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Isogenic cell models of cystic fibrosis-causing variants in natively expressing pulmonary epithelial cells

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ABSTRACT

Background: Assessment of approved drugs and developmental drug candidates for rare cystic fibrosis (CF)-causing variants of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) requires abundant material from relevant models.
Methods: Isogenic cell lines harboring CFTR variants in the native genomic context were created through the development and utilization of a footprint-less, CRISPR/Cas9 gene editing pipeline in 16HBE14o- immortalized bronchial epithelial cells.
Results: Isogenic, homozygous cell lines for three CFTR variants (F508del and the two most common CF-causing nonsense variants, G542X and W1282X) were established and characterized. The F508del model recapitulates the known molecular pathology and pharmacology. The two models of nonsense variants (G542X and W1282X) are sensitive to Nonsense Mediated mRNA Decay (NMD) and responsive to reference compounds that inhibit NMD and promote ribosomal readthrough.
Conclusions: We present a versatile, efficient gene editing pipeline that can be used to create CFTR variants in the native genomic context and the utilization of this pipeline to create homozygous cell models for the CF-causing variants F508del, G542X, and W1282X. The resulting cell lines provide a virtually unlimited source of material with specific pathogenic mutations that can be used in a variety of assays, including functional assays.

1. Introduction

Mutations in the CFTR gene cause the monogenetic disease cystic fibrosis (CF) [1–3]. Nearly 70% of disease-causing alleles are the F508del variant [4]. None of the several hundred other known disease-causing alleles exceed a frequency of 2.6% [4]. Evaluating the responsiveness of specific CF-causing CFTR variants to approved, available drugs and novel drug discovery require cells expressing rare, CF-causing variants. However, existing model systems to study CF have significant liabilities. Patient-derived material, i.e. human bronchial epithelial (hBE) cells isolated from lung explant tissue or nasal epithelial (hNE) cells, are a limited resource and this is particularly acute for cells homozygous for a given rare mutation. An alternative model, immortalized cell lines heterologously overexpressing CFTR variants from cDNA [5], have fundamentally limited relevance. These systems lack the genomic context/chromatin environment, the native CFTR promoter, and intronic sequences.

G542X and W1282X, two nonsense mutations causing premature termination codons (PTCs) in the CFTR gene, are the second and sixth most common pathogenic variants [4], respectively, but there is no therapy for any nonsense mutation approved or in later stage clinical development. These nonsense mutations cause at least two defects: 1) cells harboring these mutations produce very little or no full length functional CFTR protein and 2) Nonsense Mediated mRNA Decay (NMD) degrades the mRNA coding for CFTR [6,7]. A common trigger for NMD is the presence of exon-junction protein complexes (EJCs) downstream from PTCs on newly spliced mRNA [8]. Drug discovery approaches for novel nonsense mutation therapies can target both PTC readthrough and NMD inhibition because these two mechanisms should complement each other for greater combined efficacy [7,9]. However, PTC containing CFTR mRNA expressed from cDNA is not a substrate of the EJC-mediated NMD pathway; therefore, such cell lines have limited utility in the discovery and exploration of potential therapeutics.

Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; PTC, premature termination codon; NMD, non-sense mediated mRNA decay; EJC, exon-junction complex; AUC, area under the curve; SNP, single nucleotide polymorphism; HDR, homology directed repair; NHEJ, non-homologous end joining; CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated system.

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CF-causing nonsense variants, as well as variants with mutations outside of the open reading frame (such as intronic splice mutations) require more faithful models of native CFTR biogenesis for therapeutic development.

With the flexibility and speed of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated system (Cas), it is now feasible to create cell lines that express CFTR variants in the native genomic context using gene editing. CRISPRs are part of naturally occurring bacterial immune responses adapted for directed gene editing in mammalian cells [10,11]. In the CRISPR system that utilizes Cas9 from *S. pyogenes*, a guide RNA directs the Cas9 endonuclease to a DNA target at which the Cas9 creates a double stranded break. Non-homologous end joining (NHEJ) or homology directed repair (HDR) mend the double stranded break. NHEJ creates small, imprecise insertions or deletions, while HDR uses a donor template to precisely repair the DNA. The rates of HDR are generally low compared to that of NHEJ [12], although cell type, gene locus, and nuclease can all affect HDR and NHEJ rates [13]. The lower frequency HDR pathway is required to precisely create CF-causing variants. The CRISPR/Cas9 system has been used to create gene knock outs, generate disease models, affect chromatin, screen for genes of interest, and more, in a variety of organisms including bacteria, zebrafish, mice, and human cells [12]. However, significant hurdles remain. In particular, for modeling CF-causing variants, a limited number of cell lines establish sufficient transepithelial resistance when grown on filter supports to assess CFTR function [14], and the editing platforms and protocols designed for other cell types, such as the commonly used HEK-293 cells, do not always translate well. Because of these challenges, CF model systems have not yet fully exploited the potential of CRISPR technology.

Herein, we describe an adaptable CRISPR-based gene editing pipeline to efficiently and seamlessly (without antibiotic selection, additional mutations, or residual DNA elements such as loxP sites) create isogenic models of CF-causing variants in the genomic context of 16HBE14o- [15] immortalized bronchial epithelial cells. Additionally, we describe the utilization of this pipeline to establish three cell lines homozygous for the CFTR variants F508del, G542X, or W1282X. The resulting cell lines provide an abundant supply of cells that can be used in functional and other assays. In particular, the G542X and W1282X expressing cell lines allow efficacy evaluation of readthrough modulation. Furthermore, this pipeline enables the production of additional CFTR variants, especially those for which there are currently no therapies to overcome the basic defect.

2. Materials and methods

2.1. Reagents

Primers, ssODN HDR donor templates, quantitative real-time PCR assays (human CFTR, Hs.PT.58.28207352; human TBP, Hs.PT.58v.39858774; human GusB, Hs.PT.58v.27737538; rat GusB, Rn.PT.58.35199448), and Alt-R® CRISPR Cas9 reagents (*S.p.* Cas9 Nuclease 3NLS; crRNA; tracrRNA; and tracrRNA, ATTO^{550}) were purchased from Integrated DNA Technologies (IDT). SNP discrimination assays were custom ordered from IDT [F508del: primer 1 5′-ATTATGCCT GGCACCATTA-3′; primer 2 5′-TGATGACGCTTCTGTATCTA-3′; probe (FAM) 5′-AATATCATCTTTGGTGTTTCCT-3′; probe (HEX) 5′-AATATC ATTGGTGTTTCCTATGA-3′] or purchased from Thermo Fisher Scientific (W1282/X assay: C__32545014; G542/X assay: C__11399026; M470/V470 assay: Custom TaqMan SNP Genotyping Assay, human). WT (ND03719), W1282X (GM11723), and G542X (GM11496) samples for genotype control were purchased from Coriell Biorepository. Geneticin (G418) was purchased from Sigma-Aldrich (A-1720) and SMG1i was custom-synthesized by Kalexsyn (Kalamazoo, MI).

2.2. Cell culture

Minimum Essential Medium (MEM), Fetal Bovine Serum (FBS), Penicillin/Streptomycin, LHC-8 basal medium, Bovine serum albumin 7.5%, and TrypLE Express were purchased from Gibco. 16HBE cells were grown at 37 °C/5% CO_2 in MEM supplemented with 10% FBS and 1% Penicillin/Streptomycin. Plates/flasks were coated by incubating a thin layer of coating solution [LHC-8 basal medium, 1.34 μl/ml Bovine serum albumin 7.5%, 10 μl/ml Bovine collagen solution, Type 1 (Advanced BioMatrix), 10 μl/ml Fibronectin from human plasma (Thermo Fisher Scientific)] at 37 °C/5% CO_2 for 2–3 h followed by thorough removal of coating solution and storage at 4 °C. Cells were dissociated with TrypLE Express and centrifuged at 120 x *g* for 5 min before seeding. Medium was changed three times weekly. For cryopreservation, cells were frozen in a solution of 40% MEM, 50% FBS, and 10% DMSO. Replication deficient 16HBE14o- feeder cells were created by treating cells with 10 μg/ml mitomycin C (Sigma-Aldrich) for 60 min at 37 °C/5% CO_2. Following treatment, cells were washed with DPBS five times before being harvested and cryopreserved. Fisher Rat Thyroid (FRT) cells [5] and primary human bronchial epithelial cells [16] were cultured as previously described.

2.3. RNP assembly and nucleofection of gene editing components

Cas9 RNP was prepared fresh for each experiment. 100 μM stock tracrRNA (or tracrRNA, ATTO^{550}) and crRNA were combined at equimolar concentrations for a final duplex concentration of 50 μM, incubated at 95 °C for 5 min, and then cooled to room temperature. RNP complex was prepared by mixing tracrRNA/crRNA duplex at a 1:1.2 M ratio with Cas9 and incubating for 10–20 min at room temperature. 16HBE14o- cells were nucleofected with the Lonza 4D-Nucleofector system using the Lonza SG cell line 4D X Kit S and program CM-137. Each reaction contained approximately 200,000 16HBE14o- cells in 20 μl of supplemented SG buffer, 4 μl of RNP complex, and 1 μl of 100 μM ssODN HDR template. Guide and ssODN sequences can be found in Table 1.

2.4. Analysis of gene editing events in bulk

Genomic DNA was extracted 24 h post nucleofection using Quick Extract DNA Extraction Solution (Epicenter). Genomic DNA was quantified with a NanoDrop One spectrophotometer (Thermo Fisher Scientific). Gene editing events were analyzed by the EnGen Mutation Detection Kit (New England Biolabs) and/or digital droplet PCR (ddPCR).

2.5. Flow cytometric sorting of gene edited cells

Previously prepared replication deficient 16HBE14o- feeder cells were seeded into coated, black-walled, clear bottom 96-well plates (Thermo Scientific) at 10,000 cells/well. After the feeder cells attached, cells nucleofected 3 days prior with gene editing components were dissociated, transferred to a conical tube, and pelleted by centrifugation at 120 × *g* for 5 min, re-suspended in DPBS with Ca^{2+} and Mg^{2+} (Hyclone), and treated with DNaseI (Sigma) at a final concentration of 100 μg/ml for 15 min. Following incubation, cells were again pelleted by centrifugation at 120 × *g* for 5 min, re-suspended in ice-cold DPBS supplemented with 10% FBS, and filtered through a 5 ml polystyrene round-bottom tube with cell-strainer cap (Falcon). Each sample was split into two pools, of which one was incubated with DAPI NuncBlue Fixed Cell Stain Ready Probes Reagent (Invitrogen). Single, ATTO^{550}+ (RNP delivered), DAPI^{-} (live) cells were sorted into individual wells containing feeder cells using the Sony SH800S cell sorter. Immediately following sorting, plates were centrifuged at 120 × *g* at room temperature for 5 min. Media was replaced 7 and 14 days after sorting.

Table 1
Guide RNA, ssODN HDR donor, and sequencing primer sequences for F508del, M470, G542X, and W1282X CFTR variant creation and analysis.

CFTR variant	Guide RNA (targeting portion)	Sequence of ssODN HDR donor template(s)	Sequencing primer F	Sequencing primer R
F508del	5′ – ATTAAAGAA AATATCATCTT – 3′	5′ – CACATAGTTTCTTACCTCTTCTAGTTGGCATGCTTTGATGACGCTTCTGTATCTATATTCATCATA GGAAACACCAATGATATTTTCTTTAATGGTGCCAGGCATAATCCAGG – 3′ 5′ – CACAGTGGAAGAATTTCATTCTGTTCTCAGTTTTCCTGGATTATGCCTGGCACCATTAAAGAAAAT ATCATTGGTGTTTCCTATGATGAATATAGATACAGAAGCGTCATCAAAGCATGCCAAC – 3’	5′ – CTGAATCATGT GCCCCTTCT – 3’	5′ – TCTCTGCTGGC AGATCAATG – 3’
M470	5′ – TTCACTTCTAA TGGTGATTA – 3′	5′ – GTGAATCCTGAGCGTGATTTGATAATGACCTAATAATGATGGGTTTTATTTCCAGACTTCACTTCT AATGATGATTATGGGAGAACTGGAGCCTTCAGAGGGTAAAATTAAGCACAGTG – 3’		
G542X	5′ – GAGAAAGAC AATATAGTTCT – 3′	5′ – GCAAATGCTTGCTAGACCAATAATTAGTTATTCACCTTGCTAAAGAAATTCTTGCTCGTTGACCTC CACTCAGTGTGATTCCACCTTCTCAAAGAACTATATTGTCTTTCTCTGCAAACTTGGAGAT – 3’	5′ – ATGGAAGCCCA GTGAAGATAC – 3’	5′ – CTAGCCATAAA ACCCCAGGA – 3’
W1282X	5′ – ATTCAATAACT TTGCAACAG – 3′	5′ – TCAAATAGCAGTAAAAAATATAATTTAGTTGCCTTTTTTCTGGCTAAGTCCTTTTGCTCACCTGTG GTATCACTCCAAAGGCTTTCCTTCACTGTTGCAAAGTTATTGAATCCCAAGACACACCATC – 3’	5′ – GCTTCTGGCTT GAGCCTATG – 3’	5′ – TGAGAAAACTG CACTGGAGAAA – 3’

2.6. Identification of gene edited clones

Approximately 14–17 days after sorting, cells were incubated with media containg 10% AlamarBlue (Thermo Fisher Scientific) for 24 h before obtaining fluorescence measurements for each well with a Synergy H4 hybrid reader (BioTek) (excitation: 556/13.5; emission 596/13.5; optics: bottom). Wells with greater than background fluorescence measurements were passaged, as described above, into matching wells of two 96-well plates. The next day, plate 1 was processed for gDNA with QuickExtract DNA Extraction Solution (Epicenter). Screening qPCR assays were composed of: 2× TaqMan Genotyping Master Mix (Thermo Fisher Scientific) (1× final), 40× genotyping assay (1× final), 1 μl of gDNA, and nuclease free water in 10 μl total reaction volume. Cell clones with signal in the qPCR assay for the editing event, but not WT, were identified as preliminary hits. Up to 48 preliminary hits were PCR-amplified around the region of interest (primers in Table 1), purified with the Pure Link Quick PCR Purification Kit (Invitrogen), and Sanger sequenced by Quintara Bio. Preliminary hit clones with clean sequencing for the desired edit around the region of interest (no mixed peaks, homozygous) were considered confirmed, clonal cell lines. Clonal cell lines were expanded from 96-well plate 2, cryopreserved, and characterized.

2.7. Clone selection and characterization

Parental 16HBE14o- cells were received at internal count passage (P) 1 from which they were expanded and cryopreserved. 16HBE14o- cells at P7 or greater were transfected with gene editing reagents and passaged ~9–10 times before initial cryopreservation of clones. At least 5 clones for each CFTR variant were subject to biochemical and electrophysiological characterization. All clones behaved comparably in molecular analyses and one clone was chosen for additional characterization, use, and distribution based upon transepithelial resistance and data quality (stable baseline; characteristic responses to forskolin, potentiator (VX-770), and CFTR inhibition (Inh-172)) in electrophysiological assays. The clone chosen for use and distribution was subjected to full CFTR gene sequencing by next generation sequencing (NGS) (data not shown). 16HBE gene edited (16HBEge) cells with engineered CFTR variants have been used in assays 1–25 passages after cryopreservation and have demonstrated consistent, reproducible responses even at high passage.

2.8. Western blot analyses

Western blot analyses were performed as described previously [5], except the loading control, Na+/K+-ATPase, was detected with an antibody from Santa Cruz Biotechnology (sc-48345).

2.8.1. Quantitative PCR analyses

RNA was isolated from treated cells with the Aurum Total RNA Mini Kit (Bio-Rad). One Step reverse transcriptase and quantitative PCR was performed with the iTaq Universal Probes One Step Kit. All qPCR was run on the CFX Touch Deep Well real-time PCR detection system (Bio-Rad) and analyzed with Bio-Rad CFX Maestro 1.0 software (version 4.0.2325.0418). ddPCR assays were composed of: cDNA or digested genomic DNA, 2× ddPCR Supermix for Probes (no dUTP) (1× final) (Bio-Rad), 40× assay (1× final), and nuclease free water in 20 μl total reaction volume. cDNA synthesis was performed with the iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad). Droplets were generated with QX200 Droplet Digital PCR System (Bio-Rad), cycled in the CFX Touch Deep Well real-time PCR detection system (Bio-Rad), and analyzed with the QX200 Droplet Digital PCR system Droplet Reader (Bio-Rad). Data was analyzed with the Quantasoft software version 1.7.4.

2.8.2. Electrophysiology

16HBE cells were seeded at a density of 4.5×10^5 cells cm^{-2} onto HTS Transwell 24-well filter inserts (Corning, 3378) pre-coated with human collagen type IV (Sigma-Aldrich, C5533). Cells were grown as submerged cultures in MEM (Gibco, 11095) containing 10% FBS (Hyclone, SH30071.03) and 1% Pen/Strep, and incubated at 37 °C and 5% CO_2. Cells were treated from both the basolateral and apical side with fresh medium containing either control (vehicle) or test article for the treatment time indicated in the figure legends. After a total of 7 days, 16HBE cells typically formed electrically tight epithelia with a transepithelial resistance (R_t) of 200–600 $\Omega \cdot cm^2$ and CFTR-mediated Cl^- equivalent current (I_{eq}) was determined as described below.

Prior to functional (I_{eq}) studies, MEM was replaced with fresh HEPES-buffered (pH 7.4) solutions (assay buffer). A driving force for chloride ions was established through application of a basolateral to apical chloride ion gradient (see buffer composition below). Cell plates were mounted onto an automated robotic assay platform and equilibrated at ~36 °C for 90 min. After equilibration, transepithelial voltage (V_t) and resistance (R_t) were monitored at ~5 min intervals using a 24-channel transepithelial current clamp amplifier (TECC-24, EP Design, Bertem, Belgium). Electrode potential differences for each pair of Ag/AgCl voltage electrodes were also monitored at 5 min intervals by taking voltage measurements from a control plate with matching buffer solutions and 16HBE cells that were left untreated. I_{eq} was calculated from values of V_t and R_t using Ohm's law after correcting for series resistance and (electrode) voltage offsets unrelated to V_t. I_{eq} traces are plotted as mean ± SD ($n = 3$). The first 4 data points reflect baseline I_{eq} currents prior to sequential stimulation of CFTR with forskolin (10 μM) and VX-770/ivacaftor (1 μM). The last six data points were recorded in the presence of CFTR inhibitor CFTRinh-172 (20 μM). Agonists/antagonists were pre-diluted to 10-fold concentrations in assay buffer and added to either the basolateral (forskolin) or apical (forskolin, VX-770/ivacaftor, and CFTRinh-172) side of the membrane (assay plates only). CFTR-mediated changes in I_{eq}, (i.e. delta forskolin, delta VX-770, delta CFTRinh-172, or the area under the curve (AUC) between forskolin and CFTRinh-172 addition) are used as a measure of functional CFTR surface expression or treatment-related functional rescue of mutant CFTR.

Assay buffer: CFTR-mediated transepithelial currents were recorded using a Cl^- concentration gradient. The basolateral solution contained (mM): 137 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and D-Glucose, adjusted to pH 7.4 with NaOH/HCl ($[Cl^-]_{total}$: 146.6 mM). The apical solution was matched to the basolateral except for (mM): 137 Na-gluconate replaced 137 NaCl ($[Cl^-]_{total}$: 9.6 mM).

2.8.3. Statistical analyses

Statistical significance was analyzed with Microsoft Excel using an unpaired, two-tailed, two-sample equal variance student's *t*-test.

2.8.4. Distribution

We have, and will continue to, distribute these and additional cell lines. Cells can be requested through www.cff.org.

3. Results

3.1. Engineering CFTR variants in cultured human airway cells

Of the six most common CF-causing CFTR variants, F508del, G542X, G551D, N1303K, R117H, and W1282X, only three have approved therapies [4] – F508del, G551D, and R117H. Limited availability of patient-derived cells (most of which are heterozygous, usually with F508del as the second allele), hampers ongoing drug discovery efforts targeting the G542X and W1282X variants. It is difficult to deconvolute the experimental data from heterozygous cells because a) the nonsense allele is sensitive to NMD and has low basal expression compared to the F508del allele and b) the F508del allele responds to approved therapies, which are often added to increase signal in drug screening for unknown compounds. In F508del/G542X heterozygous primary hBE cells, NMD reduces the mRNA levels of CFTR G542X to ~25% of the F508del allele (Fig. 1a). In contrast, in isogenic Fisher Rat Thyroid (FRT) cells stably expressing CFTR cDNA, G542X mRNA abundance is higher than WT or F508del mRNA (Fig. 1b). This inconsistency between primary cells and available single genotype, cDNA expressing cell lines highlights the differences between how CFTR-G542X transcripts are processed in these two systems, particularly with regard to NMD, and the fundamental limitations of cDNA models for nonsense variants. To address these challenges, a gene editing pipeline to create isogenic models of homozygous CFTR variants on both genomic CFTR alleles (data not shown) of 16HBE14o- cells [15] was developed, optimized (Fig. 1c), and used to create 16HBE gene edited cells (16HBEge) for F508del, G542X, and W1282X CFTR variants (Supplementary Fig. 1a-c).

3.2. 16HBE14o- gene edited CFTR F508del cells respond to known pharmacological agents

M470V is a known polymorphism of the CFTR gene, which is not disease-causing by itself and many disease-causing CF variants exist both in combination with M470 or V470. However, the F508del mutation occurs exclusively paired with M470. It has been found that in the context of F508del the M470 polymorphism results in a more severe channel defect [17]. The parental 16HBE14o- cells are homozygous for the V470 polymorphism, which is retained in the G542X and W1282X models. To model the actual F508del disease allele, we created a CFF-16HBEge CFTR F508del cell line also homozygous for M470 through two rounds of gene editing, first to create F508del and subsequently to create M470.

This F508del model exhibited the characteristic responses to treatment with lumacaftor (VX-809), which supports the use of gene edited 16HBE14o- models of CFTR variants for drug discovery and development. As expected, CFTR mRNA levels were unaffected by lumacaftor (VX-809) in both WT and F508del cells (Fig. 2a). Western blot showed that F508del produced reduced levels of mature (band C) CFTR, which could be increased with lumacaftor (VX-809) treatment (Fig. 2b). Electrophysiological assays remain the "gold standard" for evaluating CFTR

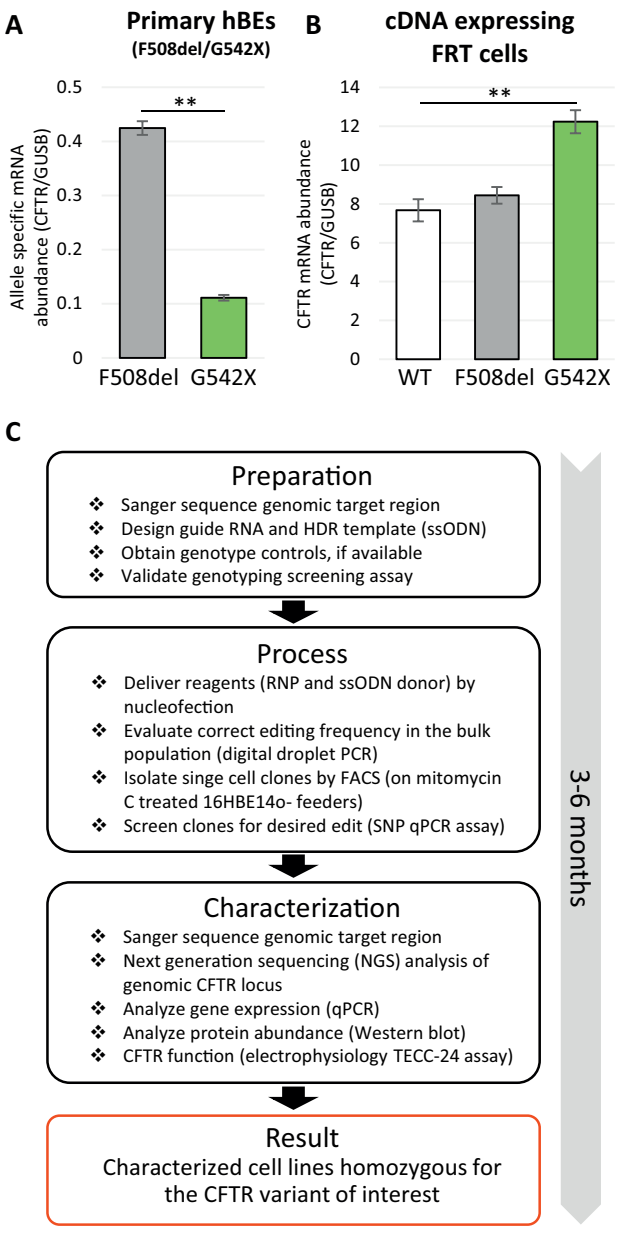

Fig. 1. Comparison of existing models to study CF. (A) Digital droplet PCR (ddPCR) analysis of CFTR gene expression of each allele normalized to GUSB of F508del/G542X primary hBE cells. (B) CFTR gene expression normalized to GusB from isogenic Fisher Rat Thyroid (FRT) cells stably expressing WT, F508del, or G542X CFTR cDNA. c) Schematic of 16HBE14o- CFTR variant gene editing pipeline. All error bars represent standard deviation ($n = 3$; unpaired *t*-test, $^*p < .05$, $^{**}p < .01$).

functional rescue in drug discovery and development. Characteristic current responses to forskolin, CFTR potentiator (ivacaftor, VX-770), and CFTR inhibitor (Inh172) of wildtype CFTR expressing, unedited parental 16HBE14o- cells were apparent in the presence, but not in the absence of a chloride gradient. The lack of an agonist/antagonist response in the absence of a chloride gradient can be explained by a diminished chloride ion driving force in symmetric chloride buffer solutions ($V_t \approx 0$ mV). Lumacaftor (VX-809) treatment also increased the function of F508del CFTR, as quantified by total area under the curve (AUC) (Fig. 2c). Electrophysiological traces are shown in Fig. 2d. 16HBE cells cultured as described above had no detectable Epithelial Na^+ Channel (ENaC) activity (data not shown). In summary, the 16HBEge CFTR F508del cell line described here exhibits the expected molecular pathology and pharmacology and suggests that these edited 16HBE14o- cells are a relevant model for therapeutic development.

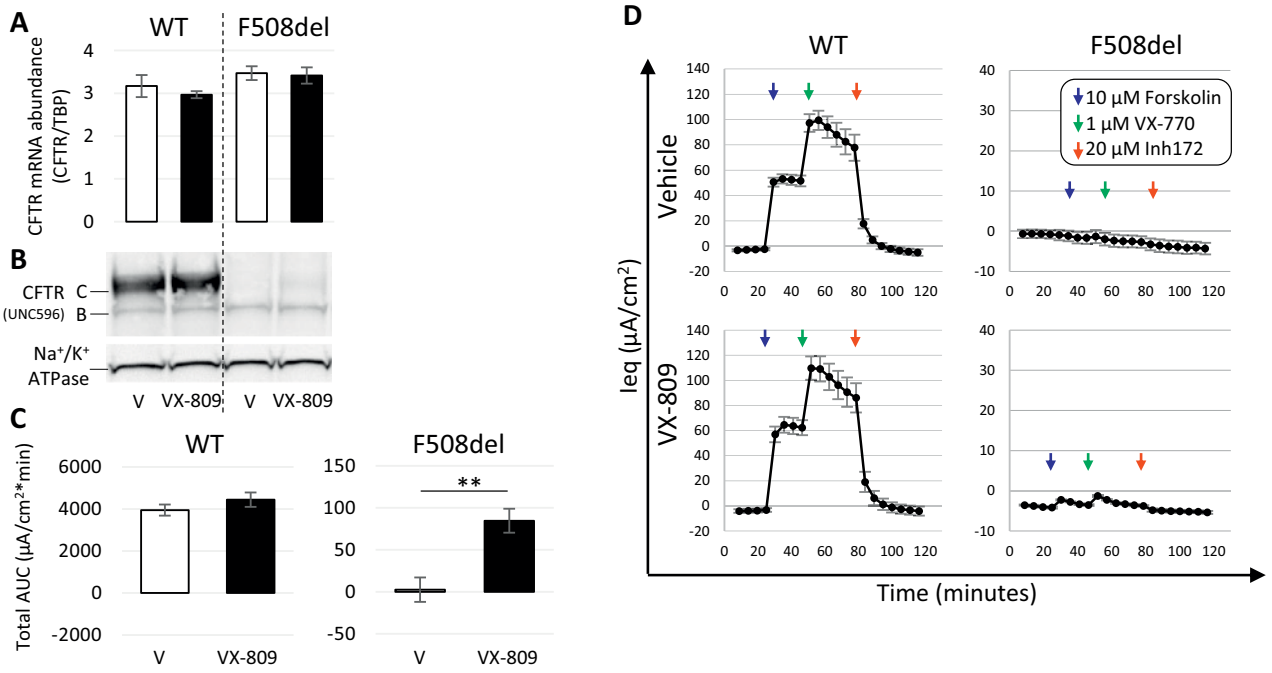

Fig. 2. CFF-16HBEge CFTR F508del cells show the characteristic response to lumacaftor treatment. (A) CFTR mRNA abundance normalized to TBP (house-keeping gene) and (B) Western blot analysis of CFTR (UNC596) and Na^+/K^+-ATPase loading control for WT and CFF-16HBEge CFTR F508del cells on filters treated with DMSO (vehicle) or lumacaftor (VX-809, 3 μM) for 24 h. (C) Total area under the curve and (D) average traces from TECC-24/Ieq assays of WT and CFF-16HBEge CFTR F508del cells. Prior to assay, cells were treated for 24 h with DMSO (vehicle) or lumacaftor (VX-809, 3 μM). Traces shown are the average of the three replicates. All error bars represent standard deviation (n = 3; unpaired t-test, *p<.05, **p<.01).

3.3. A CFTR G542X 16HBE14o- model

Successful validation of the 16HBEge CFTR F508del cell line in biochemical and functional assays suggest that CRISPR/Cas9 based gene editing of the WT CFTR gene in 16HBE14o- cells may be a viable approach to create, screen, and study rare CFTR variants in this model cell line. G542X is a class I CF mutation, defined by little or no synthesis of full-length CFTR. G542X mRNA is sensitive to degradation through the NMD pathway [6]; therefore, modeling this variant in the genomic context is of particular importance. In the absence of approved therapeutics for nonsense mutations, two reference compounds were used to evaluate the G542X (CFF-16HBEge CFTR G542X) cell line. G418 is an aminoglycoside that promotes ribosomal readthrough of premature stop codons [18] and SMG1i is a small molecule inhibitor of the NMD effector SMG1 (CFF Compound Panel N1, [19]). Normalized CFTR mRNA expression was reduced to ~20% in the G542X line compared to WT cells (Fig. 3a), which is comparable to G542X mRNA levels observed in primary hBEs (~25% of F508del mRNA, Fig. 1a). G542X CFTR mRNA abundance was significantly increased with G418 treatment. In contrast, WT CFTR mRNA abundance was significantly decreased with G418 treatment. G542X CFTR mRNA abundance was dramatically increased with SMG1i and the combination of G418 and SMG1i, while WT CFTR mRNA abundance was not affected by SMG1i. The combination of G418 and SMG1i decreased WT CFTR mRNA levels, as was observed with the G418 treatment alone (Fig. 3a). These data suggest that in the G542X cell line CFTR mutant mRNA is subject to NMD. No full length, readthrough product could be detected by Western blot. Lower levels of WT CFTR protein were observed by Western blot with G418 and G418 + SMG1i combination treatments, which is consistent with the effects of these compounds on WT mRNA abundance (Fig. 3b). In electrophysiological assays, WT CFTR function increased slightly with SMG1i treatment and SMG1i + G418 combination treatment, but declined by ~50% with G418 treatment alone. CFTR function (total AUC) was undetectable in the G542X cells, as expected. The channel function of CFTR G542X was partially restored (to ~3.4% of WT) by treatment with the reference readthrough compound G418, and further increased by the inclusion of SMG1i treatment (to ~4.4% of WT) (Fig. 3c). Electrophysiological traces are shown in Fig. 3d. Together, these data demonstrate that the CFF-16HBEge CFTR G542X model recapitulates the NMD sensitivity of the CFTR transcript, is amenable to small molecule-induced readthrough, and therefore is a relevant model for drug discovery and development.

3.4. A CFTR W1282X 16HBE14o- model

The W1282X CFTR variant is the second most common CFTR nonsense (class I) mutation and, like G542X, does not have an FDA-approved therapy that targets the basic molecular defect. In contrast to CFTR G542X, however, the truncated W1282X protein is produced, albeit at much reduced levels, and undergoes complex glycosylation indicative of trafficking to the plasma membrane, but its Cl^- transport activity is dramatically decreased compared to WT CFTR [20]. Like the CFF-16HBEge CFTR G542X cells, the W1282X cell line (CFF-16HBEge CFTR W1282X) displays significantly reduced CFTR mRNA abundance due to NMD (~20% of parental WT). G418, SMG1i, and the combination of G418 + SMG1i all increased W1282X CFTR mRNA levels (Fig. 4a). As was previously observed, WT CFTR mRNA levels were decreased with G418 treatment, were not different with SMG1i treatment, and trended down with combination G418 + SMG1i treatment. For untreated cells, truncated W1282X protein could not be detected by Western blot; however, both truncated core-glycosylated (band B) and complex glycosylated (band C) CFTR W1282X protein were detected after treatment with SMG1i, alone or in combination with G418 (Fig. 4b). As expected, in electrophysiological assays CFTR W1282X function was greatly reduced compared to WT. The function of CFTR W1282X was partially restored to ~1.6% or ~1.1% of WT by G418 or SMG1i treatment alone, respectively. Restoration of function was greatest (~2.7% of WT) with the combination treatment

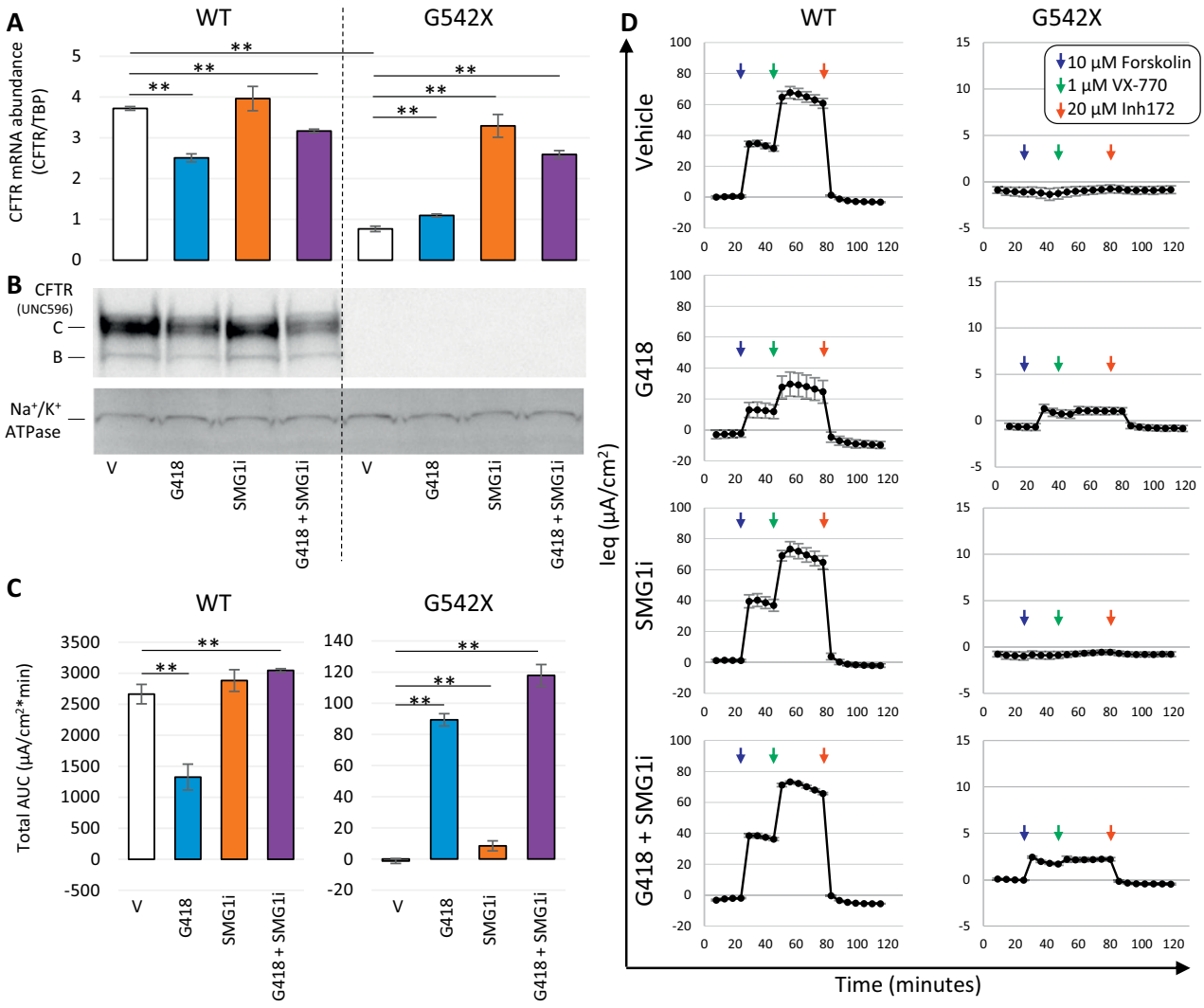

Fig. 3. Characterization of the CFF-16HBEge CFTR G542X cell line. (A) CFTR mRNA abundance normalized to TBP (house-keeping gene) and (B) Western blot analysis of CFTR (UNC596) and Na^+/K^+-ATPase loading control on WT and CFF-16HBEge CFTR G542X cells on filters treated with DMSO (vehicle), G418 (100 μM), SMG1i (0.3 μM), or a combination, as indicated, for 96 h (72 h + 24 h). (C) Total area under the curve and (D) average traces from TECC-24/Ieq assays of WT and CFF-16HBEge CFTR G542X cells. Prior to assay, cells were treated for 96 h total (72 h + 24 h) with DMSO (vehicle), G418 (100 μM), SMG1i (0.3 μM), or a combination, as indicated. Traces shown are the average of the three replicates. All error bars represent standard deviation (n = 3; unpaired t-test, *p<.05, **p<.01).

of G418 and SMG1i (Fig. 4c). Electrophysiological traces are shown in Fig. 4d. These data suggest that the CFF-16HBEge CFTR W1282X model recapitulates the NMD sensitivity of the CFTR transcript and that expression and function can both be partially restored by pharmacological intervention.

3.5. Sequencing analysis

For the ultimate confirmation of the intended genetic edits, next generation sequencing was performed on the ~189 kilobase human CFTR gene plus ~30 kilobases upstream and downstream. No off-target mutations or large insertions or deletions caused by CRISPR editing were identified within CFTR (data not shown); however, off-target effects were not analyzed genome-wide. Notably, an insertion of unknown size was identified at position (hg38) Chr7:117536118 in intron 6 of one CFTR allele in the 16HBE14o- parental cells. This insertion, which is also found in all gene-edited cell lines, contains homology to the SV40 genomic sequence, which was used in the immortalization process to create the 16HBE14o- cells [15]. Of the two CFTR alleles, the one containing this insertion appears to yield an aberrant CFTR transcript that does not generate functional CFTR. Thus, the parental 16HBE14o- cells and all derived cell lines are functionally mono-allelic.

4. Discussion

Cell lines that naturally express CFTR and harbor engineered pathogenic CFTR variants provide relevant models and a sustainable source of cell biomass for drug discovery and development efforts. In the present work, we describe an optimized pipeline for CRISPR/Cas9-mediated gene editing in 16HBE14o- cells and the utilization of this pipeline to create three isogenic CF-causing CFTR variants: F508del, G542X, and W1282X. Opportune guide/PAM sequences and strategic guide design (Table 1) enabled the seamless creation of the desired CFTR variant without the need for PAM mutations or any other synonymous codon changes. The three cell lines presented here are isogenic, as will be any additional cell lines that are made with the same starting material in this pipeline. Isogenic cell lines expressing different CFTR variants allow the engineered CFTR variant to be isolated and analyzed within a consistent genetic background. This is important because there are approximately 88 million identified variations in the human genome, mostly single nucleotide polymorphisms (SNPs) [21]. CF patients with the same CF-causing CFTR mutations can have distinct disease pathologies (e.g. pancreatic sufficient versus pancreatic insufficient), disease progression, and outcomes [22]. Consequently, primary hBE cells derived from different CF patients have genetic differences that can

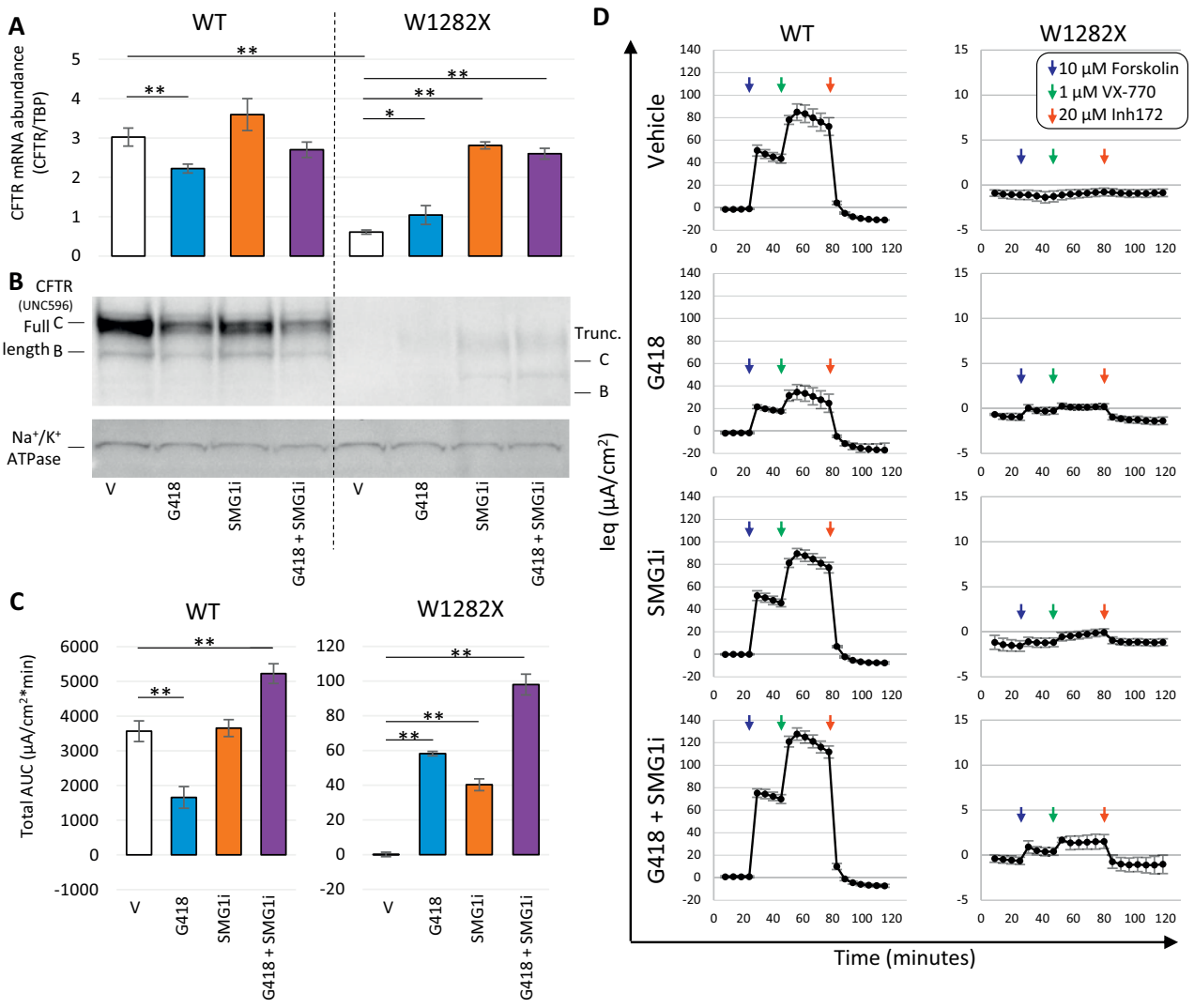

Fig. 4. Characterization of the CFF-16HBEge CFTR W1282X cell line. (A) CFTR mRNA abundance normalized to TBP (house-keeping gene) and (B) Western blot analysis of CFTR (UNC596) and Na^+/K^+-ATPase loading control on WT and CFF-16HBEge CFTR W1282X cells on filters treated with DMSO (vehicle), G418 (100 µM), SMG1i (0.3 µM), or a combination, as indicated, for 96 h (72 h + 24 h). (C) Total area under the curve and (D) average traces from TECC-24/Ieq assays of WT and CFF-16HBEge CFTR W1282X cells. Prior to assay, cells were treated for 96 h total (72 h + 24 h) with DMSO (vehicle), G418 (100 µM), SMG1i (0.3 µM), or a combination, as indicated. Traces shown are the average of the three replicates. All error bars represent standard deviation (n = 3; unpaired t-test, *p<.05, **p<.01).

affect CFTR function and CF disease, even within the context of a given CF-causing CFTR variant. The pipeline developed here could also be exploited to identify/confirm disease modifying mutations. Currently available therapies that treat the basic defect of CF are CFTR variant specific; therefore, isolating the effects of the variants and understanding how they affect CFTR physiology and pharmacology is critical for matching variants with effective therapies. The identified SV40 insertion into one CFTR allele of the 16HBE14o- cells warrants further characterization, but these cells have been an important model in CF research for decades. It appears that the cells' utility for pharmacological and functional studies is not impaired, but the insertion may result in limitations for the assessment of gene-editing therapeutic approaches targeting the CFTR gene.

The CFTR variant F508del has known pharmacological modulators; therefore, before creating rare CF-causing CFTR variants that lack approved therapies and for which there is limited ability to evaluate their relevance, we created a model for the true F508del disease allele, including the M470 polymorphism. In the absence of pharmacological modulation, the F508del variant model yielded only minimal CFTR-mediated Cl^- currents. 24-hour lumacaftor (VX-809) treatment and acute ivacaftor (VX-770) potentiation significantly increased the total CFTR-mediated Cl^- current to ~2–3% of that measured for the WT CFTR expressing parental 16HBE14o- cells (Fig. 2c–d). The Cl^- current magnitudes for parental and F508del cells reflect well the amounts of band C protein observed by Western blot (Fig. 2b and d). A very similar response was observed for several single cell-derived cell line clones, which is lower than the 7.1–25.5% range for F508del primary HBEs with lumacaftor (VX-809) reported by Van Goor and colleagues [23]. Multiple studies have shown significant variations in the magnitude of F508del CFTR functional rescue in vitro between cells from different patients [23–25]. Clinically, the observed variations for improvement in ppFEV1 in patients homozygous for F508del CFTR were even larger, with some patients experiencing no benefit at all [26]. It is impossible to know where the pharmacological response of an individual immortalized cell will fall with respect to the naturally occurring range of responses observed in cells from non-age-matched donors. The data reported here were generated using cells derived from the isolated bronchial epithelial cells of a single one-year old non-CF donor [15]. Nevertheless, the F508del variant model exhibits a significant, albeit small, reproducible response to lumacaftor (VX-809), which demonstrates that these gene edited 16HBE14o- cells are a relevant model for therapeutic testing and development.

After validating the utility of gene edited 16HBE14o- cells for drug discovery and development with the F508del model, we have initially focused our efforts on CFTR nonsense mutations. Nonsense mutations are CF-causing CFTR variants with the highest allele frequency for which there are no approved therapies that treat the basic defect, thereby representing the largest patient population with unmet medical need [4]. In the studies presented here, two common CFTR nonsense mutations were successfully created in the native gene: G542X and W1282X. Both nonsense models were evaluated with non-therapeutic reference compounds due to the current unavailability of validated, therapeutic compounds that promote readthrough or suppress NMD of CFTR PTC containing transcripts. Basally, the abundance of G542X and W1282X CFTR transcripts is ~20% of WT CFTR, consistent with sensitivity of CFTR mRNA to NMD, a major improvement over cDNA models. The reduced CFTR transcript abundance was consistent across three or more clones tested for each nonsense variant (data not shown). SMG1 is an effector protein in the NMD pathway and its inhibition by the small molecule SMG1i increased the levels of both G542X and W1282X CFTR mRNA. This result demonstrates that the G542X and W1282X CFTR mRNA are acutely sensitive to modulation of the NMD pathway activity, an attribute critically important for identifying and developing therapeutics that address the NMD process that contributes to the pathology of nonsense mutations.

The aminoglycoside G418 promotes ribosomal readthrough of premature stop codons. Theoretically, readthrough by G418 should increase the abundance of PTC-containing mRNAs and this was observed in both the CFTR G542X and W1282X models. The functional improvement from G418 was only 3.4% and 1.6% of WT function for G542X and W1282X, respectively, although SMG1i in combination with G418 further increased function for both nonsense mutations. In contrast, in WT cells G418 decreased WT CFTR mRNA and protein abundance and function. SMG1i treatment alone increased W1282X function to 1.1% of WT. These results suggest that while truncated $CFTR_{1281}$ protein does retain some function, both W1282X and G542X will likely require readthrough for meaningful therapeutic benefit.

In summary, physiologically relevant models of CF-causing CFTR variants in the genomic context of 16HBE14o- cells can be produced with the gene editing pipeline presented here. In CFF-16HBEge F508del cells the known molecular pathology and pharmacology is recapitulated. We cannot ultimately rule out that pharmacologies of compounds targeting other CFTR variants, including nonsense mutations, may not equally well mirror responses that would be observed in primary bronchial epithelial cells or humans. However, the effects of two non-therapeutic reference compounds (G418 and SMG1i) were validated in the models for the two most common CFTR nonsense mutations (G542X and W1282X). A key advantage over primary cells is that these 16HBEge cell lines can provide virtually unlimited cell biomass with specific pathogenic mutations for use in many therapeutic development assays, including functional assays. These models can contribute to the development of therapies that address the currently unmet medical need of CF patients with rare mutations.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcf.2018.12.001.

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Declarations of interest

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