

# Efficient Gene Editing at Major CFTR Mutation Loci

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**Cystic fibrosis (CF) is a lethal autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Nuclease-mediated precise gene editing (PGE) represents a promising therapy for CF, for which an efficient strategy that is free of viral vector, drug selection, and reporter enrichment (VDR free) is desirable. Here we compared different transfection methods (lipofectamine versus electroporation) and formats (plasmid DNA versus ribonucleoprotein) in delivering the CRISPR/Cas9 elements along with single-stranded oligodeoxynucleotides (ssODNs) to clinically relevant cells targeting major CFTR mutation loci. We demonstrate that, among different combinations, electroporation of CRISPR/Cas9 and guide RNA (gRNA) ribonucleoprotein (Cas9 RNP) is the most effective one. By using this VDR-free method, 4.8% to 27.2% efficiencies were achieved in creating dF508, G542X, and G551D mutations in a wild-type induced pluripotent stem cell (iPSC) line. When it is applied to a patient-derived iPSC line carrying the dF508 mutation, a greater than 20% precise correction rate was achieved. As expected, genetic correction leads to the restoration of CFTR function in iPSC-derived proximal lung organoids, as well as in a patient-derived adenocarcinoma cell line CFPAC-1. The present work demonstrates the feasibility of gene editing-based therapeutics toward monogenic diseases such as CF.**

## INTRODUCTION

Cystic fibrosis transmembrane conductance regulator (CFTR) is a cyclic AMP (cAMP)-dependent chloride channel at the apical membrane of epithelial cells.<sup>1</sup> Mutations in the human CFTR gene often lead to cystic fibrosis (CF), a lethal autosomal recessive inherited disease.<sup>2</sup> Of over 1,900 mutations that have been discovered, more than 300 are disease causing. The most common CF-causing mutation is a 3-bp deletion resulting in the loss of phenylalanine (F) residue at amino acid position 508 (dF508 or dF), which

accounts for 70% of CF patient alleles, followed by G542X (2.5%) and G551D (2.1%).

CFTR is among the first monogenic disease genes identified almost 30 years ago.<sup>3</sup> A small molecule compound drug, ivacaftor, has gained U.S. Food and Drug Administration (FDA) approval for treating G551D patients with significant benefits;<sup>4</sup> however, the combination use of ivacaftor and lumacaftor<sup>5</sup> or tezacaftor and ivacaftor<sup>6</sup> for treating dF/dF homozygous patients only leads to modest benefits.

Evolved from the conventional gene therapy concept in which one or more copies of a functional gene are inserted into the genome, often with problems such as uncontrollable integration sites and copy number,<sup>7</sup> precise gene editing (PGE) in patient or patient-derived cells represents a promising therapeutic approach toward the cure of monogenic diseases such as CF.<sup>8</sup> On the other hand, targeted mutations in major CFTR loci can be used to establish *in vitro* and animal models of the disease for basic research and drug development. To achieve these goals, a high PGE rate is a prerequisite. Furthermore, especially for future *in vivo* gene correction-based therapeutics, it is desirable that the correction is achieved in one step without using viral vectors, drug selection, or reporter enrichment (VDR free).

Thanks to the development of gene-editing nucleases, first zinc-finger nucleases (ZFNs), then transcription activator-like effector nucleases

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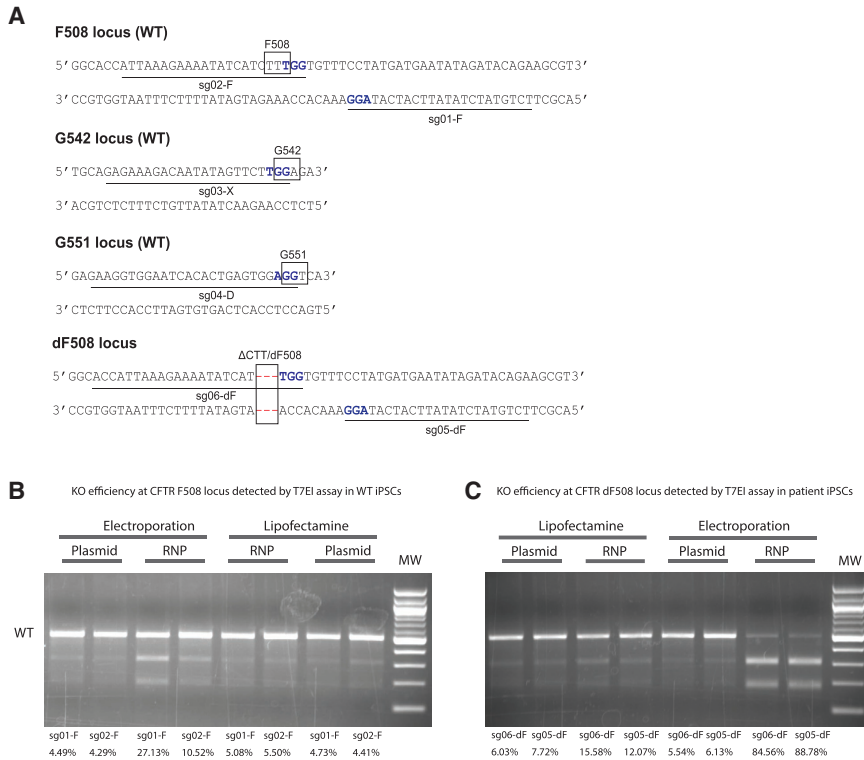
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**Figure 1. Comparison of Different Transfection Protocols to Deliver CRISPR/Cas9 Elements to Human iPSCs**

(A) Illustration of gRNA sequences and positions used in this report. Underlined, gRNA target sequence; blue letters, protospacer adjacent motif (PAM) sequence; boxed, targeted mutation locus. (B) Indel rates at the CFTR F508 locus detected by T7EI assay in wild-type iPSCs. (C) Indel rates at the CFTR dF508 locus detected by T7EI assay in patient cell-derived iPSCs.

ments, either as plasmid DNA (pDNA) or RNP, in clinically relevant cells. We proceeded with the RNP electroporation method to create different CFTR mutations, to correct the dF508 mutation in patient-derived cells, and to test if gene correction of dF508 in patient-derived cells leads to a predicted gain of CFTR function.

## RESULTS

### Electroporation of RNP Is Highly Efficient in Delivering CRISPR/Cas9 Elements to iPSCs

CRISPR/Cas9 elements can be delivered in the format of pDNA or Cas9 RNP. Lipofectamine and electroporation represent the two primary transfection methods. We compared the  $2 \times 2$

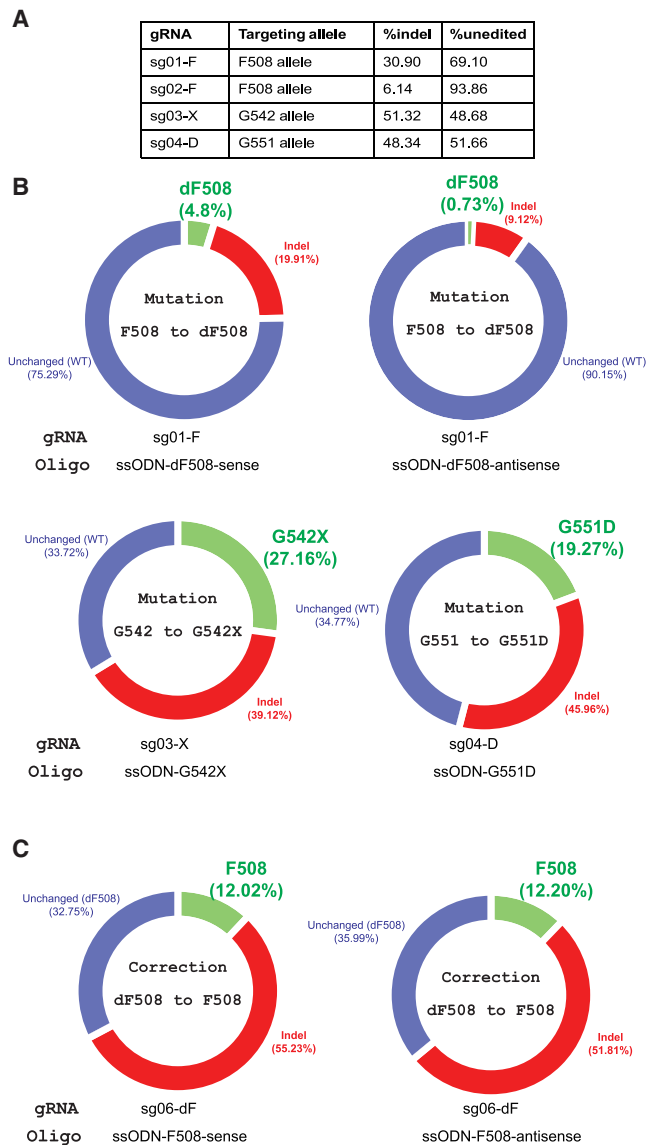
combinations of the transfection methods (lipofectamine  $\times$  electroporation) and the delivery formats (pDNA or Cas9 RNP) in targeting either the F508 locus (by sg01-F or sg02-F) in a wild-type iPSC line or the dF508 locus (by sg05-dF or sg06-dF) in a CF patient-derived iPSC line. A list of guide RNAs (gRNAs) used in the present study is shown in Figure 1A. The T7 endonuclease I (T7EI) assay was employed to detect the insertion and deletion (indel) events. The highest indel rates were achieved by electroporation of Cas9 RNP in both wild-type (27.13% using sg01-F; Figure 1B) and patient iPSCs (88.78% using sg05-dF; Figure 1C), higher than those achieved by other combinations (Figures 1B and 1C). These findings are in agreement with prior reports, including our recent one<sup>17</sup> that electroporation of Cas9 RNP is highly efficient in delivering CRISPR/Cas9 elements to cultured cells.

### Efficient Generation of Major CF-Causing Mutations in Human iPSCs

We next used the electroporation Cas9 RNP method to create three of the most prevalent CF-causing mutations, namely, dF508, G551D, and G542X, in a wild-type iPSC line. Deep sequencing (deepseq) was employed to determine the frequencies of indels, precise mutations, and unchanged (i.e., wild-type). First we validated the targeting efficiencies of gRNAs (sg01-F and sg02-F targeting F508, sg03-X targeting G542, and sg04-D targeting G551) by using Cas9 RNP without donor single-stranded oligodeoxynucleotides (ssODNs) (Figure 2A). Consistent with the T7EI data (27.13%), sg01-F was efficient, leading to a 30.90% indel rate. sg02-F, however, was inefficient with only a

(TALENs), and most recently CRISPR/Cas9,<sup>9,10</sup> highly efficient gene knockout (KO) in human cells and model animals has become a norm; however, the knockin efficiency remains to be further improved. In the context of CF, several groups have attempted to genetically correct the dF508 mutation with limited success. Without any drug selection, Schwank et al.<sup>11</sup> reported an  $\sim 0.2\%$  correction rate in human intestine stem cells using CRISPR/Cas9, and Suzuki et al.<sup>12</sup> obtained an  $\sim 0.1\%$  correction rate using TALEN in iPSCs in the first step, which was increased to  $>10\%$  after 5–6 rounds of enrichment. Even with puromycin selection, Camarasa and Gálvez<sup>13</sup> only achieved a  $<0.01\%$  correction rate using TALEN in iPSCs. Crane et al.<sup>14</sup> corrected dF508 mutation in patient-derived iPSCs using ZFN with puromycin selection, but the efficiency was not reported. Most recently in 2018, Valley et al.<sup>15</sup> reported the establishment of a CRISPR/Cas9-based gene-editing pipeline for creating CF-causing mutations (e.g., dF, G542X, and W1282X) in primary cells, but the editing efficiency was not reported. The highest known rate of correction (16.7%) was achieved by Firth et al.<sup>16</sup> using CRISPR/Cas9 in CF patient-derived iPSCs; notably, however, the correction was achieved in two steps and utilized both puromycin selection and a GFP reporter. It is clear that a one-step VDR-free method to efficiently correct CFTR mutation is yet to be established.

We recently reported efficient PGE by electroporation of CRISPR/Cas9 ribonucleoprotein (RNP) to human stem and primary cells.<sup>17</sup> In the present work, we compared the electroporation method with lipofectamine-mediated transfection in delivering CRISPR/Cas9 ele-



**Figure 2. Creating Mutations at Major CFTR Loci**

(A) Indel efficiencies of gRNAs targeting the F508, G542, or G551 locus determined by deepseq. (B) Efficiencies in creating dF508, G542X and G551D mutations determined by deepseq. (C) Efficiencies in correcting dF508 mutation determined by deepseq.

6.14% indel rate. High indel rates were achieved by sg03-X (51.32%) and sg04-D (48.34%).

We proceeded with sg01-F, sg03-X, and sg04-D along with their corresponding ssODNs (Table S2) to create CF-specific mutations. The deepseq results revealed that 4.8% of alleles were mutated to dF508 using sg01-F and ssODN-dF508-sense, while the remainder consisted of 19.91% various indels and 75.29% wild-type. Interestingly, using the same gRNA (sg01-F) but a different oligonucleotide (oligo; ssODN-dF508-antisense), we only got <1% dF508 mutation rate, signifying

the necessity in testing multiple donor templates for knockin experiments (Figure 2B). It also appears that certain loci are more amenable for nuclease-mediated PGE: 27.16% of alleles were mutated to G542X by using sg03-X and ssODN-G542X, and 19.27% alleles were mutated to G551D by using sg04-D and ssODN-G551D (Figure 2B), both higher than the mutation rates achieved at the F508 locus.

### Efficient Correction of dF/dF Mutations in Patient-Derived iPSCs

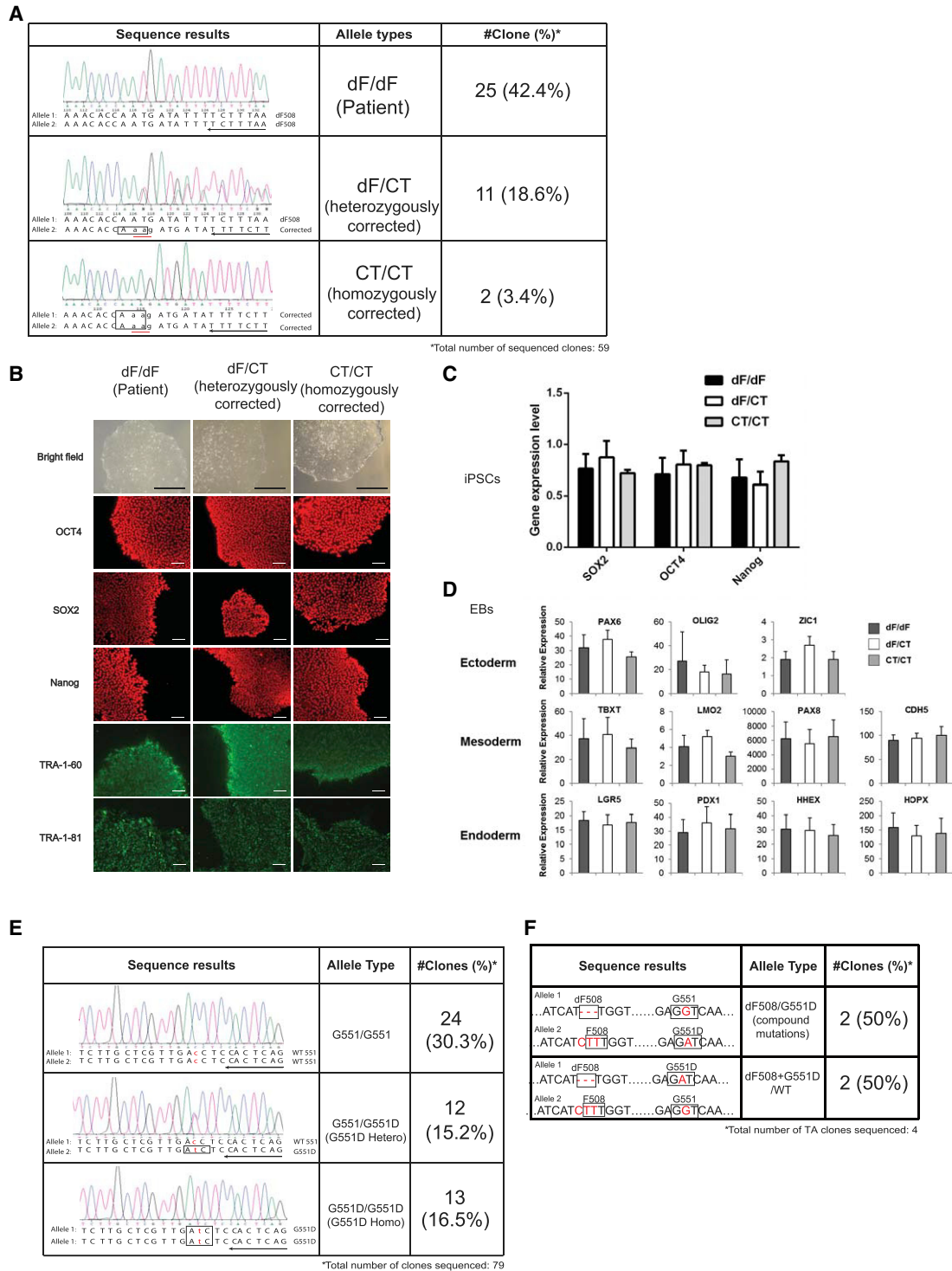
We then investigated if efficient correction of the most common dF508 mutation can be achieved by electroporation of Cas9 RNP. A patient-derived iPSC line (ACS-1004, ATCC) carrying the homozygous dF mutation was used. sg06-dF, which has been validated with greater than 80% indel rates (Figure 1C), was used as the targeting gRNA. Two different donor ssODNs (ssODN-F508-sense and ssODN-F508-antisense; Table S2) were compared (Figure 2C). Consistent with the T7EI assay, deepseq results showed that a high percentage (>60%) of alleles were edited (indel or PGE) at the dF locus and efficient correction of the dF mutation was achieved at comparable levels by both the sense (12.02%) and antisense ssODN (12.20%). These results demonstrate that greater than 10% PGE rates of the dF alleles in patient-derived iPSCs can be achieved by the electroporation of Cas9 RNP method.

### Establishment of Isogenic iPSC Lines Carrying Different CFTR Mutations

We next worked to establish gene-corrected iPSC lines from the patient-derived dF/dF iPSCs using the sg06-dF and sense oligo (ssODN-F508-sense) combination. After electroporation, the cells were cultured and subsequently subjected to limited dilution to establish single-cell clones. Genomic DNA from individual clones was analyzed for PGE events. Of the total 59 clones that were sequenced, we obtained 11 (18.6%) heterozygously corrected (dF/CT) and 2 (3.4%) homozygously corrected (CT/CT) lines (Figure 3A). The correction efficiency was 12.7% at the allele level (15/118) and 22.0% (13/59) at the cellular level.

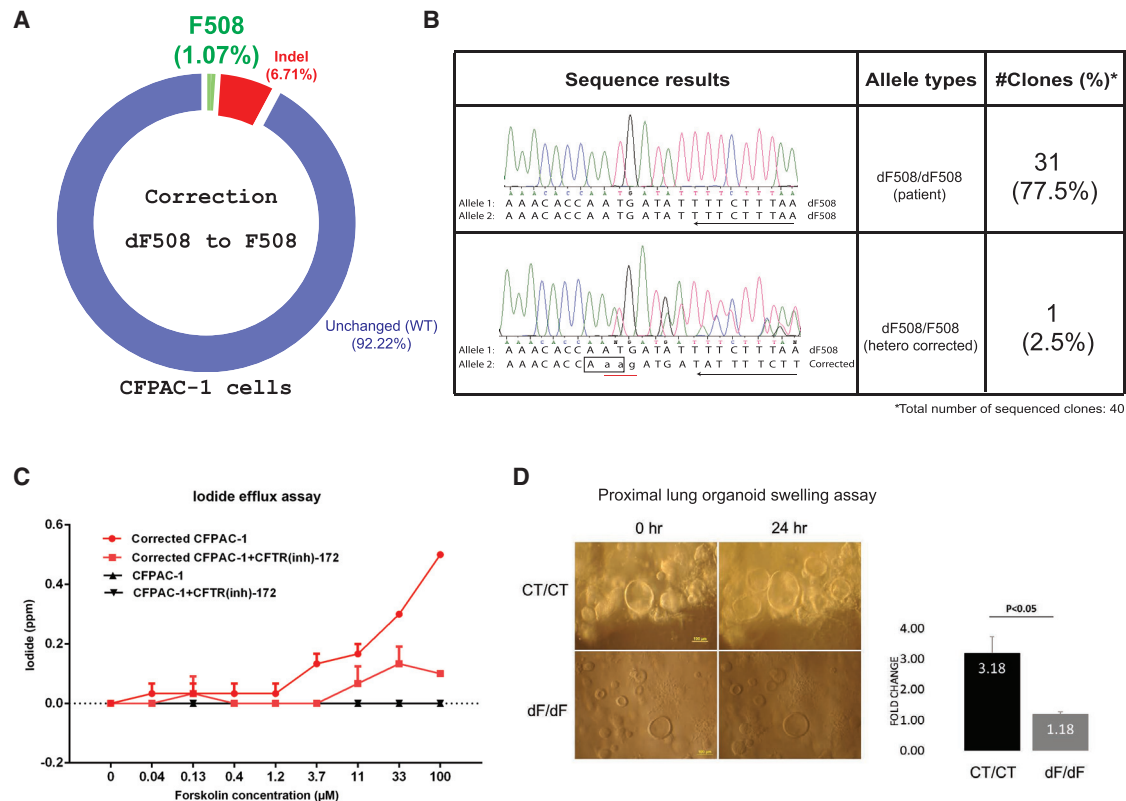
We selected one dF/CT and one CT/CT iPSC line for continuous culture, and we examined typical pluripotency markers in these cells. Both lines expressed pluripotent markers OCT4, SOX2, Nanog, TRA-1-60, and TRA-1-81, as determined by immunocytochemistry staining (Figure 3B). Consistently, qRT-PCR revealed that the expression profiles of pluripotency genes Oct4, Sox2, and Nanog in the dF/CT and CT/CT iPSCs are indistinguishable from those of the original patient-derived dF/dF iPSCs (Figure 3C). The differentiation capacity of gene-corrected (dF/CT and CT/CT) iPSC lines, in comparison with the original dF/dF iPSC line ( $n = 1$  for each genotype), was evaluated by *in vitro* differentiation (Figure S1A). Germ layer-specific markers, as determined by qRT-PCR (Figure 3D) and immunostaining (Figure S1B), were at similar levels among dF/dF, dF/CT, and CT/CT embryoid bodies (EBs). These results indicate that nuclease-mediated gene correction did not compromise the stem cell quality.

To further exploit this system, we subjected dF/CT iPSCs to sg05-D and the corresponding donor oligo ssODN-G551D with the goal of establishing an iPSC line carrying the compound heterozygous dF and G551D mutations. We screened 79 single-cell-derived clones,



**Figure 3. Establishment of Gene-Corrected and Compound Mutation iPSC Lines from dF/dF Patient-Derived iPSCs**

(A) Efficiencies in generating heterozygously (dF/CT) and homozygously (CT/CT) corrected iPSC clones from a patient- (dF/dF) derived iPSC line. (B) Immunofluorescent staining of pluripotency markers in iPSCs. Scale bar, 100  $\mu$ m. (C) Expression of pluripotency genes in iPSCs. Data are presented as mean  $\pm$  SEM. (D) Germlayer-specific transcript levels in EBs derived from iPSC lines. Data are presented as mean  $\pm$  SEM. (E) Efficiencies in generating G551D mutation clones from a dF/CT iPSC line. (F) Percentage of clones that carry the G551D/dF heterozygous compound mutations among four heterozygous G551D clones.



**Figure 4. Restoration of CFTR Function in Gene-Corrected CFPAC-1 Cells and iPSC-Derived Proximal Lung Organoids**

(A) Correction efficiencies in patient-derived CFPAC-1 cells determined by deepseq. (B) Efficiency in establishing a heterozygously corrected (dF/CT) CFPAC-1 cell clone. (C) Iodide efflux assay using uncorrected CFPAC-1 (dF/dF) and heterozygously corrected (dF/CT) CF-PAC1 cells. Data are presented as mean  $\pm$  SEM. (D) Forskolin-stimulated swelling assay using gene-corrected iPSC-derived proximal lung organoids. Data are presented as mean  $\pm$  SEM. Left, bright-field images of pLOs; right, quantitative fold change of pLO size between CT/CT and dF/dF genotypes. Scale bar, 100  $\mu$ m.

of which 12 (15.2%) carried the G551D mutation in one allele and 13 (16.5%) in both alleles (Figure 3E). The total mutation rate was 24.1% (38 of 158) at the allele level. We isolated total RNAs from four G551D heterozygous clones, obtained cDNA, and used them for TA cloning to exclude the ones where G551D and dF508 mutations fell on the same allele. Sequence analysis of TA clones confirmed that two of the four analyzed clones (50%) carried the compound heterozygous dF/G551D mutations, with the G551D (and only G551D) mutation on one allele and the dF508 (and only dF508) mutation on the other allele (Figure 3F). We selected one clone for continued culture and established a dF/G551D iPSC line.

Through this work, we established isogenic iPSC lines that carry dF/CT, CT/CT, and dF/G551D alleles from the original patient-derived dF/dF iPSC line. These cells may serve as useful tools for basic research and drug screening.<sup>18</sup>

#### Gene Correction Restores CFTR Function in CFPAC-1 Cells and iPSC-Derived Proximal Lung Organoid Cells

To test whether genetic correction of the dF allele would restore the chloride channel function in CFTR-expressing cells, we first worked

on a patient-derived dF/dF adenocarcinoma cell line, CFPAC-1 (CRL-1918, ATCC). The same strategy of gene correction (i.e., electroporation, RNP, sg06-dF, and ssODN-F508-sense) that was validated in the dF/dF iPSCs was employed. The deepseq results show that 1.07% of alleles were precisely corrected (Figure 4A), lower than that achieved in iPSCs (12.02%; Figure 2C). Nevertheless, we successfully obtained one heterozygously corrected (dF/CT) CFPAC-1 clone of the 40 that were screened (2.5%) (Figure 4B). The corrected (dF/CT) and the uncorrected (dF/dF) CFPAC-1 cells were then subjected to the iodide efflux assay (IEA) (Figure 4C), which is a commonly utilized simple method to monitor CFTR channel function because few channels other than CFTR can conduct iodide ions.<sup>19</sup> As expected, little iodide efflux, indicated by iodide concentrations in the supernatant, could be detected after the uncorrected dF/dF CFPAC-1 cells were stimulated with CFTR agonist Forskolin, at concentrations ranging from 0 to 100  $\mu$ M. On the contrary, in the corrected dF/CT cells, the iodide efflux was obvious in a Forskolin dose-dependent pattern (Figure 4C). Furthermore, such efflux was partially or totally blocked when CFTR inhibitor CFTR(inh)-172 was used, confirming that the functional correction was achieved through the genetic correction of the dF mutation.

To confirm the IEA, we conducted the Premo Halide Sensor Assay, a fluorescent protein-based method to measure CFTR chloride channel activity,<sup>20</sup> in the dF/dF and CFTR-corrected (dF/CT) CFPAC-1 cells. Consistent with IEA results, the decrease in fluorescence, an indicator for CFTR channel function, was only observed in the corrected CFPAC-1 + Forskolin group, but not in the uncorrected CFPAC-1 cells with or without Forskolin stimulation and not in the corrected CFPAC-1 cells without CFTR agonist Forskolin (Figure S2). Collectively, these results show that the CFTR channel function was rescued by CRISPR/Cas9-mediated gene correction in the CFPAC-1 cells.

We next followed a recently published method,<sup>21</sup> and we established proximal lung organoids (pLOs) using the patient-derived dF/dF and gene-corrected CT/CT iPSCs. It has been reported that pLOs carrying wild-type CFTR alleles would respond to Forskolin stimulation by expansion in the organoid sphere size in a swelling assay, whereas dF/dF pLOs would have only minimal changes in size.<sup>21</sup> Indeed, we observed a greater than 3-fold size expansion in the CT/CT pLOs 24 h after Forskolin stimulation, in sharp contrast to the little change (1.18-fold) in the dF/dF pLOs (Figure 4D). These observations are consistent with those by McCauley et al.,<sup>21</sup> in which they reported a 1.7- to 2.1-fold increase in wild-type and dF/CT pLOs versus a 1.0- to 1.2-fold size change in the dF/dF pLOs 20 h post-Forskolin stimulation. Our work, for the first time, demonstrates that homozygous (CT/CT) correction restored CFTR function in iPSC-derived pLO cells.

## DISCUSSION

One clinically relevant finding of the present work is that greater than 20% of dF/dF patient-derived iPSCs can be repaired, either heterozygously (18.4%) or even homozygously (3.6%), by the CRISPR/Cas9 method. The repair is achieved in a one-step VDR-free manner by electroporation of Cas9 RNP, the first to our knowledge to reach this efficiency and a magnitude higher than those achieved by many prior efforts.

In this work, we also demonstrate that as high as 27% precise editing rates can be achieved in major CF-causing loci. We consider that the following factors may have contributed to the high PGE efficiencies. First, we used Cas9 RNP and ssODN. It has been widely accepted that, compared to pDNA, Cas9 RNP and ssODN are easier to deliver to the cells thanks to their smaller sizes.<sup>22,23</sup> Second, we introduced protospacer adjacent motif (PAM) mutations in all the donor ssODNs (Table S2), which have been shown to significantly improve the overall mutation efficiencies.<sup>17</sup>

Several novel cell lines were generated in this project, including the isogenic dF/dF, dF/CT, CT/CT, and dF/G551D iPSC lines. Although iPSCs express little CFTR, upon proper differentiation they may be used to produce lung organoids, airway epithelial cells, or other cell types that can be used in regenerative medicine therapy for CF or as tools for basic and translational research. These isogenic lines are different only in the CF-causing loci; hence, they are expected to reduce or eliminate compounding genetic variations that are often

encountered when cell lines derived from different individuals are used in research and development. Of note, we generated an iPSC line carrying the compound heterozygous mutations of dF508 and G551D for the first time, which may contribute to the drug development for the rare percentage of CF patients carrying such compound heterozygous mutations.

One concern for CRISPR/Cas9-based therapy is the off-target editing. We evaluated potential off-target mutations that fall on intragenic regions in the genetically corrected lines in the present work (Table S3). No off-target mutations were detected. Although this result indicates that CRISPR/Cas9-mediated corrections of the dF508 mutation comes with low off-target risks in the present work, we agree that whole-genome sequencing is needed for clinical applications of CRISPR/Cas9-corrected cells.<sup>24</sup>

In sum, we show that major CFTR mutation loci can be efficiently edited, often at efficiencies greater than 10%, in clinically relevant cells by electroporation of Cas9 RNP. Using this one-step, VDR-free PGE method, we corrected the dF508 mutation in human iPSCs and CFPAC-1 cells. Several lines of isogenic iPSC lines carrying different CFTR mutations, including a compound heterozygous dF508/G551D line, were generated. Our work demonstrates the feasibility of gene-editing therapeutics toward monogenic diseases such as CF.

## MATERIALS AND METHODS

### Cell Culture

#### Human iPSCs

Human wild-type (ACS-1030) or dF/dF (ACS-1004) iPSCs were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in feeder-free condition in mTeSR1 (85850, STEMCELL Technologies, Vancouver, Canada) on Matrigel-354277, Corning, Corning, NY, USA) coated cultureware surface. For transfection, cells were dissociated with Accutase (07920, STEMCELL Technologies) to single cells. To improve survival, 10  $\mu$ M rho-associated coiled-coil kinase (ROCK) inhibitor Y27632 (Y-5301, LC Labs, Woburn, MA, USA) was added to the transfected cells and removed after the cells were attached.

#### CFPAC-1 Cells

CFPAC-1 cells were purchased from ATCC (CRL-1918). Cells were cultured in DMEM (11995065, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) (FBS1824-003, Nucleus Biologics, San Diego, CA, USA). For transfection, cells were dissociated with 0.25% trypsin (25200-056, Thermo Fisher Scientific) to single cells. Special caution was taken not to agitate the cells by hitting or shaking the flask while waiting for the cells to detach to avoid clumping.

#### gRNA Synthesis

All primers were ordered from Integrated DNA Technologies (Coralville, IA, USA). Primer pairs were annealed to PCR assemble the gRNA DNA template and then synthesize the gRNA by *in vitro* transcription, using a T7-Scribe Standard RNA IVT Kit (C-ASF3107,

Cellsript, Madison, WI, USA). The gRNA was then purified using the GeneJET RNA purification column supplied with the kit, and the concentration was measured.

### Electroporation

Single-cell suspensions were prepared as indicated in [Cell Culture](#). Cells were resuspended in electroporation buffer (13-0104, Cellectris, Manassas, VA, USA) to  $25 \times 10^6$  cells/mL. For Cas9 RNP transfection, 10  $\mu$ g Cas9-NLS protein (1074182, Integrated DNA Technologies, Coralville, IA) was pre-mixed with 3.3  $\mu$ g gRNA at room temperature for 10 min first, and then the formed RNP complex was mixed with the cells and transferred to a 120- $\mu$ L electroporation tube (12-0104, Cellectris). For plasmid DNA transfection, 1.2  $\mu$ g pDNAs expressing Cas9 (42230, Addgene, Watertown, MA, USA) and 0.4  $\mu$ g pDNAs expressing sgRNA (53188, Addgene), instead of Cas9 RNP, were added to the cells. In knockin experiments, 10  $\mu$ g ssODN was added to the mixture. A list of ssODNs is provided in [Table S2](#). The electroporation conditions were 620 V for 30 ms using a tube electroporator machine (CTX-1500A LE, Cellectris). After electroporation, the cells were immediately transferred back to warm medium to continue culture.

### Lipofectamine Transfection

Single-cell suspensions were prepared as indicated in [Cell Culture](#). Approximately 300,000 iPSCs were resuspended in 1 mL mTeSR1 medium (85850, STEMCELL Technologies) supplemented with Y-27632 before they were used for transfection. Lipofectamine transfection was conducted using Lipofectamine 2000 Transfection Reagent (11668-019, Thermo Fisher Scientific), following the manufacturer's instructions with minor modification. For plasmid transfection, a total of 1.6  $\mu$ g pDNAs consisting of 1.2  $\mu$ g pDNAs expressing Cas9 and 0.4  $\mu$ g pDNAs expressing sgRNA were used. The Lipofectamine (4  $\mu$ L) and pDNAs (1.6  $\mu$ g) were each diluted in 100  $\mu$ L Opti-MEM I Reduced Serum Medium (31985070, Thermo Fisher Scientific), before they were mixed together (the lipid-DNA complex) and incubated for 15 min at room temperature. The lipid-DNA complex (200  $\mu$ L) was then added to the resuspended cells, and the total mix was transferred to one well of a 12-well plate. For Cas9 RNP transfection, 2  $\mu$ g Cas9 protein and 0.67  $\mu$ g sgRNA were mixed to form the RNP. Next, the RNP and 4  $\mu$ L Lipofectamine were each diluted in 100  $\mu$ L OPTI-MEM, before they were mixed together. The remaining steps were similar to that of plasmid transfection. At 2 days after transfection, we collected cells to isolate genome DNA.

### T7EI Assay

The T7EI assay was conducted as previously described.<sup>17</sup> Briefly, the purified PCR products were denatured and re-annealed and digested with T7EI (M0302L, New England Biolabs, Ipswich, MA, USA) for 30 min at 37°C, and then run in 2% agarose gel. Non-perfectly matched DNA (presumably indel sites) would be recognized and cleaved by T7EI, leading to two cleaved bands, whereas the perfectly matched DNA would not be recognized and cleaved by T7EI, hence leading to only one band (the wild-type band). The ratio of the inten-

sity of the average of the two cleaved bands over that of the wild-type band was used as a quantitative estimate for indel efficiency.

### Immunofluorescent Staining

#### iPSCs

The iPSCs were fixed with 4% paraformaldehyde (PFA) in DPBS for 20 min for immunofluorescent staining, following our routine protocol.<sup>25</sup> Primary antibodies included the following: OCT4 (1:100, 09-0023, ReproCELL, Beltsville, MD, USA), SOX2 (1:100, 09-0024, ReproCELL), Nanog (1:100, 09-0020, ReproCELL), TRA-1-60 (1:100, 09-0010, ReproCELL), and TRA-1-81 (1:100, 09-0011, ReproCELL). Secondary antibodies included Alexa Fluor goat anti-mouse 488 (A11029, Thermo Fisher Scientific) and goat anti-rabbit 647 (ab150079, Abcam, Cambridge, MA, USA).

#### EBs

The EBs were fixed with 4% PFA in DPBS for 20 min for immunofluorescent staining, following our routine protocol.<sup>26</sup> Primary antibodies included the following: Alexa Fluor 488 CD56 (1:200, MHCD5620, Thermo Fisher Scientific), VE-Cadherin (1:200, AF938, R&D Systems, Minneapolis, MN), and SOX17 (1:400, 698501, BioLegend, San Diego, CA, USA). Secondary antibodies included the following: Alexa Fluor 488 Donkey anti-Goat IgG (H+L) Secondary Antibody (1:200, A11055, Thermo Fisher Scientific) and Alexa Fluor 488 Goat anti-Mouse IgG (H+L) Secondary Antibody (1:200, A11029, Thermo Fisher Scientific).

### In Vitro Differentiation

The EBs were derived from iPSCs by *in vitro* differentiation, as previously described.<sup>26</sup> Briefly, gently scraped human iPSCs were transferred to an ultra-low attachment plate. Cells were maintained in suspension to generate EBs in EB medium consisting of 20% KnockOut SR (10828010, Thermo Fisher Scientific), 2 mM GlutaMAX Supplement (35050061, Thermo Fisher Scientific), 0.1 mM MEM Non-Essential Amino Acids Solution (11140076, Thermo Fisher Scientific), and 0.1 mM 2-Mercaptoethanol (31350010, Thermo Fisher Scientific) in KnockOut DMEM/F-12 (12660012, Thermo Fisher Scientific). EB medium was changed every day. After 5 days of growth suspension, EBs were transferred onto Matrigel- (354277, Corning) coated plates (353047, Corning) to allow for attachment. The entire EB plate was harvested on day 8 for qRT-PCR to confirm germlayer markers.

### IEA

IEA was conducted as previously described.<sup>19</sup> Briefly, 24-well plates containing cells were incubated with iodine-loading buffer (150 mM NaI, 2 mM CaCl<sub>2</sub>, 0.8 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, and 5.4 mM knock-in (KI), adjusted to pH 7.4 with 1 N sodium hydroxide) for 4 h at 37°C. After washing, CFTR activator Forskolin (S2449, Selleck Chemicals, Houston, TX, USA) at concentrations ranging from 0 to 100 mM was added in the culture medium, with or without the presence of 20  $\mu$ M CFTR inhibitor CFTR(inh)-172 (C2992, Sigma-Aldrich, St. Louis, MO), for 5 min. Supernatant iodine concentration was measured with a modified Sandell-Kolthoff reaction and expressed as a function of Forskolin concentration.<sup>19</sup>

### Premo Halide Sensor Assay

Premo Halide Sensor Assay in dF/dF CFPAC-1 or gene-corrected dF/CT CFPAC-1 cells was conducted using a commercial kit (P10229, Thermo Fisher Scientific), following the manufacturer's instructions. Briefly, 5.5 mL Premo Halide sensor transduction solution was added to  $5 \times 10^6$  cells to allow baculovirus-mediated gene delivery of a Venus variant of *Aequorea victoria* GFP, whose fluorescence is sensitive to local halide concentration, in particular iodide. The cells were incubated at room temperature for 4 h with gentle rotation, before culture medium supplemented with 1:1,000 dilution of the enhancer was added after removing the transduction solution. The cells were then cultured in the enhancer solution for 2 h at 37°C. After removing the enhancer medium, the cells were cultured in culture medium overnight, before they were treated with or without stimuli (i.e., Forskolin). The fluorescence was measured at 1 min prior to (−1 min), immediate upon (0 min), and every min until 13 min (1–13 min) after the stimulation. The relative fold change of fluorescence over that at 0 min was calculated and used as an indicator of CFTR channel function.

### pLO Swelling Assay

pLOs were established following the protocol that McCauley et al.<sup>21</sup> recently described. Briefly, iPSCs were sequentially differentiated into definitive endoderm, anterior foregut endoderm, lung epithelium, and eventually pLOs. In the pLO swelling assay, organoids were incubated with 10 μM Forskolin (S2449, Selleck Chemicals) for 24 h, and they were monitored by time-lapse microscopy. The areas of individual pLOs at 0 and 24 h were measured by ImageJ.<sup>27</sup> The fold change was calculated as the ratio of pLO size at 24 h over that at 0 h and used as an indicator of CFTR function.<sup>21</sup>

### Genotyping and Deepseq

Cells were harvested 48 h after electroporation transfection, and genomic DNA was extracted with the Wizard Genomic DNA Purification Kit (A1120, Promega, Madison, WI, USA). Targeted regions were PCR amplified using high-fidelity PCR master mix (F532L, Thermo Fisher Scientific), with the corresponding primers listed in Table S1. The products were gel purified using Qiaquick gel purification kit (29706, QIAGEN, Germantown, MD, USA) and sequenced. For deepseq, the purified PCR products were sent to the DNA Core at Massachusetts General Hospital (<https://dnacore.mgh.harvard.edu/new-cgi-bin/site/pages/index.jsp>).

### Statistical Analysis

Microsoft Excel (Seattle, WA, USA) was used for all statistical analysis. The Student's t test was used to determine the significances between treatment and control groups; p values < 0.05 were considered significant.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and three tables and can be found with this article online at <https://doi.org/10.1016/j.omtn.2019.02.006>.

### AUTHOR CONTRIBUTIONS

J.X. conceived the idea. J.X., Y.E.C., and F.S. designed the experiments. J.R., H.H., D.Y., L.M., X.H., H.J., H.W., C.R., H.M., G.W., and J.Z. conducted the experiments. J.R., H.H., and J.X. analyzed the data. J.R., H.H., K.L., F.S., and J.X. wrote the manuscript.

### CONFLICTS OF INTEREST

The authors have no potential financial or non-financial conflicts of interest.

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