Title

TALENs Facilitate Single-step Seamless SDF Correction of F508del CFTR in Airway Epithelial Submucosal Gland Cell-derived CF-iPSCs

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TALENs Facilitate Single-step Seamless SDF Correction of F508del CFTR in Airway Epithelial Submucosal Gland Cell-derived CF-iPSCs

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Cystic fibrosis (CF) is a recessive inherited disease associated with multiorgan damage that compromises epithelial and inflammatory cell function. Induced pluripotent stem cells (iPSCs) have significantly advanced the potential of developing a personalized cell-based therapy for diseases like CF by generating patient-specific stem cells that can be differentiated into cells that repair tissues damaged by disease pathology. The F508del mutation in airway epithelial cell-derived CF-iPSCs was corrected with small/short DNA fragments (SDFs) and sequence-specific TALENS. An allele-specific PCR, cyclic enrichment strategy gave ~100-fold enrichment of the corrected CF-iPSCs after six enrichment cycles that facilitated isolation of corrected clones. The seamless SDF-based gene modification strategy used to correct the CF-iPSCs resulted in pluripotent cells that, when differentiated into endoderm/airway-like epithelial cells showed wild-type (wt) airway epithelial cell cAMP-dependent Cl ion transport or showed the appropriate cell-type characteristics when differentiated along mesoderm/hematopoietic inflammatory cell lineage pathways.

Introduction

Cystic fibrosis (CF) is caused by mutations in the CF trans-membrane conductance regulator (*CFTR*) gene.[1] Of the >2,000 disease-causing mutations detected in the *CFTR* gene (http://www.genet.sickkids.on.ca/cftr/), a trinucleotide (CTT) deletion that spans codons 507 and 508 and results in loss of a phenylalanine at amino acid 508 (F508del) of the CFTR protein is found in ~70% of all CF alleles.[2] CF patients typically exhibit a variety of pathologies that include abnormal mucus accumulation in airways and lungs, accompanied by opportunistic bacterial infections that appear to be associated with both airway epithelial cell (AEC) and immune cell dysfunction. Recent studies suggest that CFTR is a component of the monocyte and macrophage response to infection in CF patients.[3,4] Since CF-associated pathologies result in extensive tissue damage, treatment of CF will require a comprehensive strategy that both corrects the underlying genetic defect and repairs/regenerates damaged tissues. In this context, the ability to reprogram mature somatic cells into

induced pluripotent stem cells (iPSCs)[5,6] has opened the door for development of a comprehensive, personalized cellular therapy for CF.[7] These patient-specific iPSCs have the potential of generating transplantable, autologous cells/tissues that circumvent rejection by the host immune response, enhancing the potential for successful engraftment and tissue repair and avoiding the need for immunosuppressive drugs.[8–10] Several studies have already indicated that embryonic stem cells and fibroblast-derived CF-iPSCs can be differentiated into cells that have properties of endoderm[11–13] and airway epithelium.[14–18] Ultimately, further refinement of such cell differentiation protocols should be able to produce cells that will successfully rebuild damaged airways.

An important component of a comprehensive therapy for CF is the repair of the disease-causing CF mutation(s). Restoration of wild-type (wt) CFTR function in the repaired tissues will be critical in ameliorating the dysfunction associated with the *CFTR* mutation. The sequence-specific gene-editing approach, small/short fragment homologous replacement (SFHR), has been applied to numerous genomic targets,

including *CFTR*, in multiple cell systems both *in vitro* and *in vivo*.[19–22] SFHR is mediated by polynucleotide small/short DNA fragments (SDFs), but until now, has not been applied to correct *CFTR* mutations in human CF-iPSCs. While SFHR-driven homologous exchange (HE) efficiencies as high as ~10% have been observed with microinjection,[22,23] the efficiency of HE can range between 0.05 to ~5%, depending on the cells, the method of nucleic acid delivery or other transfection parameters.[19,24] Since transcription activator-like effector nucleases (TALENs)[25–28] and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 nuclease[29–31] mediate DNA double strand breaks (DSBs) by enhancing the efficiency of homologous recombination between donor plasmid DNA and a genomic target, we reasoned that this induction of DSBs could facilitate SDF-mediated HE as well. In this study, TALENs were used to minimize off-target effects associated with the CRISPR/Cas9 system[32,33] and enhance SDF-mediated correction of the *F508del CFTR* in CF-iPSCs.

Results
Generation of CF-iPSCs
Primary airway submucosal gland AECs (CFSME101) from a CF patient homozygous for the F508del mutation were reprogrammed by transduction with four individual retroviruses, each containing one canonical transcription factor (*OCT4*, *SOX2*, *KLF4*, or *c-MYC*) and grown for 3–4 weeks on mitomycin C inactivated mouse embryo fibroblast feeders until candidate iPSC colonies appeared. Initially, five candidate iPSC colonies were generated (CF1-iPS1, CF1-iPS2, CF1-iPS3, CF1-iPS4, and CF1-iPS5) giving a reprogramming efficiency of ~2×10^{-4}. Of the five clones generated, four were selected for further analysis and experimentation.

The *F508del/F508del CFTR* genotype of the parental CFSME101 primary cells and the CF1-iPSC lines was confirmed by allele-specific PCR (AS-PCR; **Supplementary Figure S1a**) and DNA sequence analysis of PCR products generated by non-AS-PCR (**Supplementary Figure S1b**). Immunocytochemical analysis showed that the CF1-iPSC clones expressed pluripotent markers SSEA3, SSEA4, TRA-1–60, TRA-1–81, and NANOG (**Supplementary Figure S1c**, **Supplementary Table S1**). Pluripotence was further demonstrated *in vitro* by expression of α-fetoprotein (endoderm), TUJ1 (ectoderm), and α-smooth muscle actin (mesoderm) in embryoid body cells (**Supplementary Figure S1d**, **Supplementary Table S1**) and *in vivo* by tissues derived from teratomas generated in immunodeficient NGS mice representing the three primordial germ layers (**Supplementary Figure S1e**). Cytogenetic analysis of cell lines CF1-iPS1, -iPS4, and -iPS5 between P5.6-P5.8 (where passage number PX.Y.etc = X passages before transduction/reprogramming, Y passages since candidate colony isolation) showed a normal diploid female karyotype (46,XX; **Supplementary Figure S1f**).

TALEN enhanced correction of *F508del CFTR*
A 491 bp wtCFTR donor SDF (491z-SDF) was used for correction, since this SDF alone has been previously shown to mediate HE at the genomic F508del *CFTR* locus in AECs.[19,20,22] Sequence-specific *CFTR*-TALEN pairs (CFTAL-1B and CFTAL-2B; **Supplementary Figure S2a**) were designed to

enhance 491z-SDFs-mediated HE and also cut only in the mutant DNA. CF1-iPS4 cells were transfected with either 10^7 or 2×10^7 SDFs/cell in absence or presence of CFTAL-1B and CFTAL-2B expression vectors. The advantage of this TALEN pair is that one of the pair is allele specific for the mutant allele and, as such, has enhanced binding to and cutting of the mutant allele. Cells were harvested after 3 days with Accutase or Dispase, subcultured, and analyzed by AS-PCR for *wtCFTR* DNA. Subcultured cells were harvested again on days 7 and 9 for analysis. CF1-iPS4 cells cotransfected with SDFs and TALENs appeared to have significantly more *wtCFTR* DNA than those transfected with SDFs alone (**Figure 1a** and **Supplementary Figure S2b,c**), indicating enhancement of SDF-mediated HE at the *CFTR* locus by TALENs.

Isolation and characterization of corrected CF-iPSCs
Corrected CF1-iPS4 cell clones were isolated by a cyclic dissociation/PCR enrichment protocol involving dissociation of the cells with Dispase, subculturing as cell clumps into individual wells of a 12-well plate and screening by AS-PCR (see **Supplementary Materials and Methods**).[34] Enhancement of SDF-mediated HE was observed in the corrected cell subpopulation at day 9 after cotransfection with 2×10^7 SDFs/cell and TALENs CFTAL-1B and CFTAL-2B (**Figure 1a**, lane 21).

Approximately 100-fold enrichment in the corrected CF1-iPS4 cell population (~10% corrected cells) was achieved after five to six enrichment cycles (ECs) (**Figure 1b** and **Supplementary Figure S2d**). At the sixth enrichment cycle (EC_6), cells in well $EC_6 C_{2.1}$ (P5.55.15), derived from well C_2 (P5.55.14), had the same apparent ratio of *F508delCFTR:wtCFTR* as in well $EC_6 C_2$ (P5.55.14) (**Figure 1a**). The $EC_6C_{2.1}$ cells were plated at single cell densities on a 60-mm dish. Of the 20 clones isolated, one clone (c1) (P5.55.17) indicated both *wt* and *F508delCFTR* by AS-PCR (**Figure 1c** and **Supplementary Figure S3**). A subsequent isolation from EC_8, $EC_8 B_{3.1}$ (P5.55.19), yielded 6 heterozygote clones of the 30 isolated (20%). DNA sequence analysis of CF1-iPS4 clone 1 (CF1-iPS4c1) confirmed heterozygosity (**Figure 2a**). Allele-specific RT-PCR analysis (**Supplementary Table S2**) of *CFTR* mRNA expression at P5.55.21 showed expression of both *wt* and *F508delCFTR* mRNA, whereas untransfected CF1-iPS4 p5.14 showed only *F508delCFTR* mRNA expression (**Figure 2b**). While iPSCs express low levels of *CFTR* mRNA (S Suzuki, DC Gruenert, unpublished observations), it was still possible to differentiate between *wt* and *F508delCFTR* mRNA expression in cultures also expressing pluripotence markers. The pluripotence of the corrected cells (**Figure 2c**, **Supplementary Table S1**) was further indicated by their ability to form three primordial germ layers in embryoid bodies *in vitro* (**Figure 2d**, **Supplementary Table S1**).

Off-target events due to TALEN/SDF-mediated genome editing were evaluated by Southern blot hybridization for detection of random integration and by screening the most likely off-target TALEN-binding sites that differ from the intended target site by up to seven mismatches per TALEN half-site. Southern blot analysis showed no spurious donor DNA random integration bands in two distinct corrected clones (CF1-iPS4c1 and CF1-iPS4D2) when compared to untransfected parental CF1-iPS4 cells (PC) (**Figure 2e**). All three samples gave a single band at 8746- or 7530-bp, following gDNA digestion with EcoRV and XbaI, respectively,
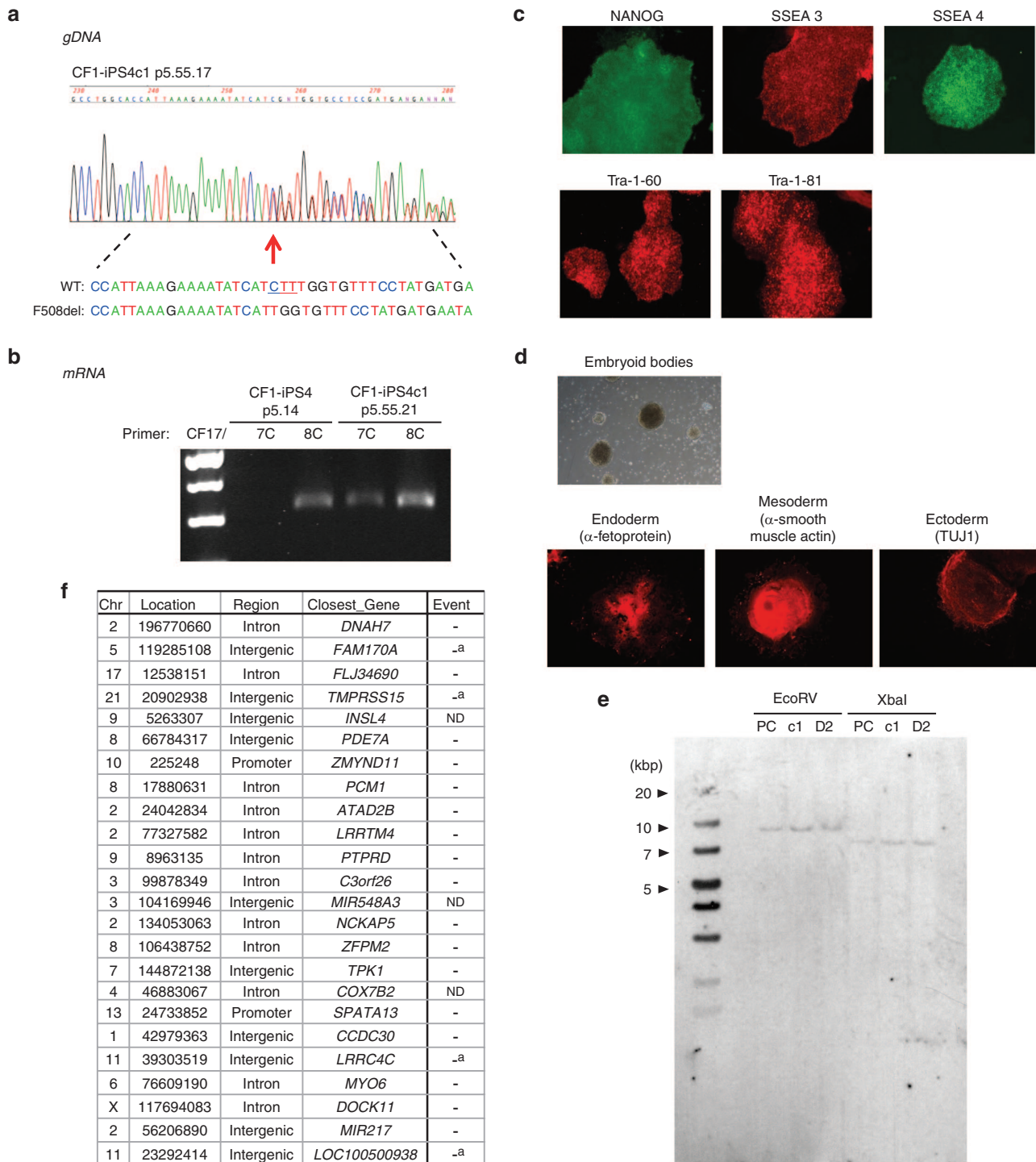
Figure 1 Isolation of a corrected clone after TALEN/SDF-mediated correction of genomic *F508del CFTR* in CF1-iPS4 cells.
(**a**) Enrichment for corrected CF1-iPS4 cells co-transfected with 2×10^7 491z-SDFs/cell and the CFTAL-B expression vectors (lane 21). These cells showed the highest proportion of *wtCFTR* containing cells by AS-PCR and were distributed equally into a new multiwell plate. (**b**) AS-PCR analysis with primer pair CF1B/CF7C (**Supplementary Table S2**) (wt, top and middle) or CF1B/CF8C (**Supplementary Table S2**) (F508del, bottom) was again used to identify those cells with the highest proportion of *wtCFTR* DNA at each individual enrichment step followed by equal distribution of the cells from the well with the highest proportion of *wtCFTR* DNA into a new multiwell plate, etc (lanes 9 to 16), (first step: A4, second step: A4, third step: A3, fourth step: B2, fifth step: C2, sixth step: C1, seventh step: B3, eighth step: C2). (**a** and **b**) Lanes 2 to 8 reflect a control PCR titration analysis, mixing genomic DNA from a non-CF-iPS (*wtCFTR*) cell line (SC2-iPS$_{rs}$3) with CF1-iPS4 (*F508delCFTR/F508delCFTR*) at varying percentages. (**c**) Limiting dilution was used to isolate single cell-derived clonal populations of corrected cells from population C2 of the sixth enrichment step (EC$_6$). AS-PCR analysis was performed with primer pairs CF1B/CF7C or CF1B/CF8C on DNA from single colonies following limited dilution. Banding patterns for the *wtCFTR/wtCFTR*, heterozygote (*wtCFTR/F508delCFTR*), and homozygous mutant (*F508delCFTR/F508delCFTR*) were reconstructed by mixing the genomic DNA of a non-CF-iPS cell line (SC2-iPS$_{rs}$3) with CF1-iPS4 (F508del) at varying percentages (lanes 2 to 7). Clone c1 shows a pattern consistent with that of the *wtCFTR/F508delCFTR* heterozygote (lanes 9 and 10), while clones 2 to 20 all show a *F508delCFTR/F508delCFTR* pattern (**Supplementary Figure S3**, **Supplementary Table S2**).

indicating that there was no random integration of the SDF with TALEN-enhanced correction.

Candidate off-target sequences were selected based on sequence homology and repeat-variable diresidue-binding preferences using the PROGNOS algorithms. The 24 top-ranked sites were assessed by Sanger sequence analysis of genomic DNA from parental, CF1-iPS4 P5.50, and corrected CF1-iPS4c1 P5.55.29 cells (**Supplementary Table S3**). No mutations could be attributed to TALEN-induced off-target events (**Figure 2f**). Based on the reference hg19 genome, the several single-nucleotide polymorphisms present in both corrected and uncorrected CF1-iPS4 were identical. Sequence analysis at three sites failed and was not further considered. While this is not a comprehensive off-target investigation of the entire genome, it is, nonetheless, encouraging that no off-target events were observed at any of the 21 independent sites evaluated.

Differentiation and phenotypic characterization of corrected CF1-iPSc1 cells

Corrected CF1-iPS4c1 cells were also evaluated for CFTR function and the ability to differentiate into cells showing airway epithelial-like features to determine their potential usefulness in cell therapy for CF. Transepithelial Cl ion transport, a hallmark of CF, was assessed in CF1-iPS4c1 cells grown to confluence on cell culture inserts with an air–liquid interface. After 19 and 20 days in culture, respective transepithelial resistances (R$_t$) in Ussing chambers were 45 and 63 Ωcm^2 and forskolin-activated, CFTRinh$_{172}$-blocked, CFTR-specific Cl currents were –0.5 and –1.0 µA/cm^2 (mean = –0.75 µA/cm^2) (**Figure 3b** i top). Expression of zonula occludens-1 (ZO1) protein, a marker of epithelial cell differentiation, was found widely expressed in the cultures, although it was not uniform throughout the monolayer (**Figure 3b** i bottom, **Supplementary Table S1**), suggesting incomplete epithelial differentiation. A previous study with human mesenchymal stem cells showed transdifferentiation of mesenchymal stem cells co-cultured with human AECs under air–liquid interface conditions that led to AECs with functional CFTR expression.[35] In that context, CF1-iPS4c1 cells were co-cultured with CFBE41o-AECs and assayed for differentiation and CFTR function. CF1-iPS4c1/CFBE41o- co-cultures were evaluated up to 20 days for transepithelial Cl secretion and ZO1 expression (**Figure 3a**, **Supplementary Table S1**). After 7 days, cAMP-dependent Cl currents, activated by forskolin and blocked by CFTRinh$_{172}$, were similar to those observed

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a

gDNA

CF1-iPS4c1 p5.55.17

WT: CCATTAAAGAAAATATCATCTTTGGTGTTTCCTATGATGA

F508del: CCATTAAAGAAAATATCATTGGTGTTTCCTATGATGAATA

b

mRNA

c

d

e

f

Chr	Location	Region	Closest_Gene	Event
2	196770660	Intron	DNAH7	-
5	119285108	Intergenic	FAM170A	-a
17	12538151	Intron	FLJ34690	-
21	20902938	Intergenic	TMPRSS15	-a
9	5263307	Intergenic	INSL4	ND
8	66784317	Intergenic	PDE7A	-
10	225248	Promoter	ZMYND11	-
8	17880631	Intron	PCM1	-
2	24042834	Intron	ATAD2B	-
2	77327582	Intron	LRRTM4	-
9	8963135	Intron	PTPRD	-
3	99878349	Intron	C3orf26	-
3	104169946	Intergenic	MIR548A3	ND
2	134053063	Intron	NCKAP5	-
8	106438752	Intron	ZFPM2	-
7	144872138	Intergenic	TPK1	-
4	46883067	Intron	COX7B2	ND
13	24733852	Promoter	SPATA13	-
1	42979363	Intergenic	CCDC30	-
11	39303519	Intergenic	LRRC4C	-a
6	76609190	Intron	MYO6	-
X	117694083	Intron	DOCK11	-
2	56206890	Intergenic	MIR217	-
11	23292414	Intergenic	LOC100500938	-a

Figure 2 Characterization of the CF1-iPS4c1 corrected cell line. (**a**) DNA sequencing histograms of non–allele-specific PCR products using CF1 and CF5 primers (**Supplementary Table S2**) from *CFTR* exon 11 in CF1-iPS4c1. The arrow indicates where the wt and F508del alleles on each chromosome diverge. (**b**) AS RT-PCR of RNA isolated from the parental CF1-iPS4 and from the CF1-iPS4c1 cells. The primer pair CF17/CF7C is selective for *wtCFTR* expression and CF17/CF8C (**Supplementary Table S2**) indicates expression of *F508delCFTR* mRNA. (**c**) Antibody staining for expression of pluripotence markers (**Supplementary Table S1**) with the CF1-iPS4c1–corrected cells (P5.55.21). (**d**) CF1-iPS4c1 cultures generated directly from the embryoid bodies demonstrate the potential for the corrected cells to differentiate into three germ layers *in vitro*. Immunostaining indicated endoderm (α-fetoprotein), mesoderm (α-smooth muscle actin), and ectoderm (TUJ1 (**Supplementary Table S1**)). (**e**) Southern blot analysis of EcoRV- and XbaI-digested genomic DNA (gDNA) isolated from untransfected CF1-iPS4 (parental cells, PC) as well as the CF1-iPS4c1 and D2-corrected clones. The *wtCFTR*-SDF (491z-SDF) was used as a probe for random integration or the endogenous *CFTR* locus. (**f**) Summary of off-targeting event analysis. The first four columns list predicted off-target sites for TALEN pair CFTAL-B as described in **Supplementary Table S3**. Column 5 indicates the results of sequencing. (-) = Corrected clone, CF1iPS4c1, P5.55.29, has no mismatches and Indels (deletions and/or insertions) compared with the CF1-iPS4 P5.50, parental cell line; (ND) = not done due to sequence analysis failure; a = CF1-iPS4 and corrected clone have identical nucleotide polymorphisms when compared to hg19 (UCSC human genome 19).
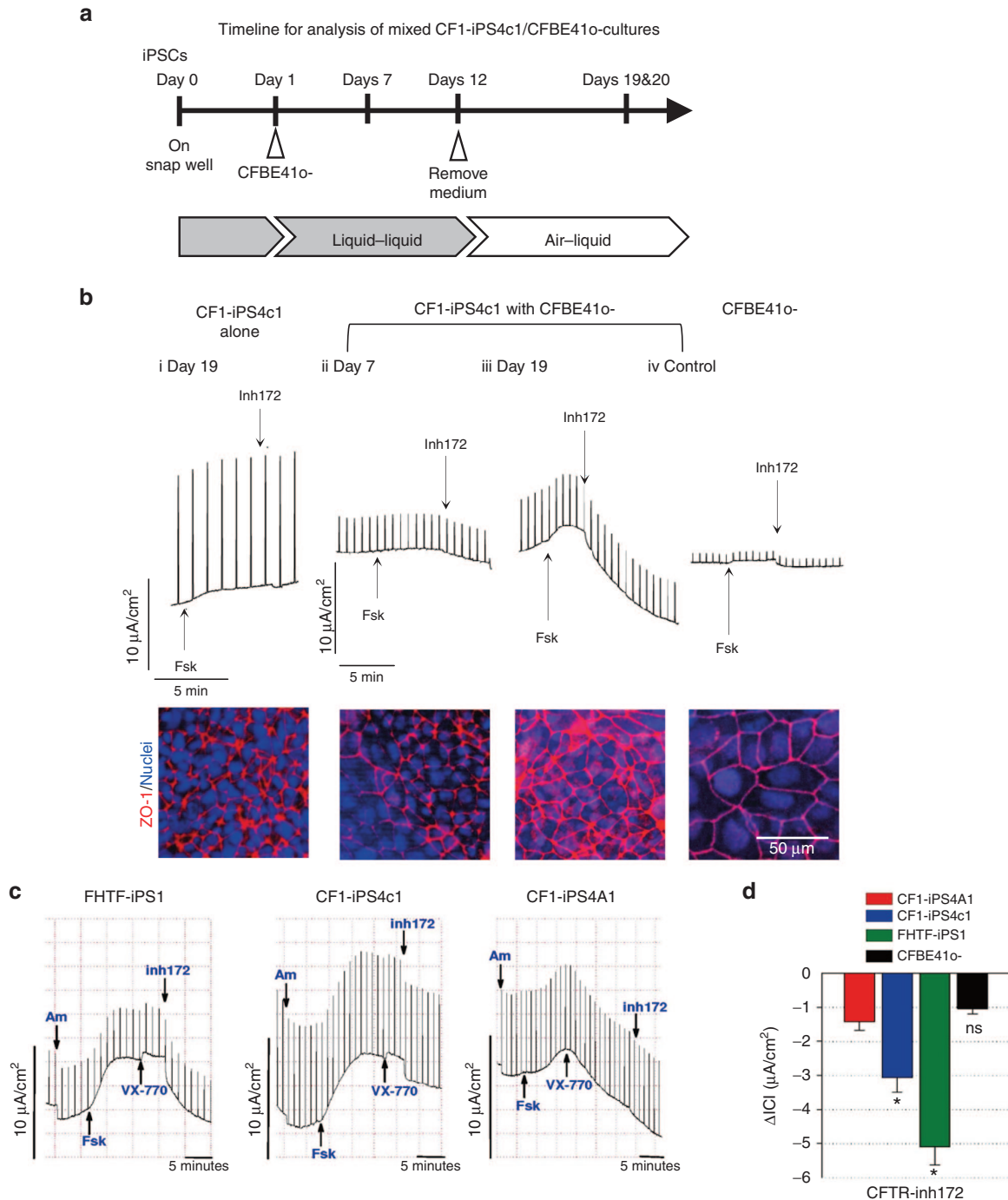
Figure 3 Functional analysis of corrected CF1-iPS4c1 cells. (**a**) CFTR Cl transport and expression of ZO-1 in CF1-iPS4c1 cells grown to confluent cell sheets in the presence or absence of CFBE41o- cells. Cells were grown under ALI culture conditions on day 12 and later. (**b**) Transepithelial Cl current recordings across CF1-iPS4c1 cells alone (i), co-cultures of CF1iPS4c1/CFBE41o-cells (ii, iii), and CFBE41o-cell monolayers, (control, d11) (iv); Fsk, 20 μmol/l forskolin; inh172, 50 μmol/l CFTRinh$_{172}$. Corresponding immunostaining for tight junction formation (**Supplementary Table S1**) in CF1-iPS4c1 islands or CFBE41o- cells alone was indicated below. (**c**) Representative transepithelial Cl currents (I_{Cl}) in positive control FHTF-iPS1 cells (*wt/wt*), CF1-iPS4c1 cells (*wt/F508del*), or negative control CF1-iPS4A1 cells (*F508del/F508del*) grown in CFBE41o- co-culture for 13 days. Current deflections indicate 1 mV pulses applied every 60 seconds to monitor transepithelial resistance. Amiloride (20 μmol/l) was added to the apical surface to inhibit Na absorption, while forskolin (20 μmol/l) and VX-770 (5 μmol/l) were added to the serosal and apical surface, respectively, to stimulate CFTR Cl currents. CFTRinh$_{172}$ (50 μmol/l) was added to the apical surface to verify inhibition of functional CFTR. (**d**) The bar chart reflects the inhibition of CFTR Cl currents by CFTRinh$_{172}$ in CF1iPS4A1, CF1iPS4c1, and FHTFiPS1 grown in CFBE41o- co-culture for 13 and 15 days, and CFBE41o- cells. Cl current changes (ΔI_{Cl}) were determined from transepithelial recordings of Cl currents (I_{Cl}) after standardizing the drift. Results represent the mean ± SE ($n = 4$; *$P < 0.05$ versus CF1-iPS4A1; ANOVA followed by Holm-Sidak test). ALI, air–liquid interface.

for CFBE41o- alone (**Figure 3b** i.v., $R_t > 1,000$ Ωcm^2, $I_{Cl} = -0.80$ $\mu A/cm^2$). However, from days 12 to 20, the average CFTRinh$_{172}$ blocked CFTR current was -4.70 ± 2.0 $\mu A/cm^2$ (**Figure 3b** iii) (significantly larger than Cl currents in CF1-iPS4 alone). All CF1-iPS4c1 co-culture islands showed prominent ZO1 expression, indicating epithelial differentiation, tight junction formation, and cell polarity. Characteristic membrane localized peripheral ZO1 staining of CF1-iPS4c1 islands became more prominent with time in co-culture; however, the CF1-iPS4c1 cells still showed tight junction discontinuities when compared to pure CFBE41o- cultures (**Figure 3b**, **Supplementary Table S1**). Overall, these observations indicate that CF1-iPS4c1 cells begin to adopt features of the CFBE41o- AECs and show CFTR expression and function. HE-mediated correction of CFTR-associated Cl current was further demonstrated by comparing three iPSCs with distinct *CFTR* genotypes: (i) non-CF, FHTF-iPSCs - *wt/wt CFTR*, (ii) corrected CF1-iPS4c1 cells - *wt/F508del*, and (iii) uncorrected CF1-iPS4A1 cells - *F508del/F508del* (**Supplementary Figure S4a**). When co-cultured with CFBE41o- cells (**Supplementary Figure S4b**), assessed for their *CFTR* mRNA expression (**Supplementary Figure S4c**) and assayed for transepithelial Cl ion transport (**Figure 3c**), *CFTR (wt* and/or *F508del)* mRNA expression in all three cell lines was much higher on day 9 than on day 6 and was maintained (FHTF-iPSCs) or increased (CF1-iPS4c1 and A1) with additional days in co-culture (**Supplementary Figure S4c**). While there were no changes in short circuit current ($\Delta I_{SC} \approx 0$) on day 6 in response to Cl transport activators, significant changes in I_{SC} were observed on days 9, 13 (**Figure 3c**), and 15. At day 9, uncorrected cells showed 6.1 ± 1 $\mu A/cm^2$ and CFTR-corrected cells showed 8.2 ± 1.6 $\mu A/cm^2$ of amiloride-blocked currents. Treatment of the co-cultures with CFTR-specific VX-770 that stimulated or CFTRinh$_{172}$ that blocked CFTR-dependent Cl current, in FHTF-iPS and CF1-iPS4c1 cells, but not in CF1-iPS4 A1 cells after 13 and 15 days in co-culture (**Figure 3c,d**), indicating that the TALEN/SDF-mediated genetic correction in CF1-iPS4c1 cells led to wtCFTR functional activity.

Using a more controlled, defined airway differentiation protocol (**Figure 4a**, **Supplementary Table S1**), lineage specific proximal airway-like epithelial cell progression of CF1-iPS4c1 cells was achieved. With the defined airway differentiation protocol, cultures showed enrichment for cells expressing NKX2.1 and FOXJ1, markers of ciliated AECs (days 43–61) (**Figure 4b**, **Supplementary Table S1**) as well as concomitant expression of CFTR protein with wtCFTR function (day 62) (**Figure 4c**). These observations are consistent with proximal airway-like differentiation into ciliated cells and functional correction of the CF1-iPS4c1.

Because CF pathology has a significant inflammatory component associated with macrophages,[36–39] CF1-iPS4c1 cells were directed to differentiate along a hematopoietic lineage-specific pathway into monocyte- and macrophage-like cells (**Supplementary Figure S5a**). After day 7, CF1-iPS4c1 cells expressing CD34 and KDR, but not CD45, were observed by flow cytometry, indicating the early stages of hematopoietic/endothelial cell differentiation (**Supplementary Figure S5b**, **Supplementary Table S1**). By day 16, cells with endothelial markers KDR, CD34, CD31, and CD144, as well as a small

population of cells expressing hematopoietic markers, CD45 and CD34 (including myeloid progenitors expressing CD45 and CD33) (**Supplementary Figure S5c**, **Supplementary Table S1**) was detected. On day 49, flow analysis, indicated the presence of some CD34^{low} expressing cells, and a distinct CD45$^+$ CD33$^-$ population that included a small proportion of CD14 expressing monocytes (**Supplementary Figure S5d**, **Supplementary Table S1**). The flow data was corroborated by qRT-PCR analysis. On day 49, *CD14* mRNA levels were significantly increased, consistent with the presence of cells in the monocyte/macrophage lineage pathway (**Supplementary Figure S5e**, **Supplementary Table S2**).

Discussion

The differentiation of CF-iPSCs into cells with an inflammatory cell features provides the potential for assessing and comparing the parental CF1-iPS4–derived inflammatory cells to the corrected CF1-iPS4c1 cells. As with airway-like epithelial cell differentiation, differentiation into inflammatory cells will aid in development of a cellular therapy for regenerating an intact hematopoietic system in CF patients, as well as in development of a disease model system to assess potential pharmacological therapeutic agents. While further studies are required to identify the appropriate engraftable progenitor cells to be used in repopulating the airways and hematopoietic system, these studies lay the foundation for developing a seamless gene correction strategy in iPSCs generated with a nonintegrating reprogramming vehicle. In addition, the airway and hematopoietic differentiation studies are a critical part of developing and refining xeno-free cellular therapies for CF.

One recent study in CF adult intestinal stem cell organoids showed apparent functional correction of F508del CFTR using CRISPR/Cas9 to facilitate homologous recombination.[32] Genetic correction of these multipotent adult stem cells resulted in swelling properties similar to those observed in organoids derived from healthy individuals. However, due to off-target events and the selection marker remaining in the genomic DNA, the approach described is not footprint free and has the risk of unintended adverse mutagenesis. In addition, the cells that were corrected were not pluripotent and had a limited potential in terms of their ability to differentiate into other cell types within the body. The TALEN/SDF strategy described here shows no apparent off-target events (**Figure 2e,f**) and appears to be seamless in the sense that there are no other alterations in the targeted region. Moreover, cells/tissues associated with CF pathology, will need to have pluripotence for a multi-tissue therapeutic value, a requirement that intestinal stem cell organoids will not satisfy. Two other recent papers demonstrate functional correction of CFTR in iPSCs using ZFNs[16] or CRISPR/Cas9s.[18] However, unlike the present study, the correction of the *CFTR* mutation utilizes a donor plasmid that contains a selection marker gene and relies on a protocol that is not footprint free and a seamless single-step strategy for isolating cells with corrected genomic DNA. Therefore, the overall strategy presented here of generating patient-derived iPSCs combined with seamless, single-step, mutation correction as well as co-culture-induced and/or directed multilineage differentiation
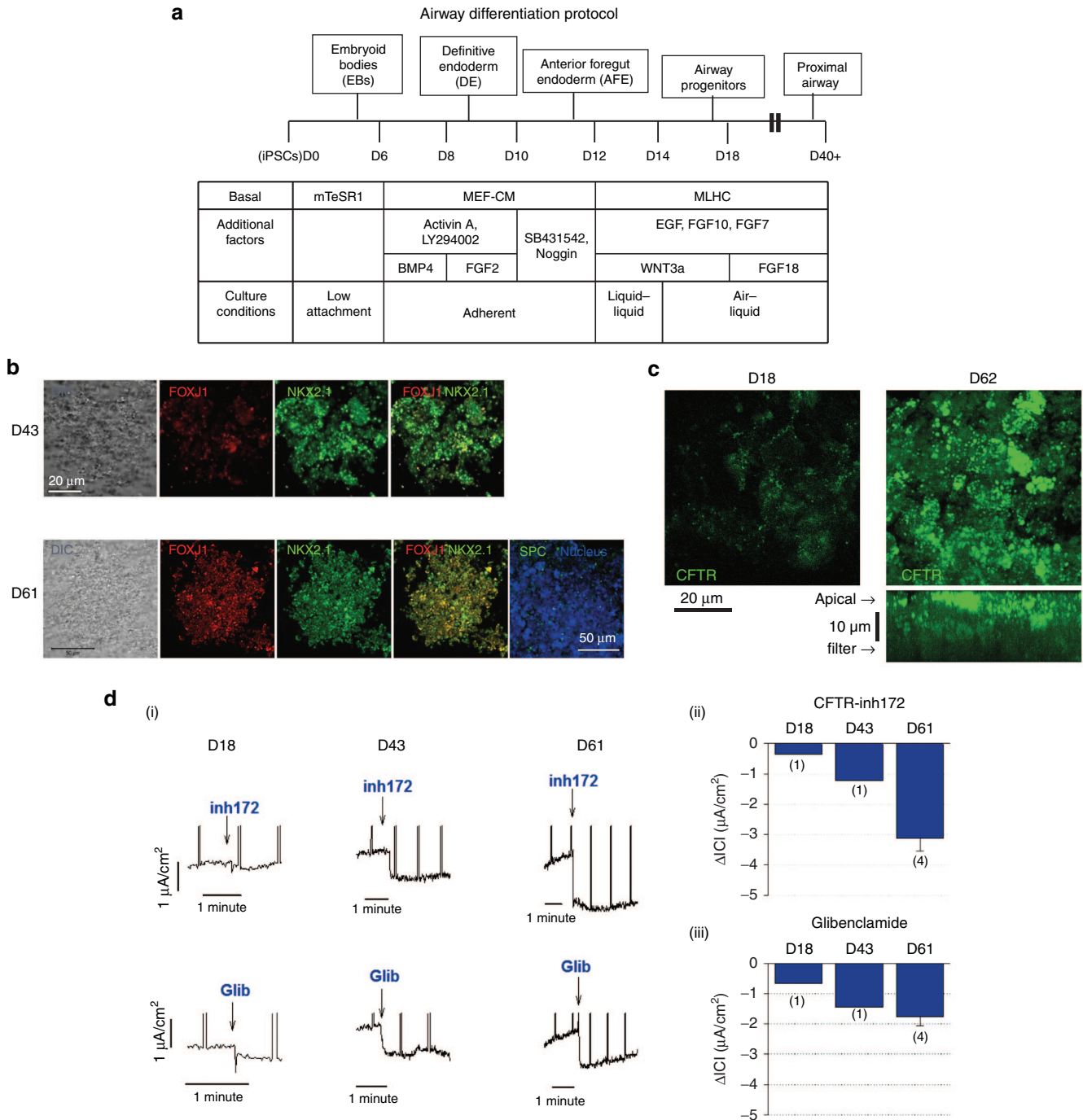
Figure 4 Directed airway differentiation and functional CFTR expression. (**a**) Time course of a defined airway differentiation protocol. (**b**, **Supplementary Table S1**) Immunostaining for FOXJ1 (red, ciliated cell marker) and NKX2.1 (green, airway cell marker) with the overlay at days 43 and 61. Increased co-expression (orange) from day 43 to day 61 indicates prominent proximal airway differentiation and the presence of ciliated cells. Surfactant protein C (distal airway and alveolar type II cell marker, green) was detected in a few cells at day 61. Nuclei are stained with Hoechst 33342 (blue). (**c**, **Supplementary Table S1**) Immunostaining of CFTR protein expression of confluent CF1-iPS4c1cells grown on Snapwell inserts (filter) on day 18 and day 62. Orthogonal XZ image at day 62 shows abundant CFTR expression at the luminal surface. (**d**) Corresponding transepithelial I_{Cl} measurement from days 18, 43, and 61. Ussing assay was performed with pure cultures of CF1-iPS4c1 grown on Snapwell inserts in presence of a serosal-to-mucosal Cl gradient (120 mmol/l: 0 or 60 mmol/l) and in presence of amiloride (Amilo, 20 mmol/l), forskolin (Fsk, 20 mmol/l), and VX-770 (3 mmol/l). Transepithelial I_{Cl} was detected in response to CFTR-inh 172 (50 mmol/l) and glibenclamide (1 mmol/l). Voltage pulses (1 mV) were applied every 60 seconds and are cut off. Details for inhibition of I_{Cl} by CFTR-inh172 and glibenclamide are indicated in cultures for days 18, 43, and 61. Acute inhibition demonstrates CFTR activity in the *CFTR*-corrected iPSCs. **ii.** Average changes in chloride current (ΔI_{Cl}) in response to CFTR-inh172 and **iii.** Glibenclamide at increasing times of differentiation. Data were pooled from measurements that were done on two consecutive days (days 61 and 62). Number of experiments is indicated in parenthesis (*n* = 1–4).

will provide an effective starting platform for building a personalized cell and gene-based CF therapy.

In summary, the studies described are distinct from previous studies, showing that human airway submucosal gland epithelial cells can be reprogrammed into CF-iPSCs and corrected by a seamless DNA fragment (polynucleotide)-based SFHR strategy that combines *wtCFTR*-SDFs (not a plasmid donor DNA) with *CFTR*-specific TALENs. Through a straightforward cyclic enrichment protocol[34] corrected clones that retain their pluripotence were isolated without selection. Another unique aspect of this study is the demonstration that a corrected CF-iPSCs can be "differentiated" into airway epithelial-like cells through co-culture with immortalized AECs. These corrected CF-iPSCs showed wild-type cAMP-dependent Cl ion transport properties after co-culture and directed differentiation along an endodermal lineage pathway into cells that had proximal airway epithelial-like properties. In addition, the corrected CF1-iPS4c1 cells were also able to differentiate into inflammatory cells expressing macrophage and monocyte markers.

Materials and Methods

Cells and culture conditions. All human tissue studies were approved by the UCSF Committee on Human Research (CHR) and California Pacific Medical Center (CPMC) Institutional Review Board (IRB). Primary CF tracheobronchial submucosal gland epithelial cells (CFSME101), homozygous for the *F508del CFTR* mutation, were obtained from the UCSF CF Cell Culture Core Laboratory (Director, Dr WE Finkbeiner) and grown in MLHC8e medium.[40] Immortalized CFBE41o- cells,[41–44] used for the co-culture studies, are homozygous for the *F508del CFTR* mutation were routinely grown in supplemented Eagle's Minimal Essential Medium and subcultured with polyvinylpirrolidone/ethylene glycol tetraacetic acid/trypsin. Co-culture with iPSC-derived cells was carried out on Transwell inserts in mTeSR1 medium.

Human iPSCs were generated by retroviral reprogramming[5] according to guidelines developed by the CPMC Research Institute Stem Cell Research Oversight Committee and the UCSF Gamete and Embryonic Stem Cell Research Committee. The iPSCs were grown and subsequently subcultured by mechanical isolation and enzymatic dissociation of individual iPSC colonies with collagenase type IV (Invitrogen, Carlsbad, CA)[45] or Dispase (StemCells, Inc, Vancouver, British Columbia, Canada).

Cell passage number is denoted as $Pn_1.n_2.n_3. - n_z$, where n_1 = number of passages as primary cells before reprogramming, n_2 = number of passages since reprogramming, n_3 = number of passages after transfection with SDF, etc., where each period delineates the onset of a specific protocol or treatment that alters the character of the cells.[46]

Generation of sequence-specific TALENs. TALEN targeting sequences were designed using Web-based software, TALE-NT 2.0 (https://boglab.plp.iastate.edu/).[47] The following sequences were selected: TALEN pairs CFTAL1B: 5′-TCTCAGTTTTCCTGGATTAT, spacer: gcctggcaccattaaagaa, CFTAL2B: AATATCATTGGTGTTTCCT A-3′. CFTAL2B is allele specific and preferentially binds to the mutant genomic

sequence, and will therefore not bind and contribute to cleavage of donor and repaired DNA (**Supplementary Figure S2a**). We were unable to generate a comparable CRISPR/Cas9 nickase (Cas9n) pair for this locus.[48] CFTR-B TALEN plasmids were assembled by following Golden Gate TALEN assembly method[25] with the Golden Gate TALEN plasmid kit (Kit # 1000000024, Addgene, Cambridge, MA). In the second Golden Gate reaction, a novel backbone plasmid (MR015; MH Porteus and M Rahdar, unpublished data) was used for optimal mammalian cell expression.

SDF-mediated correction with TALENs. CF1-iPS4 cells, P5.55, were nucleofected with the 491z-SDF at 10^7 SDFs/cell or 2×10^7 SDFs/cell in absence or presence of CFTAL-B TALENs. Genomic DNA was isolated on days 3, 7, and 9 posttransfection and amplified with AS-PCR primers CF1B/CF7C or CF1B/CF8C (**Supplementary Table S2**). The resulting PCR products were analyzed on a 2% agarose gel. The well containing the highest relative amount of corrected cells as indicated by band intensity was then subjected to a multiple enrichment cycle protocol similar to those described previously.[34,49] This protocol involved (i) dissociation with dispase and approximately equal distribution of cells into each well of a multiwell plate (Falcon-Beckton Dickenson Labware, Franklin Lakes, NJ), (ii) further growth, (iii) *wtCFTR* AS-PCR analysis (**Supplementary Table S2**) of an aliquot from each well, and (iv) subculture/distribution of the cells in the well with the apparent highest proportion of corrected cells, into another multiwell plate.

DNA from untransfected CF1-iPS4 and non-CF-iPSCs was used as controls for the *F508del* and *wtCFTR* AS-PCR amplification, respectively. The sensitivity of the AS-PCR analysis and the proportion of corrected cells within the population of transfected cells were determined by mixing genomic DNA from a non-CF-iPSC line (SC2-iPS$_{rs}$3) with varying percentages of CF1-iPS4 DNA.[50]

Allele-specific reverse transcription PCR. Total RNA was extracted using ReliaPrep RNA Cell Miniprep System (Promega, Madison, WI) and then synthesized into cDNA using ProtoScript M-MuLV First Strand cDNA Synthesis Kit (New England BioLabs, Ipswich, MA). Allele-specific reverse transcription PCR for CFTR expression was performed with the CF7C (*wt*) or CF8C (*F508del*) paired with a non–allele-specific primer, CF17 (**Supplementary Table S2**).

Corrected CF1-iPS4c1 and CF airway epithelial cell co-cultures. Immortalized CF bronchial epithelial cells, CFBE41o-, have been shown to promote trans-differentiation of marrow-derived mesenchymal stem cells to acquire an AEC phenotype.[35] In a similar context, CFBE41o- cells were co-cultured with *CFTR* corrected CF1-iPS4c1, *CFTR* wild-type FHTF-iPS1 and *CFTR* uncorrected CF1-iPS4A1 cells to promote the differentiation of the iPSCs into AECs. CFBE41o- cells are homozygous for the *F508del* mutation, express very low levels of *F508del CFTR* mRNA, and readily form tight epithelial cell monolayers.[44]

Mixed CFBE41o-/iPSC co-cultures were generated by adding ~5×10^4 CFBE41o- cells/cm² to iPSCs plated on 12-mm clear polyester Snapwell inserts (Corning Life Sciences,

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Acton, MD). The CF1-iPS4c1/CFBE41o- co-culture was grown in mTeSR1 medium and transepithelial ion transport measurements were made up to 20 days post-plating. An air–liquid interface[51] was established at day 12 and maintained throughout the rest of the experiment.

Measurement of CFTR-dependent Cl transport. Short circuit current (I_{SC}) of CFTR-dependent Cl currents were measured in Easy Mount Ussing chambers (Physiologic Instruments, San Diego, CA) across cell monolayers grown on Snapwell inserts as previously described.[52] Transepithelial voltage was clamped to 0 mV with a standard four-electrode voltage clamp. I_{SC} was continuously recorded. At 60-second intervals, transepithelial voltage was clamped to 1 mV for 1 second to monitor transepithelial resistance (Rt). A serosal-to-mucosal Cl gradient was established to increase the electrochemical driving force for Cl secretion. Transepithelial I_{SC} measured under these conditions was termed I_{Cl}. The serosal solution comprised: (in mmol/l) 120 NaCl, 25 $NaHCO_3$, 5 KCl, 1.2 NaH_2PO_4, 5.6 glucose, 1.0 $CaCl_2$, and 1.2 $MgCl_2$, while the apical Cl-free solution comprised: (in mmol/l) 120 Na-gluconate, 20 $NaHCO_3$, 5 $KHCO_3$, 1.2 NaH_2PO_4, 5.6 glucose, 2.5 $Ca(gluconate)_2$, and 1.2 $MgSO_4$. Experiments were carried out at 37 °C, and solutions were gassed with 5% CO_2 in air for a solution pH of 7.4. Positive currents were defined as the movement of anions from the serosa to mucosa. Forskolin (Calbiochem, La Jolla, CA) (5 or 20 µmol/l) was serosally added to stimulate CFTR activity. VX-770 (5 µmol/l), a CFTR channel opener was added to the apical side. CFTRinh$_{172}$ (CF Foundation Therapeutics; 50 µmol/l), a CFTR-specific chloride channel inhibitor, was added to the apical side quantifying CFTR involvement in transepithelial Cl currents. In addition, glibenclamide (500 µmol/l), a general Cl transport inhibitor that blocks both CFTR and non-CFTR Cl transport, was used to determine the amount of non-CFTR current contributed to the overall Cl current. Recordings on matched cultures were performed side-by-side.

Supplementary Material

Figure S1. Characterization of CF1-iPSC lines.
Figure S2. Clonal enrichment of CF-iPSCs corrected with SDFs and TALENS.
Figure S3. Corrected clone isolation.
Figure S4. Assessment of control iPSCs.
Figure S5. Lineage differentiation of CF1-iPS4c1 cells into inflammatory hematopoietic cells.
Table S1. Antibodies.
Table S2. PCR primers and products.
Table S3. Potential CFTAL-B off-target binding sites and PCR analytical primers.
Materials and Methods

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Supplementary Information accompanies this paper on the Molecular Therapy–Nucleic Acids website (http://www.nature.com/mtna)