephan, Germany. The pigs had a body weight ranging from 35-90 kg. Each treatment was performed on one pig. In total five pigs were treated. The first pig (90 kg weight) was treated with FFL SNIM RNA in the PEI Formulation of Example 6 using an EFlow mesh nebulizer and measurement of luciferase activity in lung homogenates. The second pig (60 kg weight) was treated with FFL SNIM RNA in the PEI Formulation of Example 6 using an EFlow mesh nebulizer and measurement of luciferase activity in lung specimens by BLI. The third pig (80 kg weight) was treated with FFL SNIM RNA in the PEI Formulation of Example 6 using a PARI BOY jet nebulizer and measurement of luciferase activity in lung specimens by BLI. The fourth pig (60 kg weight) was treated with FFL SNIM RNA/hCFTR mRNA in the PEI Formulation of Example 6 using an Aeroneb mesh nebulizer and measurement of luciferase activity in lung specimens by BLI. The fifth pig (35 kg weight) was treated with FFL SNIM RNA in the HGT5001 Formulation of Example 6 using an Aeroneb mesh nebulizer and measurement of luciferase activity in lung specimens by BLI.

[0206] Sedation in pigs was initiated by premedication with azaperone 2 mg/kg body weight, ketamine 15 mg/kg body weight, atropine 0.1 mg/kg body weight and followed by insertion of an intravenous line to the lateral auricular vein. Pigs were anesthetized by intravenous injection of propofol 3-5 mg/kg body weight as required. Anesthesia was maintained by isoflurane (2-3%) with 1% propofol bolus injection at 4 to 8 mg/kg body weight to enhance anesthesia as required. Duration of the anesthesia was approximately 1-3 hrs. Pigs were killed with bolus injection of pentobarbital (100 mg/kg body weight) and potassium chloride via the lateral ear vein. Lungs were excised and tissue specimens were collected from various lung regions followed by incubation in cell culture medium overnight. For measurement of luciferase activity tissue specimens were either homogenized and analyzed in a tube

luminometer or incubated in a medium bath comprising D-Luciferin substrate and subjected to ex vivo luciferase BLI.

[0207] *Details and Results for Pig #1.* The experimental set up is illustrated in Figure 20. For aerosol administration an EFlow mesh nebulizer was connected in-line to the ventilation tubing of the respirator. Aerosol administration took approximately 60 min and was longer than expected from control experiments with an open system. This was apparently caused by increased back pressure during nebulisation as evidenced by aerosol outflow at the reservoir of the mesh nebulizer. Eight milliliters of the PEI Formulation of Example 6 comprising 1 mg FFL SNIM RNA in water for injection were prepared as described in WP5 and were nebulized in two separate 4 ml portions one after another. Luciferase measurement was performed in tissue homogenates of excised lung specimens of various lung regions after overnight incubation in cell culture medium. Expression values were mapped according to the origin of the lung specimens (Figure 21).

[0208] The results showed successful luciferase expression in pig lung tissue. Luciferase expression was highest in central parts of the lung and declined towards more distal regions of the lung. The expression pattern correlated with the expected deposition pattern of the inhaled FFL SNIM RNA-PEI nanoparticles according to the chosen ventilation parameters. Levels of luciferase expression were in the same range as observed in mouse experiments in WP5 using the same the PEI Formulation of Example 6.

[0209] *Details and Results for Pig #2.* Aerosol administration of FFL SNIM RNA in the PEI Formulation of Example 6 in pig #2 was performed as in pig #1 but luciferase activity was measured on lung specimens by bioluminescent imaging (BLI). This experiment was performed to establish ex vivo luciferase measurement of organ cultured lung specimens by BLI. Luciferase measurement was clearly observed in individual tissue specimens of different lung regions of the treated pig (Figure 22). The experiment confirmed results obtained from pig #1.

[0210] *Details and Results for Pig #3.* Aerosol administration in pig #1 and #2 using the EFlow mesh nebulizer revealed some technical difficulties and inadequate nebulisation time.

Therefore, pig #3 was treated using the PARI BOY jet nebulizer which was connected to the ventilation tubing via a T-connector. Aerosol administration lasted longer (approximately 80 min) than with the EFlow mesh nebulizer and aerosol administration was non-satisfying. Very low luciferase activity was detected in sliced lung samples from different lung regions of the treated pig (Figure 23).

[0211] *Details and Results for Pig #4.* The results of the previous experiments demonstrated that a mesh nebulizer is more suitable for aerosol administration to the lungs of pigs in the chosen set up than a jet nebulizer. For this reason, another mesh nebulizer was tested for this purpose which satisfactorily nebulized the PEI Formulation of Example 6 when tested in an open system. Pig #4 was treated using the Aeroneb mesh nebulizer which was connected in-line to the tubing of the respirator. In this experiment, 1 mg of hCFTR mRNA was co-delivered together with 1 mg of FFL SNIM RNA in the PEI Formulation of Example 6. This was done to test formulation stability and nebulisability of co-formulated FFL SNIM RNA/hCFTR mRNA-PEI nanoparticles with respect to repeated dosing in to be performed in Example 8. The formulation was stable and did not reveal incompatibility with nebulisation. Luciferase activity was clearly observed in individual tissue specimens of different lung regions of the treated pig (Figure 24).

[0212] The experiment confirmed results obtained from pig #1 and pig #2, although higher expression levels were obtained. The experiment showed that the Aeroneb mesh nebulizer was best suited for delivery of the the PEI Formulation of Example 6 to the lungs of pigs. Moreover, the experiment demonstrated FFL SNIM RNA was still active when co-delivered together with hCFTR mRNA.

[0213] *Details and Results for Pig #5.* Pig #5 was treated with 1 mg of FFL SNIM RNA in the HGT5001 Formulation of Example 6 aerosolized with the Aeroneb mesh nebulizer. The formulation could be aerosolized without technical difficulties. Luciferase activity was clearly observed in individual tissue specimens of different lung regions of the treated pig (Figure 25).

[0214] The experiment showed that aerosolized FFL SNIM RNA in the HGT5001 Formulation of Example 6 is active in pig lung tissue, although expression levels were approximately 15-20-fold lower than in pigs treated with the the PEI Formulation of Example 6.

[0215] *Conclusion.* Successful results were obtained using the Aeroneb mesh nebulizer with the PEI Formulation of Example 6. Four pigs were treated with the PEI Formulation of Example 6 to identify the optimal experimental setup for aerosol delivery. The results demonstrated that luciferase expression could be detected in pig lung homogenates and by BLI. Luciferase expression was highest in central parts of the lungs and hardly seen in the distal areas of the lungs. The Aeroneb mesh nebulizer was found to give the best results together with the shortest delivery time. According to these experiments another pig was treated with FFL SNIM RNA encapsulated in the HGT5001 Formulation of Example 6. Although luciferase expression was clearly observed in some parts of the pig lungs, expression levels were lower than for FFL SNIM RNA in the PEI Formulation of Example 6. The results from this work package clearly demonstrated that SNIM RNA delivery to the lungs of pigs as a large preclinical animal model was feasible using various formulations such as polymer (e.g., PEI) based Formulation and lipid (e..g, HGT5001) based formulations. The results of this example provided proof of concept for successful SNIM RNA delivery to the lungs of a large animal which closely mimics the situation in human patients by nebulizer used in clinical practice.

Example 8: *In Vivo* mRNA Delivery (Weekly Dose)

[0216] A trial was performed to evaluate practicability of an aerosol application once a week in pigs. Practicability was defined as performing three aerosol applications of modified mRNA in intervals of one week without induction of lung disease (absence of adverse events higher than grade 2). Additional objectives were to evaluate i) grade of distress of the animals, ii) adverse events occurring during laboratory or clinical assessment of the pigs, and iii) measurement of the induced proteins (luciferase and hCFTR).

[0217] Repeated aerosol administration of SNIM RNA in the PEI Formulation to the lungs of pigs was established. Groups of two pigs were treated one, two, or three times at weakly intervals with FFL SNIM RNA/hCFTR SNIM RNA in the PEI Formulation of Example 6. Two untreated pigs served as controls. Lungs were excised 24 hrs after treatment and ex vivo luciferase activity was measured in isolated lung specimens by BLI. Expression of hCFTR protein was analysed using IP/WB. Immunohistochemistry (IHC) was performed for detection of luciferase expression on the cellular level. Toxicology was investigated by measurement of inflammatory cytokines in serum and blood chemistry. Histopathology was performed on lung samples. The study protocol "Pilot project: Repeated application of modified mRNA to establish an animal model for aerosol therapy of cystic fibrosis in pigs" was approved by the local authorities before the start of the experiments (Animal experiments license Nr.: 0-045-12).

[0218] *Experimental Design*. Pigs, German Landrace, female approximately 6 weeks old (~25 kg body mass in average) at nebulisation, were purchased from Technical University Munich, Weihenstephan, Germany. Pigs were randomized and treated according to the scheme below (Table 3). Treatment groups of each two pigs were as follows:

Group 0 - Control group without treatment

Group I - Aerosol administration of 1 mg FFL SNIM RNA and 1 mg hCFTR SNIM RNA in the PEI Formulation of Example 6 on day 1.

Group II - Aerosol administration of 2 mg hCFTR SNIM RNA in the PEI Formulation of Example 6 on day 1 and 1 mg FFL SNIM RNA and 1 mg hCFTR SNIM RNA in the PEI Formulation of Example 6 on day 8.

Group III - Aerosol administration of 2 mg hCFTR SNIM RNA (6379-186) in the PEI Formulation of Example 6 on day 1 and day 8, aerosol administration of 1 mg FFL SNIM RNA and 1 mg hCFTR SNIM RNA in the PEI Formulation of Example 6 on day 15.

[0219] The scheme for treatment and evaluation of each group is shown in Table 3. In addition to the illustrated interventions, physical examination of the pigs was done on a daily basis.

[0220] **Table 3.** Time-line diagram of different treatment groups.

(Abbreviations used:

- AA Aerosol application
- Bw Blood work D day
- Euth. Euthanasia of the animal)

***Group 0** (untreated animals):*

1st Euth. Bw ↓ d1

***Group 1** (1 aerosol application; survival 1d):*

1st 1st Bw Bw AA Euth. ↓ ↓ d1, d2

***Group 2** (2 aerosol applications; survival 8d):*

1st 1st 2nd 2nd Bw Bw Bw Bw AA AA Euth ↓ ↓ ↓ ↓ d1, d2, d3, d4, d5, d6, d7, d8, d9

***Group 3** (3 aerosol applications; survival 15d):*

1st 1st 2nd 2nd 3rd 3rd Bw Bw Bw Bw Bw Bw AA AA AA Euth ↓ ↓ ↓ ↓ ↓ ↓ d1, d2, d3, d4, d5, d6, d7, d8, d9, d10, d11, d12, d13, d14, d15, d16

[0221] *Experimental procedure.* Sedation in pigs was initiated by premedication with azaperone 2 mg/kg body weight, ketamine 15 mg/kg body weight, atropine 0.1 mg/kg body

weight and followed by insertion of an intravenous line to the lateral auricular vein. Pigs were anesthetized by intravenous injection of propofol 3-5 mg/kg body weight as required. Anesthesia was maintained with continuous intravenous infusion of 1% propofol as required. Ventilation parameters were matched with endexpiratory carbon dioxide and adjusted if necessary. Anesthesia, respiratory and cardiovascular parameters were monitored continuously using pulse oximetry, capnography, rectal temperature probe and reflex status. Animals received infusion of balanced electrolyte solution at 10 ml/kg/h. Duration of the anesthesia was approximately 80-120 min. Pigs were extubated after onset of sufficient spontaneous breathing. Pigs were killed with bolus injection of pentobarbital 100 mg/kg of body weight via the lateral ear vein after sedation. Lungs were excised and sliced approximately 1 cm thick tissue specimens were collected from various lung regions followed by incubation in cell culture. For measurement of luciferase activity tissue specimens were incubated in a medium bath comprising D-Luciferin substrate and subjected to ex vivo luciferase BLI.

Luciferase expression in treatment groups by BLI.

[0222] For **Group 0** (Control group without treatment), no luciferase activity was observed in lung slices (Figure 26).

[0223] For **Group I** (Aerosol administration of 1 mg FFL SNIM RNA and 1 mg hCFTR SNIM RNA in the PEI Formulation of Example 6), Luciferase activity was clearly detected in lung specimens of one time treated pigs #3 and #6 (Figure 27). Luciferase expression was highest in central parts of the lungs.

[0224] For **Group II** (Aerosol administration of 2 mg hCFTR SNIM RNA in the PEI Formulation of Example 6 on day 1 and 1 mg FFL SNIM RNA and 1 mg hCFTR SNIM RNA in the PEI Formulation of Example 6 on day 8), Luciferase activity was clearly detected in lung specimens of twice-treated pigs #4 and #8 (Figure 28). Luciferase expression was highest in central parts of the lungs. It has to be considered that samples were stored for additional 10 hours in cell culture medium before measurement because of a power blackout on the day of the measurements and resulting technical problems with the BLI system.

[0225] For **Group III** (Aerosol administration of 2 mg hCFTR SNIM RNA in the PEI Formulation of Example 6 on day 1 and day 8, aerosol administration of 1 mg FFL SNIM RNA and 1 mg hCFTR SNIM RNA in the PEI Formulation of Example 6 on day 15), Luciferase activity was clearly detected in lung specimens of thrice-treated pigs #1 and #2 (Figure 29). Luciferase expression was highest in central parts of the lungs.

[0226] *Properties of SNIM RNA-PEI nanoparticles.* Particle size and zeta potential was measured for SNIM RNA-PEI formulations before nebulisation (Table X1). The SNIM RNA-PEI nanoparticles could be reproducibly formed with a size ranging from 25-37 nm and zeta potentials ranging from 30-49 mV.

Table X1. Particle size and Zeta Potential measurements

Pig #	Treatment #	Radius ± S.D.(nm)	Zeta potential ± S.D.(mV)
1	1	26.7±0.3	36.9±5.9
	2	33.3±0.6	42.5±5.5
	3	31.6±0.4	41.3±3.4
2	1	24.7±0.5	32.9±3.3
	2	34.9±0.2	41.5±1.4
	3	32.5±0.4	29.1±1.1
3	1	35.2±0.8	42.9±1.9
4	1	36.9±1.1	45.4±0.6
6	1	27.5±0.1	30.5±6.6
	2	33.0±0.8	49.1±3.0
8	1	25.5±0.1	44.0±2.1
	2	33.3±0.3	45.9±9.5

[0227] *Luciferase expression in treatment groups by IHC.* IHC for FFL was performed on tissue specimens of lung slices (Sophistolab AG, Eglisau, Switzerland) which were positive by BLI and compared with lung tissue of an untreated pig and luciferase-positive mouse tumor tissue as positive control. As expected a strong signal was seen in the luciferase-positive mouse tumor tissue, whereas lung tissue of the untreated pig did not show specific staining. A clearly detectable staining pattern could be observed in the lung tissue of pig #1 which received three

treatments. FFL expression was most prominent in the bronchial epithelium of large and small airways (Figure 30).

[0228] *Detection of hCFTR protein in lung tissue of treated pig by IP/WB.* Highly BLI-positive lung tissue of three times treated pig #1 was subjected to hCFTR IP/WB according to the protocol described by van Barneveld A et al., Cell Physiol Biochem. 30, 587-95 (2012) (Figure 31). Mature complex-glycosylated hCFTR appears as the disperse so-called C-band. Mannose-rich hCFTR appears as the more dense so-called B-band. Clearly hCFTR expression is observed in T84 positive control cells and lung tissue of pig #1 treated with hCFTR SNIM RNA in the PEI Formulation of Example 6. Expression of hCFTR protein was not observed in untreated pigs. A comparison of hCFTR protein expression in human lung tissue from a published study using the identical protocol (van Barneveld A et al., supra) suggested that expression of hCFTR in pig lung tissue after hCFTR SNIM aerosol treatment was similar to hCFTR expression in healthy human lung.

[0229] This finding was further confirmed by using a different set of antibodies for detection of hCFTR protein by IP/WB in treated pig lung (see Example 6). One sample from the luciferase expressing lung areas from pig #1 and another from the caudal lobe of pig #2, where no luciferase activity could be detected, thus indicating lack of mRNA delivery and/or expression were selected as positive and negative controls. Protein lysates prepared from these samples were immunoprecipitated using MAB25031 (R&D Systems) and hCFTR protein detected using AB570. As shown in Figure 32, luciferase expression correlated with the expression of hCFTR mRNA. Sample from the left caudal lobe from pig #2 where no luciferase activity was detectable, was also negative for hCFTR (lane 1), whereas hCFTR could be detected in samples from pig #1 which were positive for luciferase (lane 2).

[0230] *Toxicology: Preliminary histological assessment of lung samples.* A histological assessment of samples of the lungs taken after the euthanasia of three animals was performed. After embedding in paraffin sections lung samples were stained with Hematoxiline-Eosine for morphological evaluation. The findings were consistent across the samples from the three pigs,

two of which (pig #1 and pig #2) received three aerosol applications and the third (pig #7) was an untreated control with no aerosol application.

[0231] *Toxicology: Distress.* Only pig #2 and pig #1 showed mild signs of distress on day 2-4 after the first treatment. Thus, three aerosol applications within three weeks caused only mild distress

[0232] *Toxicology: Adverse events.* Kind and frequency of adverse events (AE) were analyzed by laboratory parameters (blood, MBS and BAL) and by physical examination of the pigs (defined as a secondary objective in this trial).

[0233] Serum and whole blood samples were taken at the time points defined by the study protocol. Twelve representative parameters (haemoglobin, hematocrit, AP, ALT, AST, CK, bilirubin, creatinine, glucose, potassium, thrombocytes, and white blood cells) being indicative to show organ specific pathology (blood, bone marrow, liver, muscle, and kidney) were selected and the test results obtained from the VetMedLab, Ludwigsburg, Germany classified according to VCOG, version 2011.

[0234] The results showed that no severe adverse events (AE) were observed in the pigs (an AE of grade 3, 4, or 5 would have qualified as severe). There was no impairment of laboratory parameters after aerosol application of SNIM RNA in the PEI Formulation of Example 6. For the slight changes in some parameters (e.g. CK or liver enzymes) it is more likely that these changes were caused by the experimental procedure per se (e.g. i.m. injections and anaesthesia). Also no negative effect from repeated application could be detected -even after the third application, the pigs of group 3 show no AE higher than AE grade 2. Even AE grade 1 or 2 were rare and showed no correlation to the aerosol application of SNIM RNA in the PEI Formulation of Example 6.

[0235] Besides the repeated blood samples two other parameters were assessed to evaluate pathological processes in the lung: i) Brocho-Alveolar-Lavage fluid (BALF) - taken after euthanasia, and ii) microbiology samples (MBS) (smear form the trachea - taken during the

anaesthesia). BALF was taken from each pig during autopsy and was stored at -80°C for further examination. Tracheal smears were taken prior to each aerosol application and microbiologically examined. These examinations revealed a broad spectrum of pathogens including *Bordetella bronchiospectica* (a common pathogen of the respiratory tract of the pig) and *Escherichia coli*. Pigs were once treated with tulathromycin i.m.-injection (1 ml Draxxin® 10%).

[0236] *Physical examination*. In addition to the laboratory parameters, physical examinations of the pigs were performed in the observation periods between the aerosol applications (for details see 1.1.2 of annex 1 and annex 4 of the study protocol). As no system for documenting, grading and assigning the attribution of the AE, either to the intervention or something else is defined for pigs, the common toxicology criteria (CTC)-system established for dogs and cats was used (published by VOCG in 2011). To grade the laboratory parameters, species specific ULN (upper limits of normal) and LLN (lower limits of normal) were used. Clinical assessments were made within the following six AE categories:

(1) allergic/immunologic events; (2) pulmonary/respiratory; (3) constitutional clinical signs; (4) dermatologic/skin; (5) gastrointestinal; and (6) pulmonary/respiratory.

[0237] The results showed that no severe AE (no grade 3, 4, or 5) were observed in the pigs. There was no impairment of parameters assessed by physical examination after the aerosol application of SNIM RNA in the PEI Formulation. The two pigs of group 3 showed grade 1 and 2 AE in three of the respiratory parameters (bronchospasm/wheezing, larynx oedema, and dyspnoea) but these mild or moderate findings were restricted to one or two days. As these observations only occurred after the first anaesthesia/intubation/aerosol application in these two pigs but not after the second or third aerosol application in these two pigs or in any other pig, it is unlikely that these findings are caused by the substance under investigation.

[0238] *Conclusion*. The results of this example demonstrated that the PEI Formulation encoding FFL and hCFTR SNIM RNA could be successfully aerosolized repeatedly to the lungs

of pigs without loss of activity after each treatment cycle and without adverse events. Luciferase expression was found in central parts of the lung tissue but hardly detected in distal lung areas. The regional pattern of luciferase expression correlated with the expected deposition pattern of the the PEI Formulation of Example 6 according to settings used for controlled ventilation. Immunohistochemistry on selected lung samples form treated pigs showed luciferase expression predominantly in the bronchial epithelium of large and small airways. IP/WB clearly demonstrated expression of complex-glycosylated C-band of mature human CFTR in treated pig lung which was absent in untreated pig lung and luciferase-negative lung specimens. Expression of hCFTR in pig lung tissue after hCFTR SNIM RNA aerosol treatment was comparable to the hCFTR expression in healthy human lung when compared to published reports using the identical protocol for hCFTR protein detection. Adverse events grade 1 or 2 were very rare and showed no correlation to the aerosol application of SNIM RNA in the PEI Formulation. Thus, expression of hCFTR protein was successfully demonstrated in lungs of pigs treated with SNIM hCFTR mRNA.

Example 9: CFTR Encoding mRNA Containing Signal Peptide

[0239] This example demonstrates that a CFTR protein may be effectivey expressed from a CFTR encoding mRNA with a signal peptide encoding sequence.

[0240] *Messenger RNA Synthesis.* For the experiment, C-terminal His_{10} tagged codon optimized human cystic fibrosis transmembrane conductance regulator (CO-CFTR-C-His_{10})(SEQ ID NO:15), a codon optimized human CFTR with a growth hormone signal sequence leader (GH-CO-CFTR)(SEQ ID NO:16) and codon optimized human CFTR (CO-CFTR)(SEQ ID NO:17) SNIM RNA were synthesized by *in vitro* transcription from a plasmid DNA template using standard methods. *Cells and CFTR transfection.* Human embryonic kidney HEK293T cells were grown in DMEM (Invitrogen Cat # 11965-092) supplemented with 10% fetal bovine serum, 2 mM L-Glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The day before

transfection, cells were plated on 6-well plates at 50-60% confluence and incubated under normal tissue culture conditions (36°C in a humidified atmosphere of 5% CO2, 95% air). In preparation for transfection, 60 µl Lipofectamine 2000 (Invitrogen Cat # 11668019) was diluted in OptiMem reduced serum media (Invitrogen Cat # 31985-062) and gently vortexed. For the experiment 4 µg of either CO-CFTR, GH-CO-CFTR or CO-CFTR-C-His_{10} SNIM RNA was diluted in 900 µl OptiMem media. The mRNA was immediately added to the diluted Lipofectamine® and incubated at room temperature for 30 minutes. The plating media was gently aspirated and replaced with 1 ml OptiMem Reduced Serum Medium and 300 µl of each respective mRNA/Lipofectamine® complex. Cells were incubated under standard tissue culture conditions.

[0241] *Western Analysis*. Approximately 48 post transfection, cells were removed from their respective plates and lysed. Whole cell lysate was subjected to separation by SDS-PAGE and probed by Western blot. As shown in Figure 33, robust expression of human CFTR protein was detected following CO-CFTR, GH-CO-CFTR and human CO-CFTR-C-His_{10} mRNA transfection, by anti-CFTR (A&B) or anti-His (C) antibodies (Figure 33).

Example 10: *In Vivo* CO-CFTR-C-His10 mRNA Delivery to CFTR Knockout Mice

[0242] *Analysis of human CFTR protein produced via intratracheal administered mRNA-loaded nanoparticles*. All studies were performed using CFTR KO mice. CFTR mRNA formulation or vehicle control was introduced using a PARI Boy jet nebulizer. Mice were sacrificed and perfused with saline, after a predetermined period of time, to allow for protein expression from the mRNA.

[0243] *Messenger RNA Synthesis.* In the example, C-terminal His_{10} tagged codon optimized human cystic fibrosis transmembrane conductance regulator (CO-CFTR-C-His_{10}) SNIM RNA and codon-optimized FFL SNIM RNA were synthesized by *in vitro* transcription from plasmid DNA templates.

[0244] *PEI Formulation.* For the approach, delivery and expression of CO-CFTR-C-His_{10} mRNA in the lungs of CFTR knockout mice was evaluated using both polymeric and lipid-based nanoparticle forluations. Polymeric nanoparticle formulations with 25 kDa branched PEI prepared as follows. The required amount of SNIM RNA was diluted just before application in water for injection (Braun, Melsungen) to a total volume of 4 ml and added quickly to 4 ml of an aqueous solution of branched PEI 25 kDa using a pipette at an N/P ratio of 10. The solution was mixed by pipetting up and down ten times and nebulized as two separate 4.0 ml fractions one after another to the mouse lungs using the indicated nebulizer.

[0245] *cKK-E12 Formulation.* For the lipid-based nanoparticle experiment, a lipid formulation was created using CO-CFTR-C-His10 SNIM RNA in a formulation of cKK-E12:DOPE:Chol:PEGDMG2K (relative amounts 50:25:20:5 (mg:mg:mg:mg). The solution was nebulized to the mouse lungs using the indicated nebulizer.

[0246] *Nebulization (Aerosol) Administration of Human CO-CFTR-C-His10 mRNA.* CFTR test materials were administered by a single aerosol inhalation via PARI Boy jet nebulizer (nominal dose volume of up to 8 mL/group). The test material was delivered to a box containing the whole group of animals (n=4) and connected to oxygen flow and scavenger system.

[0247] *Administration of Human CO-CFTR-C-His_{10} mRNA.* CFTR mRNA was prepared in the manner described above. Four CFTR knockout mice were placed in an aerosol chamber box and exposed to 2 mg total codon optimized unmodified human CFTR mRNA (comprising the coding sequence of SEQ ID NO: 3) via nebulization (Pari Boy jet nebulizer) over the course of approximately one hour. Mice were sacrificed 24 hours post-exposure.

[0248] *Euthanasia.* Animals were euthanized by CO2 asphyxiation at representative times post-dose administration (± 5%) followed by thoracotomy and exsanguinations. Whole blood (maximal obtainable volume) was collected via cardiac puncture and discarded.

[0249] *Perfusion.* Following exsanguination, all animals underwent cardiac perfusion with saline. In brief, whole body intracardiac perfusion was performed by inserting 23/21 gauge

needle attached to 10 mL syringe containing saline set into the lumen of the left ventricle for perfusion. The right atrium was incised to provide a drainage outlet for perfusate. Gentle and steady pressure was applied to the plunger to perfuse the animal after the needle had been positioned in the heart. Adequate flow of the flushing solution was ensured when the exiting perfusate flows clear (free of visible blood) indicating that the flushing solution has saturated the body and the procedure was complete.

[0250] *Tissue Collection.* Following perfusion, all animals had their lungs (right and left) harvested. Both (right and left) lungs were snap frozen in liquid nitrogen and stored separately at nominally -70°C.

[0251] *Expression of human CFTR from CO-CFTR-C-His_{10} mRNA in CFTR knockout mice.* CFTR expression was detected by Western blot analysis of tissue lysate collected from CFTR mRNA-treated mouse lungs. Mature "C" band was detected in left and right lungs of all treated mice, for both the lipid-based and polymeric-based formulations (Figure 34). Expression of the mature "C" band was verified by comparison with lysate collected from HEK 293T human CO-CFTR-C-His_{10} positive cells as described in Example 9. In contrast, no detectable signal was observed in lysate collected from wild type untreated control mice (Figure 34). Taken together, these data suggest that both polymeric and lipid based formulations (such as the cKK-E12 formulation listed above) are effective for lung delivery of CFTR mRNA, e.g., via inhalation, and that once delivered, the codon optimized CFTR mRNA can effectively express human CFTR protein.

Example 11: *In Vivo* Dose Escalation Study

[0252] *Dose Escalation of PEI encapsulated mRNA aerosol delivery to the lungs of pigs.* Aerosol administration of a combination of firefly luciferase (FFL) SNIM RNA and codon optimized human CFTR (CO-CFTR) SNIM RNA at varying concentrations to pig lungs was established by a stepwise experimental procedure. In a first step the FFL/CO-CFTR SNIM RNA

formulation was nebulized to anaesthetized pigs during controlled ventilation. In a second step, the animals were sacrificed by bolus injection of pentobarbital (100 mg/kg of body weight) and potassium chloride via the lateral ear vein after sedation 24 hours after aerosol administration was completed. Lungs were excised and sliced to approximately 1 cm thick tissue specimens. For measurement of luciferase activity, tissue specimens were incubated in a medium bath comprising D-Luciferin substrate and subjected to ex vivo luciferase BLI. After BLI, samples from luciferase-positive and luciferase-negative regions were taken for histopathology, immunohistochemistry and in situ hybridization. The residual specimens were shock-frozen in liquid nitrogen and subsequently stored at -80°C until analysis by IP/WB and Elisa.

[0253] *Messenger RNA Synthesis.* In the example, codon optimized human cystic fibrosis transmembrane conductance regulator (CO-CFTR) SNIM RNA, codon-optimized FFL mRNA SNIM RNA were synthesized by *in vitro* transcription from plasmid DNA templates using standard methods.

[0254] *Experimental Design.* Pigs of the German Landrace were obtained from Technical University Munich, Weihenstephan, Germany. The pigs had a body weight ranging from 35-90 kg. The study was designed using both age and weight-matched pigs to control for variability. A single cohort of 6 pigs (3 male and 3 female) was established for each experimental group of the 4-arm study. The first cohort was treated with water for injection (WFI) alone, which was administered using a Aeroneb mesh nebulizer. The second cohort was treated with a solution of 1 mg FFL SNIM RNA and 1 mg of codon optimized human CFTR (CO-CFTR) SNIM RNA in the PEI Formulation described below, using an Aeroneb mesh nebulizer. The third cohort received 1 mg of FFL SNIM RNA and 5 mg of codon optimized human CFTR (CO-CFTR) SNIM RNA in the PEI Formulation described below. The fourth cohort was treated with 1 mg of FFL SNIM RNA and 10 mg of codon optimized human CFTR (CO-CFTR) SNIM RNA in the PEI Formulation described below. The scheme for treatment and evaluation of each group is shown in Table 4 below.

Table 4. Experimental Design for Dose Escalation Study

Cohort	Pigs (No. and Sex)	Treatment	Formulation
1	6 (3 male + 3 female)	N/A	WFI
2	6 (3 male + 3 female)	1 mg FFL + 1 mg CO-CFTR	Branched 25 kDa PEI + WFI
3	6 (3 male +3 female)	1 mg FFL + 5 mg CO-CFTR	Branched 25 kDa PEI + WFI
4	6 (3 male + 3 female)	1 mg FFL + 10 mg CO-CFTR	Branched 25 kDa PEI + WFI

[0255] *mRNA - PEI formulation.* An exemplary standardized formulation procedure described below was performed just before treatment of the animals.

Materials:

Syringe pump (Mixing device):
Manufacturer: KD Scientific
Type: KDS-210-CE

Syringe:
Manufacturer: B.Braun
Type: Omnifix, 20mL or 30 mL / Luer Lock Solo
Ref.: 4617207V

Tubing:
Manufacturer: B.Braun
Type: Safeflow Extension Set
Ref.: 4097154

Needle:
Manufacturer: B.Braun
Type: Sterican, 20G x 1 ½ ''
Ref.: 4657519

Mixing valve:
Manufacturer: B.Braun
Type: Discofix C 3SC
Ref.: 16494C

Water for injection:
Manufacturer: B.Braun

Type: Aqua
Ref.: 82423E

[0256] Examplary method for the preparation of polyplexes containing 1mg hCFTR SNIM RNA and 1 mg FFL SNIM RNA N/P 10 in a volume of 8mL: 3mL water for injection and 3mL RNA stock solution (c: 1mg/mL in water; 1.5mL FFL mRNA + 1.5mL CFTR mRNA) were filled into a 15mL falcon tube. In a second falcon tube 5.61mL water for injection were mixed with 0.39mL brPEI stock solution (c: 10mg/mL in water). Two 20mL syringes were fixed in the mixing device. Each of them was connected to a needle via a tubing. One syringe was filled with the RNA- and the other with the PEI-solution using the withdrawal function of the syringe pump. (Settings: Diameter: 20.1mm, Flow: 5mL/min, Volume: 5.9mL). The needles were removed and the tubes connected to the mixing valve. It was important to connect the syringe containing the RNA-solution to the angled position of the valve. To control the outlet diameter, a needle was connected. The mixing was performed using the infusion function of the syringe pump (Settings: Diameter: 20.1mm, Flow: 40mL/min, Volume: 5.8mL). To achieve a reproducible polydispersity index, the samples were fractionated manually during mixing. The first few µL until the flow was stable (100-200µL) and the last few µL sometimes containing air bubbles were collected in a separate tube. The mixture was incubated for 30min at room temperature for polyplex formation and afterwards stored on ice. For different doses, the parameters were modified and adapted as shown in Table 5.

Table 5: Exemplary volumes and settings for different mixing volumes

	mRNA component			PEI component		
Cohort	V (FFL SNIM RNA 1 mg/mL) (mL)	V (hCFTR SNIM RNA 1mg/mL) (mL)	Water (mL)	V (brPEI stock; 10 mg/mL) (mL)	Water (mL)	Aerosolized volume (ml)
2	1.5	1.5	3	0,39	5.61	8
3	1.17	5.83	7	0,91	13.09	24
4	1.09	10.91	12	1,56	22.44	44

Cohort	V(withdrawal) (ml)	V(infusion) (ml)
2	5,9	5,8
3	13,9	13,8
4	23,9	23,8

V(withdrawal) and V(infusion) designate the setting on the syringe pump for aspiration and dispension, respectively, of the mRNA and PEI components.

[0257] *Transfection of HEK cells to check the functionality of the nebulized complexes.* Post nebulization, an aliquot of complexes (80µl) was used to transfect HEK cells. One day prior to transfection, $1x10^6$ cells were plated in 6 well plates. At the day of transfection, medium was removed from the cells, cells were washed with PBS once following which 80µl of complexes together with 920µl of serum free MEM medium was added per well. For each complex, three replicate wells were prepared. The cells were incubated with the complexes for 4 hours under standard cell culture conditions. At the end of incubation, complex containing medium was removed and serum containing MEM medium (1ml) was added per well. Plates were incubated under standard cell culture conditions. At 24 hours post transfection, protein lysates were prepared using the same protocol and buffers used for animal tissues with exclusion of homogenization step. Cells from three wells were pooled for analysis. Expression of human CFTR was detected using immunoprecipitation with R24.1 antibody (R&D Systems) and

Western Blot with a combination of 217, 432 and 596 antibodies (all from Cystic Fibrosis Consortium, University of Pennsylvania, PA, USA). hCFTR could be detected for all of complexes nebulized in pigs (see Figures 54-57).

[0258] *Aerosol Application.* The aerosol (WFI alone 44 ml; modified mRNA PEI formulation in WFI: 8, 24 and 44 ml) was nebulized and inhaled into the anaesthetized pig via an Aeroneb® mesh nebulizer. Sedation in pigs was initiated by premedication with azaperone 2 mg/kg body weight, ketamine 15 mg/kg body weight, atropine 0.1 mg/kg body weight and followed by insertion of an intravenous line to the lateral auricular vein. Pigs were anesthetized by intravenous injection of propofol 3-5 mg/kg body weight as required. Anesthesia was maintained by isoflurane (2-3%) with 1% propofol bolus injection at 4 to 8 mg/kg body weight to enhance anesthesia as required. Duration of the anesthesia was approximately 1-3 hrs. Pigs were sacrificed with bolus injection of pentobarbital (100 mg/kg body weight) and potassium chloride via the lateral ear vein 24 hours after completion of aerosolization. Lungs were excised and tissue specimens were collected from various lung regions. The stored samples were subjected to different assessment methods such as bioluminescence, histopathology, IP/Western Blot and Elisa.

[0259] *Bioluminescence Analysis.* For measurement of luciferase activity tissue specimens were either homogenized and analyzed in a tube luminometer or incubated in a medium bath comprising D-Luciferin substrate and subjected to *ex vivo* luciferase BLI. The data illustrates that a strong bioluminescence signal was observed for each of cohorts 2-4 (1 mg, 5, mg and 10 mgs respectively), when compared to control lung tissue samples from cohort 1 (WFI vehicle control) (Figures 35-38).

[0260] *CFTR Expression Analysis by Western Blot and Immunohistochemistry.* FFL positive tissues samples were excised (minimum of 10 samples for each pig within a cohort) and analyzed by immuneprecipitation / Western blot (IP-WB) and immunohistochemistry for human CFTR. Briefly, protein lysates were prepared from pig lungs as follows: Between 300-400mg of lung tissue was used for analysis. The tissue was homogenized in basis buffer (20mM Tris, 150

mM NaCl, pH 8.0) containing protease inhibitors using LysingMatrixA (MPBiomedicals, Ref:6910-500) and Homogeniser "FastPrep24" (MP Biomedicals). The whole tissue mix was transferred to a new 2ml safe lock pre-cooled Eppendorf tube and 25µl iodoacetamide (Sigma: I6125) and 1µl Omni cleave (1:5 diluted in Omni cleave buffer) (Epicenter: OC7810K) was added. The samples were then incubated on ice for 5 minutes, followined by addition of 26µl of 10%SDS solution. Samples were further incubated at 4°C for 60 min on a shaker. Post incubation, 260µl of lysis buffer (850µl basis buffer + 10% TritonX-100 + 5% Sodium deoxcholate) was added to the samples and they were incubated at 4°C on a shaker for 90 minutes. Finally, protein lysates were centrifuged at 13,000 rpm at 4°C for 10-20 min and the supernatant was transferred into a new Eppendorf tube. Protein concentration was quantified using the BCA Protein Assay (Pierce). Samples were aliquoted containing 10mg of total protein and end volumes were adjusted with basis buffer to 1 ml per sample. Based on the data presented in Example 6, immunoprecipitation of CFTR was carried out using antibody R24.1 and was followed by Western blot immunodetection of CFTR using a triple combination of three different antibodies obtained from Cystic Fibrosis Consortium, University of Pennsylvania, PA, USA (antibodies 217, 432, 596). To control for intra-group variability among different animals and variability in CFTR expression, the markers band in protein size standard corresponding to 150 kDa was set as reference and the band instensities of different groups were normalized to this value. As demonstrated in Figure 39, only 16% of the tissues sample analysed form the control pigs of cohort 1 resulted in a CFTR expression level greater than baseline. In contrast, cohorts 3 and 4, which represent the 5 mg and 10 mg treatment groups respectively, each resulted in greater than 30 % of their lung tissue samples testing positive for a CFTR expression level higher than baseline (Figure 39). Furthermore, the increase in CFTR expression observed within cohorts 3 and 4, was almost two fold greater than that of control.

[0261] Analysis of CFTR immunohistochemistry was performed by quantification of CFTR-positive bronchi and bronchioles. A bronchus / bronchiole was regarded as positive if at least one epithelial cell was detected within the epithelial cell layer displaying a clear membrane-

localizaed CFTR signal. A representative image of a "positive" sample is depicted in Figure 40. Coditions for CFTR immunohistochemistry were optimized by assessing specificity of available antibodies against CFTR utilizing single antibody or combinations of up to three antibodies respectively. Clear CFTR-specific signals were observed after incubation of antibody 596. The data demonstrates, that CFTR-positive epithelial cells were detected in lung tissue sections of all four cohorts, demonstrating detection of human and porcine CFTR by the immunohistochemistry procedure (Figure 41 and 45). While low (Figure 42), medium (Figure 43) and high (Figure 44) CFTR expression levels were observed for cohort 3, the overall finding demonstrates that the 5 mg treatment of codon optimized human CFTR SNIM RNA resulted in a greater number of CFTR positive cells and overall CFTR signal intensity compared to vehicle control. The data also illustrates a yet further enhancement of CFTR expression following 10 mg treatment, thus demonstrating a clear dose response effect (Figure 45). Quantification of absolute and relative numbers of CFTR-positive bronchi / bronchioles further support these findings, revealing a significant higher numbers in animals which were treated with 5 or 10 mg of human CFTR SNIM RNA compared to vehicle control (Figure 46). Indicating an overall elevation in CFTR expression levels following treatment with human CFTR SNIM RNA.

[0262] *CFTR Expression Analysis by In Situ Hybridization (ISH).* FFL positive tissues samples were excised (minimum of 10 samples for each pig within a cohort) and subjected to manual *in situ* hybridization analysis using the RNAscope® (Advanced Cell Diagnostic) "ZZ" probe technology. Probes were generated based on the codon-optimized sequence of codon optimized human CFTR SNIM RNA (SEQ ID NO:17). Briefly, the RNAscope® assay is an in situ hybridication assay designed to visualize single RNA molecules per cell in formalin-fixed, paraffin-embedded (FFPE) tissue mounted on slides. Each embedded tissue sample was pretreated according to the manufacturers protocol and incubated with a target specific human CFTR specific RNA probe. The hCFTR probe was shown bind CFTR, with cross reactivity to human, mouse, rat, pig and monkey. Once bound, the probe is hybridized to a cascade of signal amplification molecules, through a series of 6 consecutive rounds of amplification. The sample was then treated with an HRP-labeled probe specific to the signal amplification cassette and

assayed by chromatic visualization using 3,3'-diaminobenzidine (DAB). A probe specific for Ubiquitin C was used as the positive control (Figures 47A and 48A), while *dap*B was used as the negative control (Figures 47B and 48B). Positive CFTR signal was compared to that of untreated and vehicle control treated porcine lung tissue (Figure 49 A and B). Stained samples were visualized under a standard bright field microscope. The data demonstrates that treatment with 1 mg of codon optimized human CFTR SNIM RNA resulted in a dramatic increase in CFTR expression in both the right (A) and left (B) lung tissue of corhort 2, when compared to vehicle control (Figure 49 and 50 A &B) Furthermore, a further increase in CFTR expression was observed for the 5 mg and 10 mg treatment groups, as demonstrated by a dramatic increase staining observed within the right (A) and left (B) lung samples analyzed for cohorts 3 and 4 (Figures 51 and 52 A&B). Taken together, these data strongly supports the effective delivery of mRNA via inhalation and expression of human CFTR within both lobes of the lung and their various tissues.

[0263] *Conclusion.* The results demonstrated that both luciferase and CFTR mRNA can be effectively delivered *in vivo* to lung tissues. Luciferase expression was observed throughout various tissue samples collected from different regions within both the right and left lobs of the lungs. Thus suggestions, that nebulization is an effective approach for administering mRNA and results in fairly uniform distribution. Furthermore, in addition to luciferase, CFTR mRNA was also efficiently delivered to the lungs, resulting in enhanced protein expression. Expression and protein activity was verified by IP-WB, immunohistochemistry and in situ hybridization. Each approach clearly demonstrated a dose dependent increase in mRNA delivery and CFTR expression and/or activity, within the tissues of the lung. Taken together, the experiments highlight the overall practicality and feasibility for delivering CFTR mRNA to the lung of a human subject and demonstrate the effectiveness of *in vivo* CFTR protein production for therapeutic use.

Example 12: *In Vivo* Expression in the Lung

[0264] This example desmonstrates successful *in vivo* expression in the lung following aerosol delivery of mRNA-loaded nanoparticles. All studies were performed using pigs of the German Landrace, obtained from Technical University Munich, Weihenstephan, Germany. The pigs had a body weight ranging from 35-90 kg. FFL/CO-CFTR-C-His10 mRNA formulation or vehicle control was introduced using a Pari jet nebulizer. Pigs were sacrificed and perfused with saline, after a predetermined period of time, to allow for protein expression from the mRNA.

[0265] *Messenger RNA Synthesis.* In the example, codon optimized fire fly luciferase (CO-FFL) mRNA was synthesized by *in vitro* transcription from plasmid DNA templates.

[0266] *cKK-E12 Formulation.* For the lipid-based nanoparticle experiment, a lipid formulation was created using 1 mg FFL + 9 mg of CO-CFTR-C-His10 mRNA encapsulated in a formulation of cKK-E12:DOPE:Chol:PEGDMG2K (relative amounts 40:30:25:5 (mol ratio). The solution was nebulized to the Pig lungs using the indicated nebulizer.

[0267] *Aerosol Application.* The aerosol (Saline or CO-FFL cKK-E12 formulation) was nebulized and inhaled into the anaesthetized pig. Sedation in pigs was initiated by premedication with azaperone 2 mg/kg body weight, ketamine 15 mg/kg body weight, atropine 0.1 mg/kg body weight and followed by insertion of an intravenous line to the lateral auricular vein. Pigs were anesthetized by intravenous injection of propofol 3-5 mg/kg body weight as required. Anesthesia was maintained by isoflurane (2-3%) with 1% propofol bolus injection at 4 to 8 mg/kg body weight to enhance anesthesia as required. Duration of the anesthesia was approximately 1-3 hrs. Pigs were killed with bolus injection of pentobarbital (100 mg/kg body weight) and potassium chloride via the lateral ear vein. Lungs were excised and tissue specimens were collected from various lung regions followed by incubation in cell culture medium overnight. The stored samples were subjected to bioluminescence detection.

[0268] *Bioluminescence Analysis.* For measurement of luciferase activity tissue specimens were either homogenized and analyzed in a tube luminometer or incubated in a medium bath comprising D-Luciferin substrate and subjected to *ex vivo* luciferase BLI. A strong bioluminescence signal was observed for each of the (A) FFL/CO-CFTR-C-His10 mRNA

treated pigs, when compared to (B) control lung tissue samples from control pigs (Saline vehicle control) (Figure 53 A&B).

[0269] These data illustrate that FFL/CFTR mRNA were successfully delivered to and expressed in the lung by aerosol administration.

BRIEF DESCRIPTION OF SEQUENCES

SEQ ID NO 1. Wild-type CFTR amino acid sequence.

SEQ ID NO 2. Wild-type CFTR mRNA coding sequence.

SEQ ID NO 3. Non-naturally occurring CFTR mRNA coding sequence #1.

SEQ ID NO 4. CFTR mRNA 5'-UTR.

SEQ ID NO 5. CFTR mRNA 3'-UTR #1.

SEQ ID NO 6. FFL 5' UTR.

SEQ ID NO 7. FFL coding sequence.

SEQ ID NO 8. FFL 3' UTR.

SEQ ID NO 9. Non-naturally occurring CFTR mRNA coding sequence #2.

SEQ ID NO 10. Non-naturally occurring CFTR mRNA coding sequence #3.

SEQ ID NO 11. Non-naturally occurring CFTR mRNA coding sequence #4.

SEQ ID NO 12. Non-naturally occurring CFTR mRNA coding sequence #5.

SEQ ID NO 13. Non-naturally occurring CFTR mRNA coding sequence #6.

SEQ ID NO 14. Non-naturally occurring CFTR mRNA coding sequence #7.

SEQ ID NO 15. Codon Optimized Human CFTR C-terminal His_{10} fusion mRNA coding sequence.

SEQ ID NO 16. Codon Optimized Human CFTR mRNA coding sequence with a Growth Hormone Leader Sequence.

SEQ ID NO 17. Codon Optimized Human CFTR mRNA

SEQ ID NO 18. mRNA Leader Sequence #1

SEQ ID NO 19. mRNA Leader Sequence #2

SEQ ID NO 20. CFTR mRNA 3'-UTR #2.

SEQ ID NO:1

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SEQ ID NO:12
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SEQ ID NO: 13
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SEQ ID NO: 14
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SEQ ID NO: 15

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SEQ ID NO:16

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SEQ ID NO: 17
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CACUUAAGCAGGCGUUUGCCGACUGCACGGUGAUUCUCUGUGAGCAUCGUAUCGAGGCCA
UGCUCGAAUGCCAGCAAUUUCUUGUCAUCGAAGAGAAUAAGGUCCGCCAGUACGACUCCA
UCCAGAAGCUGCUUAAUGAGAGAUCAUUGUUCCGGCAGGCGAUUUCACCAUCCGAUAGG
GUGAAACUUUUUCCACACAGAAAUUCGUCGAAGUGCAAGUCCAAACCGCAGAUCGCGGCC
UUGAAAGAAGAGACUGAAGAAGAAGUUCAAGACACGCGUCUUUAA (SEQ ID NO:17)

SEQ ID NO: 18
AUGGCCACUGGAUCAAGAACCUCACUGCUGCUCGCUUUUGGACUGCUUUGCCUGC
CCUGGUUGCAAGAAGGAUCGGCUUUCCCGACCAUCCCACUCUCC (SEQ ID NO:18)

SEQ ID NO: 19
AUGGCAACUGGAUCAAGAACCUCCCUCCUGCUCGCAUUCGGCCUGCUCUGUCUCC
CAUGGCUCCAAGAAGGAAGCGCGUUCCCCACUAUCCCCCUCUCG (SEQ ID NO:19)

SEQ ID NO: 20
CGGGUGGCAUCCCUGUGACCCCUCCCCAGUGCCUCUCCUGGCCCUGGAAGUUGCC
ACUCCAGUGCCCACCAGCCUUGUCCUAAUAAAAUUAAGUUGCAUCAAGCU

Equivalents

[0270] The specification is most thoroughly understood in light of the teachings of the references cited within the specification. The embodiments within the specification provide an illustration of embodiments of the invention and should not be construed to limit the scope of the invention. The skilled artisan readily recognizes that many other embodiments are encompassed by the invention. All publications and patents cited in this disclosure are incorporated by reference in their entirety. To the extent the material incorporated by reference contradicts or is inconsistent with this specification, the specification will supersede any such material. The citation of any references herein is not an admission that such references are prior art to the present invention.

[0271] Unless otherwise indicated, all numbers expressing quantities of ingredients, reaction conditions, and so forth used in the specification, including claims, are to be understood as approximations and may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should be construed in light of the number of significant digits and ordinary rounding approaches. The recitation of series of numbers with differing amounts of significant digits in the specification is not to be construed as implying that numbers with fewer significant digits given have the same precision as numbers with more significant digits given.

[0272] The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one." The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or."

[0273] Unless otherwise indicated, the term "at least" preceding a series of elements is to be understood to refer to every element in the series. Those skilled in the art will recognize, or

be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

[0274] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described.

[0275] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

[0276] Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

CLAIMS

1. A pharmaceutical composition comprising an mRNA-loaded nanoparticle,

wherein the mRNA is an *in vitro* transcribed mRNA and has a coding sequence at least 80% identical to SEQ ID NO: 3, and

wherein the mRNA encodes a human cystic fibrosis transmembrane conductance regulator (CFTR) protein comprising the amino acid sequence of SEQ ID NO:1, wherein the pharmaceutical composition is to be administered to the lung by nebulization or aerosolization.

2. The pharmaceutical composition of claim 1, wherein the mRNA has the coding sequence at least 90% identical to SEQ ID NO: 3.

3. The pharmaceutical composition of claim 1, wherein the mRNA has the coding sequence 100% identical to SEQ ID NO: 3.

4. The pharmaceutical composition of claim 1, wherein the mRNA comprises a 5' untranslated region (UTR) and/or a 3' UTR.

5. The pharmaceutical composition of claim 4, wherein the 5'-UTR comprises SEQ ID NO: 4 and/or the 3'-UTR comprises SEQ ID NO: 5.

6. The pharmaceutical composition of claim 4, wherein the mRNA further comprises a poly-A tail.

7. The pharmaceutical composition of claim 6, wherein the poly-A tail is of at least 70, 100, 120, 150, 200, or 250 residues in length.

8. The pharmaceutical composition of claim 4, wherein the mRNA further comprises a 5' cap.

9. The pharmaceutical composition of claim 1, wherein the mRNA comprises at least one nonstandard nucleobase.

10. The pharmaceutical composition of claim 9, wherein the nonstandard nucleobase is chosen from one or more of 5-methyl-cytidine, pseudouridine, and 2-thio-uridine.

11. The pharmaceutical composition of claim 1, wherein the administration of the composition results in human CFTR protein expression in epithelial cells of the lung.

12. The pharmaceutical composition of claim 1, wherein the composition further comprises a pharmaceutically acceptable carrier.

13. The pharmaceutical composition of claim 1, wherein the nanoparticle comprises one or more organic cations.

14. The pharmaceutical composition of claim 13, wherein the one or more organic cations are selected from the group consisting of polyethyleneimine (PEI), protamine, PEGylated protamine, poly-L-lysine (PLL), PEGylated PLL, a cationic lipid and combinations thereof.

15. The pharmaceutical composition of claim 1, wherein the nanoparticle comprises a polymer.

16. The pharmaceutical composition of claim 15, wherein the polymer comprises branched PEI with a molecular weight ranging from 10 kDa to 40 kDa.

17. The pharmaceutical composition of claim 1, wherein the nanoparticle is a liposome.

18. Use of a composition comprising an mRNA-loaded nanoparticle, wherein the mRNA is an *in vitro* transcribed mRNA and has a coding sequence at least 80% identical to SEQ ID NO: 3, and

wherein the mRNA encodes a human cystic fibrosis transmembrane conductance regulator (CFTR) protein comprising the amino acid sequence of SEQ ID NO:1 in the preparation of a medicament for the treatment of cystic

fibrosis wherein the medicament is to be administered via nebulization or aerosolization.

19. The use of claim 18, wherein the mRNA has the coding sequence at least 90% identical to SEQ ID NO: 3.

20. The use of claim 18, wherein the administration of the medicament results in expression of human CFTR protein in the lung of a mammal.

21. The use of claim 20, wherein the expression of human CFTR protein is in epithelial cells of the lung.

22. The use of claim 18, wherein the mRNA has the coding sequence 100% identical to SEQ ID NO: 3.

23. The use of claim 18, wherein the mRNA comprises a 5' untranslated region (UTR) and/or a 3' UTR.

24. The use of claim 23, wherein the 5'-UTR comprises SEQ ID NO: 4 and/or the 3'-UTR comprises SEQ ID NO: 5.

25. The use of claim 23, wherein the mRNA further comprises a poly-A tail.

26. The use of claim 25, wherein the poly-A tail is of at least 70, 100, 120, 150, 200, or 250 residues in length.

27. The use of claim 23, wherein the mRNA further comprises a 5' cap.

28. The use of claim 18, wherein the mRNA comprises at least one nonstandard nucleobase.

29. The use of claim 28, wherein the nonstandard nucleobase is chosen from one or more of 5-methyl-cytidine, pseudouridine, and 2-thio-uridine.

30. The use of claim 18, wherein the composition further comprises a pharmaceutically acceptable carrier.

31. The use of claim 18, wherein the nanoparticle comprises one or more organic cations.

32. The use of claim 31, wherein the one or more organic cations are selected from the group consisting of polyethyleneimine (PEI), protamine, PEGylated protamine, poly-L-lysine (PLL), PEGylated PLL, and a cationic lipid.

33. The use of claim 18, wherein the nanoparticle comprises a polymer.

34. The use of claim 33, wherein the polymer comprises branched PEI with a molecular weight ranging from 10 kDa to 40 kDa.

35. The use of claim 18, wherein the nanoparticle is a liposome.

36. An *in vitro* transcribed mRNA comprising a coding sequence at least 80% identical to SEQ ID NO: 3 for use in treating cystic fibrosis in a mammal, wherein the mRNA comprises a coding sequence which encodes a human cystic fibrosis transmembrane conductance regulator (CFTR) protein of SEQ ID NO:1, and wherein the mRNA is to be administered to the lung of the mammal by nebulization or aerosolization.

37. The mRNA for use according to claim 36, wherein the coding sequence is at least 90% identical to SEQ ID NO: 3.

38. The mRNA for use according to claim 36, wherein the coding sequence is 100% identical to SEQ ID NO: 3.

39. The mRNA for use according to any one of the preceding claims, wherein the mRNA comprises a 5' untranslated region (UTR), a 3' UTR, a signal peptide coding sequence, a cap structure and/or a tail structure.

40. The mRNA for use according to claim 39, wherein the 5'-UTR comprises SEQ ID NO: 4 and/or the 3'-UTR comprises SEQ ID NO: 5.

41. The mRNA for use according to claim 39 or 40, wherein the tail structure is a poly-A tail of at least 70, 100, 120, 150, 200, or 250 residues in length.

42. The mRNA for use according to any one of claims 39-40, wherein the cap structure is a cap1 structure.

43. The mRNA for use according to claim 36, wherein the mRNA comprises one or more nonstandard nucleotide(s),

optionally wherein the one or more nonstandard nucleotide(s) is/are chosen from 5-methyl-cytidine, pseudouridine, and 2-thio-uridine.

44. The mRNA for use according to claim 36, wherein the human CFTR protein is expressed in the epithelial cells of the lung.

45. The mRNA for use according to claim 36, wherein the mRNA is to be administered with a carrier.

46. The mRNA for use according to claim 45, wherein the carrier comprises an organic cation, such as a cationic lipid or a cationic organic polymer.

47. The mRNA for use according to claim 46, wherein the cationic organic polymer is selected from the group consisting of polyethyleneimine (PEI), protamine, PEGylated protamine, poly-L-lysine (PLL), and PEGylated PLL.

48. The mRNA for use according to claim 47, wherein the PEI is branched PEI with a molecular weight ranging from 10 kDa to 40 kDa.

49. The mRNA for use according to any one of claims 45-48, wherein the carrier is a liposome.