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Original Article

Comparison of Cas9 and Cas12a CRISPR editing methods to correct the W1282X-CFTR mutation

Lúcia Santos[a,b], Karen Mention[b], Kader Cavusoglu-Doran[b], David J. Sanz[b], Mafalda Bacalhau[a], Miquéias Lopes-Pacheco[a], Patrick T Harrison[b], Carlos M Farinha[a,*]

[a] University of Lisboa, Faculty of Sciences, BioISI - Biosystems & Integrative Sciences Institute, Campo Grande, C8 bdg, Lisboa 1749-016, Portugal
[b] Department of Physiology, University College Cork, Cork T12 K8AF, Ireland

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ABSTRACT

Background: W1282X-CFTR variant (c.3846G>A) is the second most common nonsense cystic fibrosis (CF)-causing mutation in the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene. Even though remarkable breakthroughs have been done towards CF treatment with the approval of four CFTR protein modulators, none of these are approved for patients with nonsense mutations. CRISPR gene editing tools can be of great value to permanently correct the genetic defects caused by these mutations.

Methods: We compared the capacity of homology-directed repair (HDR) mediated by Cas9 or Cas12a to correct W1282X CFTR mutation in the CFF-16HBEge W1282X CFTR cell line (obtained from CFF), using Cas9/gRNA and Cas12a/gRNA ribonucleoproteins (RNPs) and single strand DNA (ssODN) oligonucleotide donors.

Results: Cas9 shows higher levels of correction than Cas12a as, by electroporating cells with Cas9 RNPs and ssODN donor, nearly 18% of precise editing was achieved compared to just 8% for Cas12a. Such levels of correction increase the abundance of CFTR mRNA and protein, and partially restore CFTR function in the pool of edited cells to 18% of WT CFTR function. Moreover, homozygous corrected clones produced levels of mRNA, protein, and function comparable to those of cells expressing WT CFTR.

Conclusion: Altogether, this work demonstrates the potential of gene editing as a therapeutic strategy for CF directly correcting the root cause of the disease.

1. Introduction

Cystic fibrosis (CF) is the most common life-shortening autosomal genetic disorder in Caucasians affecting about 90,000 people worldwide. Despite being a multiorgan disease, the most severe symptoms occur in the lungs, where increased mucus viscosity leads to progressive loss of lung function and ultimately to death. CF is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene which encodes an epithelial chloride and bicarbonate channel [1]. Over 2,100 variants have been so far identified in the *CFTR* gene, of which 8% are nonsense variants [2,3]. The second most prevalent nonsense variant in the *CFTR* gene is W1282X (c.3846G>A), accounting for 1.2% of the alleles worldwide [2]. W1282X-CFTR variant generates a premature termination codon (PTC) which leads to nonsense-mediated decay (NMD) and, consequently, very little or no full length functional CFTR protein is produced [3].

Over the previous decade, four different CFTR protein modulators have been approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) to treat the root cause of CF: one potentiator (VX-770) that increases the channel opening probability at the plasma membrane and is approved for 97 different variants, and three correctors (VX-809, VX-661 and VX-445) that improve protein folding and trafficking of the most prevalent CF variant F508del [4,5]. In total, this means there is a potential treatment for about 85% of people with CF. However, as these drugs act directly on the CFTR protein, they are not suitable for treating people with CF with W1282X and other PTC variants.

Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CRISPR, clustered regularly interspaced short palindromic repeats; HDR, homology-directed repair; Cas, CRISPR-associated protein; RNP, ribonucleoproteins; ssODN, single-stranded oligonucleotide; PTC, premature termination codon; NMD, nonsense mediated decay; PAM, protospacer adjacent motif; DSB, double stranded break; ICE, inference CRISPR edits; HS-YFP, halide-sensitive yellow fluorescence protein; HA, homology arm.

* Corresponding author.
E-mail address: cmfarinha@fc.ul.pt (C.M. Farinha).

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To date, therapeutic approaches for PTC variants have focused on small molecule readthrough drugs and NMD inhibitors. After early evidence that G418 elicited some readthrough, other readthrough molecules have been evaluated with one of them, PTC124 (Ataluren), progressing into clinical trials before failing to meet its clinical endpoint in Phase 3 [6,7]. More recently, ELX-02, a novel readthrough treatment, is under investigation for CF-causing PTC variants [8]. At an early stage of development, there are small molecules that block SMG-1 kinase which then inhibits NMD [9,10]. Despite these efforts, there is still no therapy approved for any nonsense variant leaving these patients in need of a targeted therapy.

An alternative to small molecule approaches is to correct the underlying genetic defect by gene editing. This would provide a one-time cure and avoid a lifelong treatment [11]. *Streptococcus pyogenes* Cas9 (SpCas9) has been the most widely used nuclease to edit the *CFTR* gene, particularly using homology-directed repair (HDR) to correct the F508del variant [12]. Nevertheless, other CRISPR-nucleases, for instance *Acidaminococcus* Cas12a (AsCas12a), have emerged as an alternative for Cas9 and its efficacy has also been demonstrated to correct a splicing variant in the *CFTR* gene [13]. Both nucleases recognize a protospacer adjacent motif (PAM) and create a double strand break (DSB) at a targeted genomic region. The default mechanism to repair the DSB is called non-homologous end joining (NHEJ) and leads to random nucleotide insertion and deletion, so called indels, in the DNA sequence (or seamless repair which is not possible to quantitate). However, in the presence of a donor sequence comprising the corrected nucleotide flanked by homology arms HDR can also occur, resulting in the removal of the mutant nucleotide and copying of the corrected nucleotide into the genome [14]. The two CRISPR systems differ in two main aspects: 1) PAM sequence recognition - while Cas9 recognizes an NGG PAM, the Cas12a recognizes a TTTV PAM; and 2) cleavage site - Cas9 creates blunt cut 3 nucleotides (nt) upstream the PAM site whereas Cas12a generates staggered cut 23 nt (target strand)/18 nt (non-target strand) downstream of the PAM recognition site [15]. Hence, comparing these two proteins is of interest in the context of *CFTR* gene editing.

Here, we compared these two CRISPR methods for correction of the W1282X-CFTR variant in the CFF-16HBEge W1282X-CFTR cell line [16]. To this end, we used HDR-mediated gene editing with HiFi-SpCas9 or AsCas12a (hereinafter Cas9 and Cas12a), both using ribonucleoproteins (RNPs) and single strand DNA oligonucleotide donor (ssODN). Our results show that Cas9 had ~2.3-fold higher percentage of precise editing than Cas12a and lower indels frequency. Characterization of the Cas9 edited cells showed that the editing resulted in an increase in CFTR mRNA abundance and restoration of full-length CFTR protein. Importantly, CFTR function to levels close to wild type were detected in the edited cells and 18% of WT in the edited pool. Altogether, our data shows that the two different CRISPR editing systems can successfully correct W1282X-CFTR and demonstrate the potential of gene editing as a possible treatment not only for patients carrying W1282X-CFTR but also other nonsense CF-variants.

2. Materials and methods

2.1. Reagents

Alt-R® CRISPR-Cas9 and Alt-R® CRISPR-Cas12a reagents (S.p. HiFi Cas9 Nuclease V3, A.s. Cas12a V3 and crRNA), and ssODN HDR donor templates (Ultramer® DNA Oligos) were purchased from Integrated DNA Technologies (IDT). Cas9 single guide RNA (sgRNA) was purchased from Synthego. Genotyping and quantitative real-time PCR primers were obtained from Eurofins Genomics and Stab-Vida, respectively. gRNA and ssODN HDR donor sequences can be find on Table 1.

Table 1
Guide RNA and ssODN HDR donor sequences for W1282X variant correction with Cas9 or Cas12a nucleases.

Nuclease	Guide RNA (5' – 3')	Sequence of ssODN HDR donor template (5' – 3')
Cas9	CAATAACTTTGCAACAGTGA	TCAAATAGCAGTAAAAAA TATAATTTAGTTGCCT TTTTTCTGGCTAAGTCCT TTTGCTCACCTGTGGTA TCACTCCAAAGG CTTTCCTCCACTGTTGCA AAGTTATTGAATCCCAA GACACACCATC
Cas12a	CAACAGTGAAGGAAAGCCTT	ACTGAACACTGAAGGAGA AATCCAGATCGATGGTGTGTCTT GGGATTCAATAACTCTGCAA CAGTGGAGGAAAGCCTTT GGAGTGATACCACAGG TGAGCAAAAGGACTTAGCC

2.2. Cell lines and culture conditions

16HBE14o$^-$ Human Bronchial Epithelial cells, expressing WT CFTR, were obtained from Children's Hospital Oakland Research Institute, UCSF, USA [17]. CFF-16HBEge W1282X CFTR cell line was obtained from the Cystic Fibrosis Foundation (CFF) [16]. Cells were grown in Minimum Essential Medium (MEM, Sigma) supplemented with 10% (v/v) of Foetal Bovine Serum (FBS, Sigma) and 1% (v/v) of L-Glutamine (Sigma) in a 37°C, 5% CO_2 humidified incubator. Culture plates and flasks were coated by incubating a coating solution [Dulbecco's Phosphate Buffered Saline (DPBS, Sigma) and 10 µL/mL of PureCol® Solution (Advanced BioMatrix)] at 37 °C/5% CO_2 for at least 2 h.

2.3. Ribonucleoparticles (RNP) formation and nucleofection

Guide RNAs for CRISPR-Cas9 and CRISPR-Cas12a genome editing were diluted to a final concentration of 44 µM and 75 µM, respectively. Each gRNA was combined with the respective nuclease protein at a 1:1.2 molar ratio for RNP complex formation. For both CRISPR editing approaches 16HBE14o- cells were nucleofected using the Neon^{TM} Transfection System (Thermo Fisher Scientific) and the Neon^{TM} Transfection System 10 µl kit. For each reaction, 200,000 cells were resuspended in 5 µl of Buffer R, mixed with 7 µl of Cas9 RNP or Cas12a complex and electroporated with or without ssODN donor using the following conditions: Voltage – 1290 V, Width – 20 ms, Pulses - 2 pulses. For the co-transfection of Cas9 RNP or Cas12a RNP and HDR template, three different ssODN donor concentrations (0.6μM, 2μM and 6μM) were added to the RNP:cells mixture before the nucleofection step. The cells were plated in a 24-well plate and 72 h after electroporation, a portion of cells was harvested for further analysis while the other portion was kept in culture.

2.4. Analysis of gene editing events in the pool of edited cells

Genomic DNA (gDNA) was extracted using DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer instructions. The target site was amplified using Q5 Hot Start High-Fidelity 2x Master Mix (New England Biolabs) using the following primers: fwd: 5'-TGGGAAGAACTGGATCAGGGAAG-3'; rev: 5'-TCGCAAAGCATTTCTCAACCTGG-3'. PCR products were purified, and Sanger sequenced (Eurofins Genomics). To analyse the gene editing events, Sanger sequencing files from unedited and edited cells

were used as input into the Inference of CRISPR Edits (ICE) webtool (2019, v2.0. Synthego).

2.5. Single cell sorting of Cas9-edited cells and identification of the gene edited clones

The pool of edited cells was single cell sorted into individual wells of a 96-well plate using the FACS Aria Fusion cell sorter (BD Biosciences). Wells containing only one cell were identified and maintained as mentioned above until reaching confluency. Approximately 20-30 days after sorting, cells were split into matching wells of two 96-well plates. At ~50% confluency, plate 1 was processed for gDNA with QuickExtract DNA Extraction Solution (Epicenter). The target site was amplified in a PCR reaction using the same primers as mentioned above and sequenced. Out of forty clones screened two homozygous corrected clones were identified, expanded from 96-well plate 2 and cryopreserved.

2.6. Quantitative PCR analysis

Total RNA was isolated from cells with the NZY Total RNA Isolation kit (NZYTech) according to manufacturer's instructions. Next, 1 µg of RNA was used to cDNA synthesis using M-MuLV Reverse Transcriptase (NZYTech) following manufacturer's instructions. *CFTR* specific products were amplified using Evagreen SsoFast PCR reagent (Bio-Rad) on the CFX96 Touch real-time PCR detection system (Bio-Rad) using the following pair of primers: CFTR (fwd:5'-ATGCCCTTCGGCGATGTTTT-3'; rev:5'-TGATTCTTCCCAGTAAGAGAGGC-3'). Endogenous levels of GAPDH were used as internal control (fwd:5'-ATGGGGAAGGTGAAGGTCG-3'; rev:5'-GGGGTCATTGATGGCAACAATA-3'). The fold difference in gene expression was calculated by the mathematical equation $2^{-\Delta\Delta CT}$.

2.7. Western-blot analysis

Whole cell lysates were size-separated onto a 10% (w/v) SDS-PAGE gel. A monoclonal anti-human CFTR antibody [570 (1:3000), CFF Therapeutics] was used to detected CFTR protein and an anti-Calnexin antibody (1:3000, BD Biosciences) was used as a loading control. CFTR quantification was performed using Image Lab software (Bio-Rad).

2.8. Generation of stable cell lines expressing Halide-sensitive Yellow Fluorescent Protein (HS-YFP) and HS-YFP assay to measure CFTR activity

Cell lines stably expressing YFP protein were generated by lentiviral transduction of the 16HBE cell lines, as described previously [18]. To this end YFP cDNA was cloned into the lentiviral expression vector pLVX-Puro that was used to transfect the packaging 293 T cell line for lentiviral particle production. These particles were then used to transduced 16HBE cell lines.

CFTR activity was measured on these newly developed cell lines as described [19]. Briefly, 24h after seeding on microplates the cells were incubated with G418 (100 µM), SMG1i (0.3 µM) or the two compounds together, or without any treatment. DMSO was used as a vehicle control. At the time of the assay, the cells were washed with PBS and stimulated with forskolin (20 µM) plus VX-770 (10 µM) for 30 min at 37 °C. The assay consisted of a continuous 14 s fluorescence reading, 2 s before and 12 s after injection of an iodide-containing solution (final concentration of I^- in the well was 100 mM). Data were normalized to the initial background-subtracted fluorescence and the fluorescence quenching rate, associated with I^- influx, was determined by fitting the final 11 s of the data for each well to an exponential function to extrapolate initial slope (dF/dt).

2.9. Statistical analysis

All graphs were plotted as the mean with error bars representing the standard error of the mean. Differences between groups were assessed by two-tailed unpaired t test. *P*-values < 0.05 were considered as statistically significant. Graph plotting and statistical analysis were performed using GraphPad Prism version 6.01.

3. Results

3.1. Design of Cas9 and Cas12a gRNAs and ssODN donors to correct the W1282X-CFTR variant by HDR

Given the different PAM requirements between the SpCas9 and AsCas12a and the creation of DSB in DNA, the design strategy of the gRNA and donor DNA template was different for the two proteins (Fig. 1A and B). According to previous studies that HDR efficiency is reduced when the target site is further away from the cutting site, we selected the PAM site closest to W1282X-CFTR for both nucleases [20]. Based on previous studies, we designed an ssODN donor with a 36-nt 3' homology arm (HA) and 91-nt 5' HA complementary to the non-target strand for Cas9 (Fig. 1A), and an ssODN donor template which is complementary to the target strand with a 5' and 3' homology arms of 77-nt and 37-nt, respectively, for Cas12a (Fig. 1B) [21,22]. The PAM site in Cas12a donor was modified to prevent cutting and indels formation after correction; a silent mutation was introduced and the Cas12a 5'-TTTV-3' PAM was changed to 5'-TCTV-3'.

3.2. CRISPR/Cas9 induces higher HDR levels than CRISPR/Cas12a to correct the W1282X-CFTR variant

To correct W1282X-CFTR by HDR, we electroporated Cas9/gRNA or Cas12a/gRNA RNPs into the W1282X-CFTR cell line and measured the level of correction and indels 72 hrs later by ICE analysis. In the absence of an ssODN donor Cas9/gRNA RNPs generated 12.7±1.7% of indels while the Cas12a gRNA only generated 2.7±0.7% of indels (Fig. 1C and D). When Cas9 RNPs were electroporated with three concentrations (0.6 µM, 2 µM and 6 µM) of the ssODN donor, ICE analysis revealed that the highest level of HDR (17.7±2.1%) and indels (20.3±3.0%) was observed with 2 µM ssODN donor (Fig. 1C). In contrast, Cas12a RNPs and donor repaired W1282X-CFTR at a much lower rate with the highest HDR efficiency being 8.7±0.5% with 6 µM of ssODN and generated a much higher indel frequency of 35.3±1.0% (Fig. 1D).

After evaluating editing at DNA level, we determined the effect on the mRNA transcripts. Compared to the DNA changes, at the mRNA level we observed a higher percentage of corrected transcripts and lower percentage of transcripts containing indels for both Cas9 and Cas12a. For Cas9 HDR we observed 60-70% of WT transcripts in all three editing conditions, whereas for Cas12a HDR, the maximum level of WT mRNA was 50% and this only occurred at the highest level of editing, a level which also resulted in the highest level of indels (Supplemental Fig. 1A and B). Thus, it was decided to further characterize these Cas9-edited cells.

3.3. Correction of W1282X-CFTR cells by HDR results in increased CFTR mRNA abundance

We first measured absolute level of mRNA expression by RT-qPCR for the pool of Cas9 RNP/2µM donor cells which yielded 17.7% of gene correction (Figs. 1C and 2A). As expected, we observed a decrease in the levels of CFTR mRNA in W1282X-CFTR cells to 43.3±0.07% of the wild type levels, consistent with previous reports (Fig. 2B) [23,24]. In the pool of edited cells, we ob-

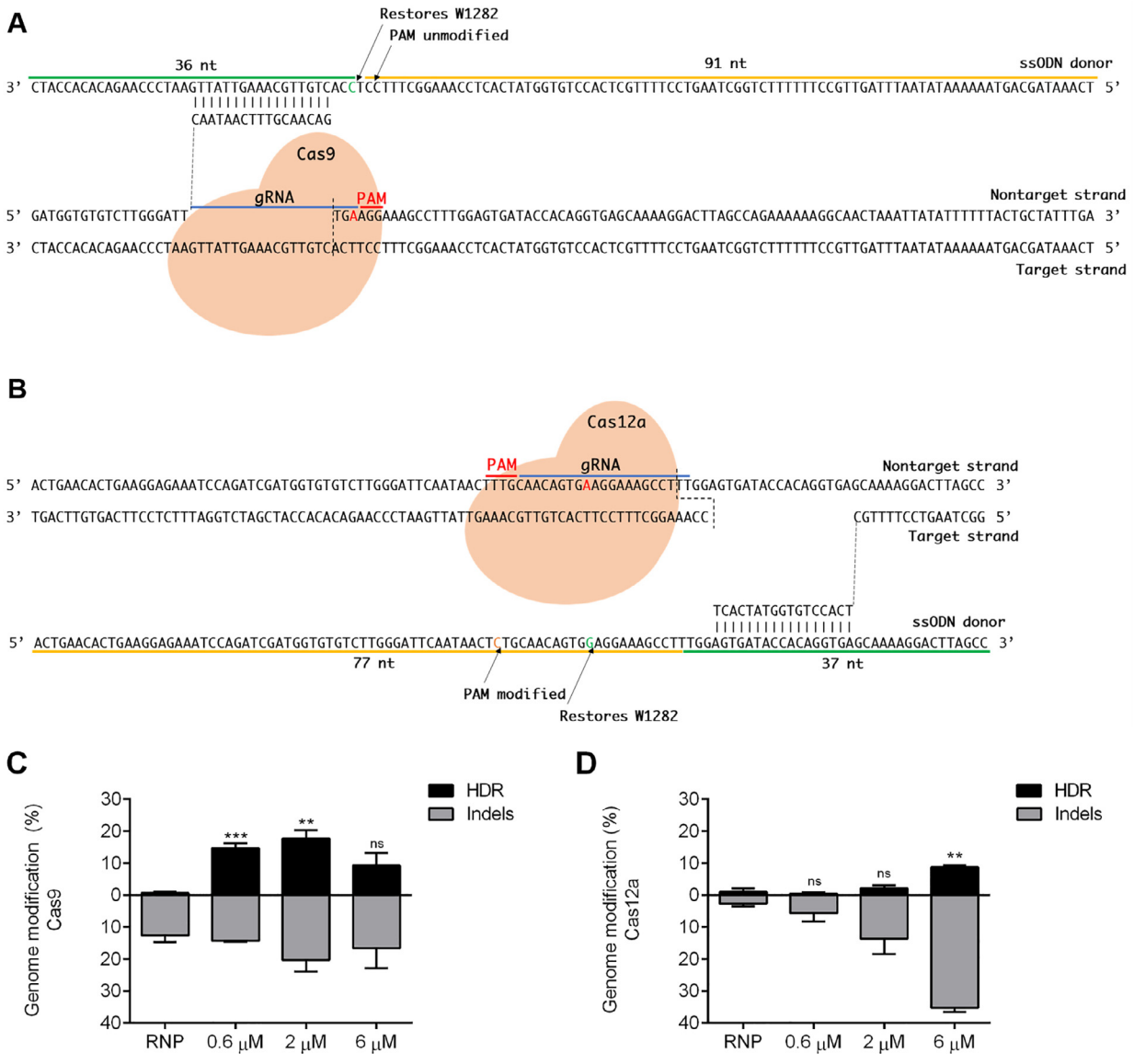

Fig. 1. Comparison of CRISPR/Cas9 and CRISPR/Cas12a gene editing capacity to correct W1282X variant in human bronchial cells. Design of gRNA and single-stranded oligonucleotide (ssODN) donor for (A) CRISPR/Cas9 and (B) CRISPR/Cas12a HDR-mediated gene editing. Cas9 or Cas12a nuclease (in light orange) recognizes PAM sequence (in red) and after gRNA (in blue) binding makes a blunt or a staggered cut, respectively, in the double-stranded DNA. Cas9 and Cas12a prefer ssODN donor templates of opposite orientations. While with Cas9 a donor template complementary to the nontarget strand was used, with Cas12a was used a donor template complementary to the target strand. Both donor templates have a longer 5' homology arm (HA, in yellow) and shorter 3'HA (in green). In the Cas12a donor the 5'-TTTV-3' PAM was changed to 5'-TCTV-3' (in orange). Genome modification generated by (C) Cas9 and (D) Cas12a after RNPs electroporation into CFF-16HBEge W1282X CFTR cell line, was assessed by ICE software analysis and shown as percentage of HDR or indels. RNP, corresponds to the electroporation of Cas/gRNA alone; 0.6 μM, 2 μM, 6 μM; corresponds to the electroporation of Cas/gRNA and the respective molar concentration of ssODN donor (mean ± SEM, n=3 biological replicates). Statistical significance of HDR *Vs.* RNP: $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, ns: not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

served a small but significant increase in the levels of CFTR mRNA expression to 53.1±0.07% of the WT (Fig. 2B)

We then decided to isolate clonal homozygous HDR corrected populations from the pool of edited cells by seeding 1 cell/well in a 96-well plate. We obtained two corrected clonal cell lines confirmed by sequencing (Fig. 2A). The CFTR mRNA levels in these two homozygous corrected clones were fully recovered to WT levels (Fig. 2B), demonstrating that gene editing can increase the abundance of CFTR mRNA.

3.4. Correction of W1282X-CFTR cells by HDR restores CFTR protein expression and function

To assess the ability of CRISPR/Cas9 gene editing to restore CFTR protein expression and function, we generated 16HBE cells stably expressing HS-YFP with all the genotypes (WT, W1282X, pool and the two corrected clones). As previously observed, full-length CFTR protein was not detected in the W1282X-CFTR cells, whereas the WT-CFTR cells and the corrected clones exhibited mature, fully glycosylated protein (band C, Fig. 3A–C) [16]. Importantly, this mature form was also detected in the pool of edited cells confirming the correction (Fig. 3A) to about 10.8±3.04% of the WT (Fig. 3B).

CFTR function was measured by the quenching rate of HS-YFP induced by iodide influx (Fig. 4A). CFTR function in W1282X-CFTR cells was greatly reduced. However, in the two homozygous clones it reached nearly the same levels as in cells expressing wild-type CFTR (Fig. 4B). Interestingly, CFTR function was restored to nearly 18% of the WT in the pool of edited cells (Fig. 4B). We also compared the editing with the ability of G418 and SMG1i, to elicit some functional correction in W1282X-CFTR and in the pool of

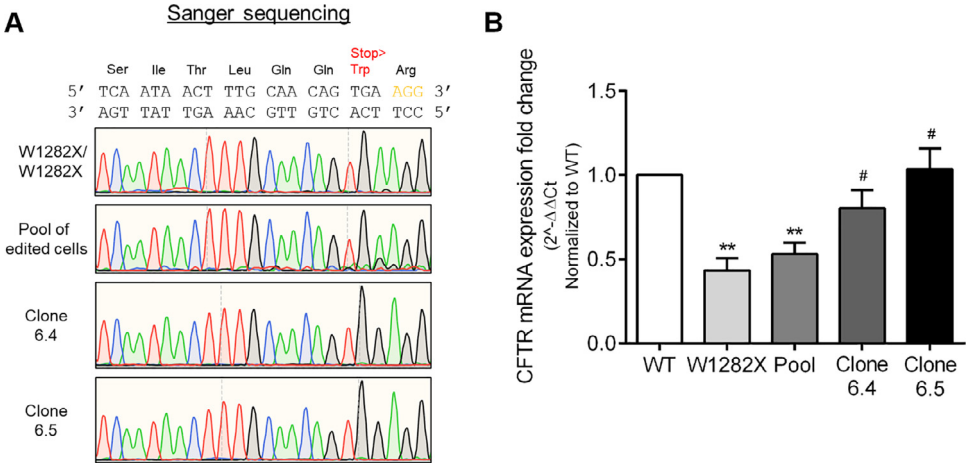

Fig. 2. CFTR mRNA levels increase after CRISPR/Cas9 gene editing. (A) Sequence correction, with gRNA sequence (PAM sequence in yellow) in the four cell populations – in the pool of edited cells there are some overlapped sequences resulting from indels and HDR coexistence; two homozygous corrected clones have the WT sequence. (B) CFTR mRNA expression levels determined by RT-qPCR. Fold-change values are mean ± SEM, relative to WT or W1282X cells (n = 3 biological replicates). *Vs.* WT: $^{**}P \leq 0.01$. *Vs.* W1282X: $^{\#}P \leq 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) .

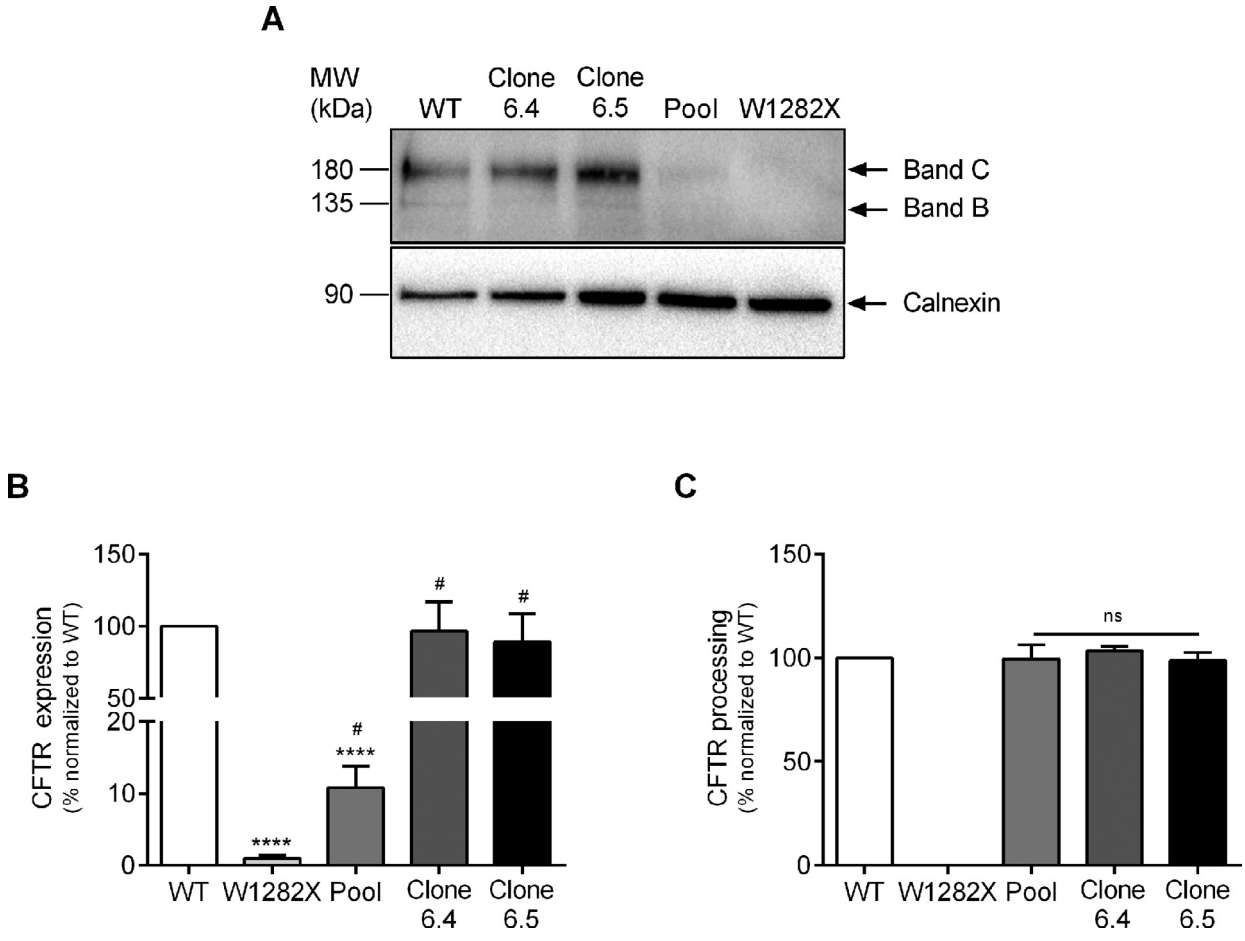

Fig. 3. Gene editing restores CFTR protein expression and processing. (A) Western Blot (WB) showing levels of CFTR protein expression in 16HBE WT, W1282X, pool of edited cells and homozygous clones (Clones 6.4 and 6.5) stably expressing HS-YFP protein. (B) Total CFTR protein levels (C+B) were quantified from (A) and normalized to calnexin (loading control). (C) CFTR processing (C/C+B) was also determined, and results shown normalized to WT-CFTR. In all cases, fold-change values are mean ± SEM, relative WT or W1282X cells ($n = 3$ biological replicates). *Vs.* WT: $^{****}P \leq 0.0001$, ns: no significant. *Vs.* W1282X: $^{\#}P \leq 0.05$.

edited cells. Although there is a trend increase in CFTR function with the combined treatment, these compounds have an almost negligible effect in rescuing W1282X-CFTR in these cells (Fig. 4C).

Overall, our findings demonstrate that editing of the *CFTR* gene by CRISPR/Cas9 is capable to restore protein expression, processing, and function without needing additional drugs.

4. Discussion

Reduced or absent expression of functional CFTR protein caused by NMD makes the approved CFTR modulators inapplicable to patients harbouring the W1282X-CFTR variant. Drug screening has been focused on agents that promote NMD inhibition and PTC

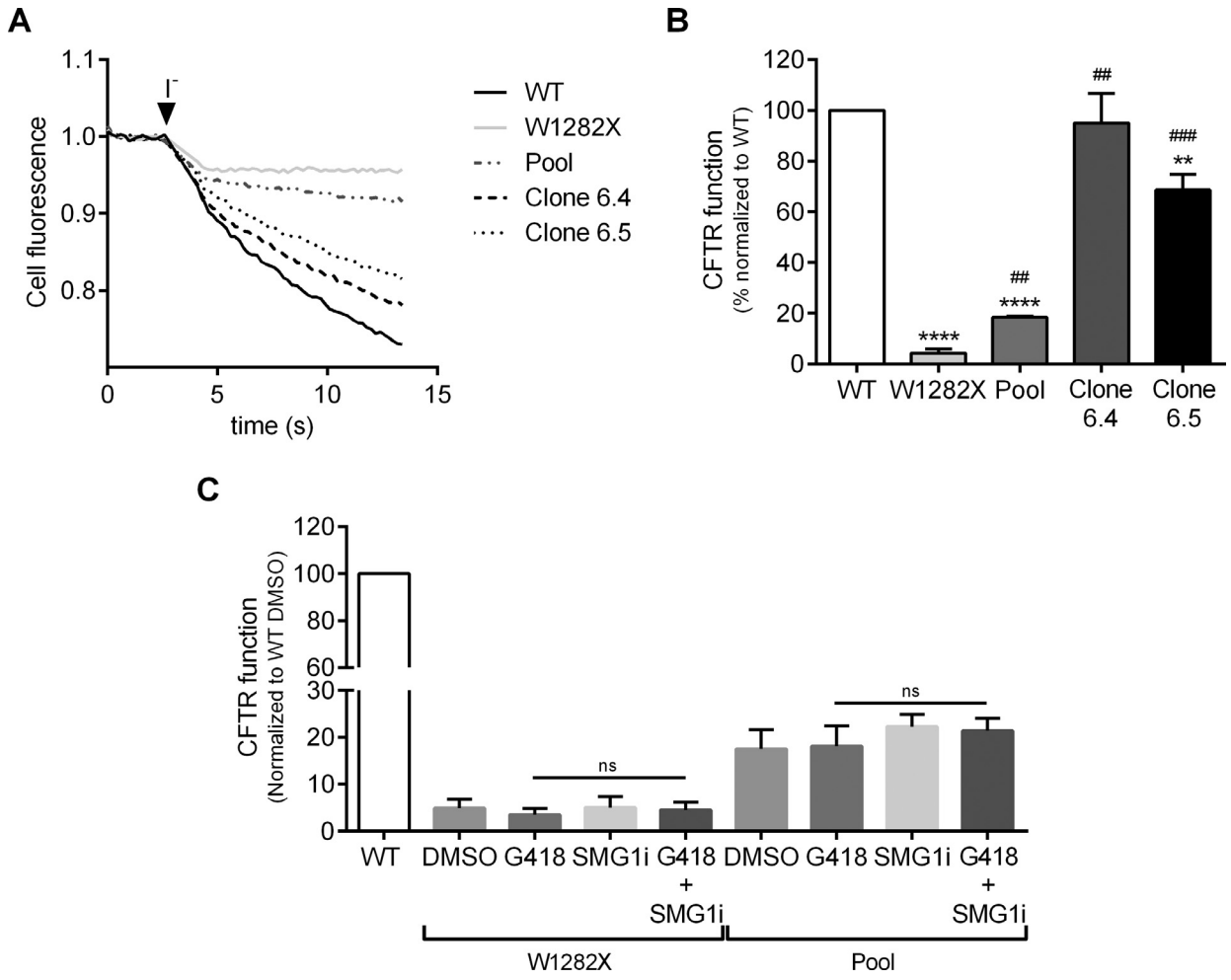

Fig. 4. Rescue of CFTR function inn edited cells. (A) Representative fluorescence decay in 16HBE cells stably expressing HS-YFP and incubated for 24 h with DMSO (vehicle), G418 (100 μM), SMG1i (0.3 μM) or the two in combination. The cells were then stimulated with Fsk (20 μM) and VX-770 (10 μM) for 30 min. (B) CFTR activity on edited cells was quantified based on the rate of YFP quenching and normalized to WT cells. (C) CFTR activity on W1282X and pool cells normalized to WT treated with DMSO. Data are shown as means ± SEM of n=3 biological replicates. *Vs.* WT: $^{**}P \leq 0.01$, $^{****}P \leq 0.0001$. *Vs.* W1282X: $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$. *Vs* DMSO: ns: not significant.

readthrough. Without candidates reaching the clinics, gene editing techniques emerge as a one-time cure from which every patient could benefit regardless the genotype. Here, we compare Cas9 and Cas12a homology directed repair of the W1282X-CFTR variant and report high editing efficiency of the mutant *CFTR* locus in human bronchial cells rescuing CFTR protein expression and function.

As we compared the efficacy of Cas9 and Cas12a to correct the W1282X-CFTR variant using RNPs and ssODNs, we observed 18% HDR with Cas9 RNP while Cas12a was only able to achieve 8% of correction. The lower HDR efficiency of Cas12a could possibly be explained by its capacity to cleave collateral ssDNA – called trans-cleavage activity - without the presence of a PAM sequence [25]. Since the donor template is a ssDNA, Cas12a undesired activity may reduce HDR efficiency. Also, the use of different donor DNA sequences for each nuclease can impact HDR efficiency. Regarding the indels formation in non-corrected alleles, Cas12a generated ~ 36% of indels while those levels with Cas9 only reached 20%. Taken all the data together our results show that compared with Cas12a, Cas9 achieved higher levels of HDR and lower levels of indels with lesser amounts of the editing reagents. However, our results do not exclude the possibility that Cas12a may perform better than Cas9 at another locus. Given its different PAM site recognition, Cas12a could be a suitable option at sites where a Cas9 PAM is not available. An example of this is the R1162X (c.3484 C>T), the fourth most common nonsense mutation in the *CFTR* gene. Even though off-target effects were not analysed genome-wide, no off-target mutations caused by CRISPR gene editing were identified within CFTR coding sequence. Prediction of potential off-target effects (Suppl. Table 1) also shows that only four are located in exonic regions, all of which have four mismatches (two of them in the core region), and without any indels detected (Suppl Fig. 2).

Characterization of the Cas9-edited cells showed complete rescue of CFTR mRNA, protein, and function in two corrected homozygous clones. To minimize the effects of a single cell cloning, we also decided to analyse the pool of edited cells. As proposed by Suzuki and colleagues, by achieving gene editing efficiency sufficient to restore CFTR function the corrected cells could be engrafted in the airways of CF-patients [26]. Assessment of editing efficiency in the pool of edited cells showed 18% of gene correction, similar to a study also using Cas9 RNP and ssODN donor to correct F508del in a patient-derived induced pluripotent cell line (iPSC) [12]. While the level of correction needed to have a clinical benefit is not yet defined, previous studies have shown that even lower levels of wild type *CFTR* transcripts, ranging between 5-8%, would be sufficient to rescue, or at least ameliorate, the CF phenotype [27,28]. According to that, our 18% of correction efficiency, resulting in 18% of wild type CFTR function, might have a therapeutic benefit. Notably, the level of CFTR function achieved with CRISPR/Cas9 gene editing, even if underestimated, greatly exceeds function resulting from G418/SMG1i treatment.

Despite this success in Cas9 editing and rescue of CFTR expression and function, a significant frequency of unwanted indels was also observed in 20% of the *CFTR* alleles. Even though the indels are likely to create a PTC whose transcripts are degraded, confirmed by its nearly absence at the mRNA level (Supplemental Fig. 1A), examination of such indels would be necessary as it may increase disease susceptibility.

Whereas there are two studies exploring the use of gene editing to precisely correct W1282X-CFTR variant, those are focused on base editing [29,30]. Importantly, one of these studies using the same W1282X-CFTR cell line reports a percentage of editing and a restoration of the CFTR protein to levels similar to ours [30]. Our study is the first comparing Cas9 and Cas12a nucleases to correct an "untreatable" CFTR mutation. Even though we show higher levels of correction with Cas9 than those with Cas12a for this particular variant, it is likely that editing efficiency could rely on the genomic context so further studies should be performed at different CFTR loci to evaluate Cas9 and Cas12a editing efficiency. In summary, our work demonstrates the therapeutic potential of gene editing to correct W1282X-CFTR which can be expanded to other nonsense mutations, and even to other monogenic diseases besides cystic fibrosis.

Declaration of Competing Interest

There is no conflict of interest related to this work.

CRediT authorship contribution statement

Lúcia Santos: Conceptualization, Investigation, Methodology, Formal analysis, Writing - original draft. **Karen Mention:** Conceptualization, Investigation, Formal analysis. **Kader Cavusoglu-Doran:** Investigation, Formal analysis. **David J. Sanz:** Investigation, Formal analysis. **Mafalda Bacalhau:** Investigation, Formal analysis. **Miquéias Lopes-Pacheco:** Investigation, Formal analysis. **Patrick T Harrison:** Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing - review & editing. **Carlos M Farinha:** Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing - review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2021.05.014.

References

[1] De Boeck K, Amaral MD. Progress in therapies for cystic fibrosis. Lancet Respir Med 2016;4:662–74 Lancet Publishing Groupp.
[2] Clinical and Functional Translation of CFTR, CFTR2 variant list history. CF Foundation, Johns Hopkins University, The Hospital for Sick Children. 2020.
[3] Clarke LA, Awatade NT, Felício VM, Silva IA, Calucho M, Pereira L, et al. The effect of premature termination codon mutations on CFTR mRNA abundance in human nasal epithelium and intestinal organoids: a basis for read-through therapies in cystic fibrosis. Hum Mutat 2019;40(3).
[4] Lopes-Pacheco M. CFTR modulators: the changing face of cystic fibrosis in the era of precision medicine. Front Pharmacol 2020;10.
[5] HIGHLIGHTS OF PRESCRIBING INFORMATION—KALYDECO® (Ivacaftor) tablets, for oral use. 2020. [Internet]. [cited 2021 Feb 6]. Available from: https://pi.vrtx.com/files/uspi_ivacaftor.pdf
[6] Konstan MW, VanDevanter DR, Rowe SM, Wilschanski M, Kerem E, Sermet–Gaudelus I, et al. Efficacy and safety of ataluren in patients with nonsense-mutation cystic fibrosis not receiving chronic inhaled aminoglycosides: the international, randomized, double-blind, placebo-controlled Ataluren Confirmatory Trial in Cystic Fibrosis (ACT CF). J Cyst Fibros 2020;19(4).
[7] Howard M, Frizzell RA, Bedwell DM. Aminoglycoside antibiotics restore CFTR function by overcoming premature stop mutations. Nat Med 1996;2(4):467–9.
[8] Kerem E. ELX-02: an investigational read-through agent for the treatment of nonsense mutation-related genetic disease. Expert Opin Investig Drugs 2020.
[9] Keenan MM, Huang L, Jordan NJ, Wong E, Cheng Y, Valley HC, et al. Nonsense–mediated RNA decay pathway inhibition restores expression and function of W1282X CFTR. Am J Respir Cell Mol Biol 2019;61(3):290–300.
[10] Laselva O, Eckford PD, Bartlett C, Ouyang H, Gunawardena TN, Gonska T, et al. Functional rescue of c.3846G>A (W1282X) in patient-derived nasal cultures achieved by inhibition of nonsense mediated decay and protein modulators with complementary mechanisms of action. J Cyst Fibros 2020;19(5):717–27 Sep 1.
[11] Hodges CA, Conlon RA. Delivering on the promise of gene editing for cystic fibrosis. Genes Dis 2019;6(2):97–108 [Internet]Available from. doi:10.1016/j.gendis.2018.11.005.
[12] Ruan J, Hirai H, Yang D, Ma L, Hou X, Jiang H, et al. Efficient gene editing at major CFTR mutation loci. Mol Ther-Nucl Acids 2019;16:73–81 Jun 7.
[13] Maule G, Casini A, Montagna C, Ramalho AS, de Boeck K, Debyser Z, et al. Allele specific repair of splicing mutations in cystic fibrosis through AsCas12a genome editing. Nat Commun 2019;10(1) Dec 1.
[14] Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. Nat Protoc 2013;8(11):2281–308.
[15] Banakar R, Schubert M, Collingwood M, Vakulskas C, Eggenberger AL, Wang K. Comparison of CRISPR-Cas9/Cas12a ribonucleoprotein complexes for genome editing efficiency in the rice phytoene desaturase (OsPDS) gene. Rice 2020;13(1) Dec 1.
[16] Valley HC, Bukis KM, Bell A, Cheng Y, Wong E, Jordan NJ, et al. Isogenic cell models of cystic fibrosis-causing variants in natively expressing pulmonary epithelial cells. J Cyst Fibros 2019;18(4):476–83 Jul 1.
[17] Haws C, Krouse ME, Xia Y, Gruenert DC, Wine JJ. CFTR channels in immortalized human airway cells. Am J Physiol-Lung Cell Mol Physiol 1992;263:6 7-6.
[18] Canato S, Santos JD, Carvalho AS, Aloria K, Amaral MD, Matthiesen R, et al. Proteomic interaction profiling reveals KIFC1 as a factor involved in early targeting of F508del-CFTR to degradation. Cell Mol Life Sci 2018;75(24):4495–509 Dec 1.
[19] Sondo E, Tomati V, Caci E, Esposito AI, Pfeffer U, Pedemonte N, et al. Rescue of the mutant CFTR chloride channel by pharmacological correctors and low temperature analyzed by gene expression profiling. Am J Physiol Cell Physiol 2011;301:872–85. [Internet]Available from: www.ajpcell.org .
[20] Elliott B, Richardson C, Winderbaum J, Nickoloff JA, Jasin M. Gene conversion tracts from double-strand break repair in mammalian cells. Mol Cell Biol 1998;18(1):93–101.
[21] Richardson CD, Ray GJ, DeWitt MA, Curie GL, Corn JE. Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. Nat Biotechnol 2016;34(3):339–44 Mar 1.
[22] Wang Y, Liu KI, Sutrisnoh NAB, Srinivasan H, Zhang J, Li J, et al. Systematic evaluation of CRISPR-Cas systems reveals design principles for genome editing in human cells. Genome Biol 2018;19(1) May 29.
[23] Linde L, Boelz S, Nissim-Rafinia M, Oren YS, Wilschanski M, Yaacov Y, et al. Nonsense-mediated mRNA decay affects nonsense transcript levels and governs response of cystic fibrosis patients to gentamicin. J Clin Invest 2007;117(3):683–92 Mar 1.
[24] Aksit MA, Bowling AD, Evans TA, Joynt AT, Osorio D, Patel S, et al. Decreased mRNA and protein stability of W1282X limits response to modulator therapy. J Cyst Fibros 2019;18(5):606–13 Sep 1.
[25] Li SY, Cheng QX, Liu JK, Nie XQ, Zhao GP, Wang J. CRISPR-Cas12a has both cis- and trans-cleavage activities on single-stranded DNA [Internet]. Cell Res 2018;28:491–3 Available from. doi:10.1038/s41422-018.
[26] Suzuki S, Crane AM, Anirudhan V, Barillà C, Matthias N, Randell SH, et al. Highly efficient gene editing of cystic fibrosis patient-derived airway basal cells results in functional CFTR correction. Mol Ther 2020;28(7):1684–95 Jul 8.
[27] Chu CS, Trapnell BC, Curristin SM, Cutting GR, Crystal RG. Extensive posttranscriptional deletion of the coding sequences for part of nucleotide-binding fold 1 in respiratory epithelial mRNA transcripts of the cystic fibrosis transmembrane conductance regulator gene is not associated with the clinical manifestations of cystic fibrosis. J Clin Invest 1992;90(3):785–90.
[28] Ramalho AS, Beck S, Meyer M, Penque D, Cutting GR, Amaral MD. Five percent of normal cystic fibrosis transmembrane conductance regulator mRNA ameliorates the severity of pulmonary disease in cystic fibrosis. Am J Respir Cell Mol Biol 2002;27(5):619–27 Nov 1.
[29] Geurts MH, de Poel E, Amatngalim GD, Oka R, Meijers FM, Kruisselbrink E, et al. CRISPR-based adenine editors correct nonsense mutations in a cystic fibrosis organoid biobank. Cell Stem Cell 2020;26(4):503–10 Apr 2e7.
[30] Jiang T, Henderson JM, Coote K, Cheng Y, Valley HC, Zhang XO, et al. Chemical modifications of adenine base editor mRNA and guide RNA expand its application scope. Nat Commun 2020;11(1) Dec 1.