EXPERIMENTAL APPROACHES TO THE TARGET EDITING OF THE *CFTR* GENE USING CRISPR-CAS9

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Cystic fibrosis is a severe autosomal recessive disease caused by mutations in the *CFTR* gene. The most common *CFTR* mutation occurring in the European population is F508del. Advances in the management of patients with cystic fibrosis aimed at blocking disease progression have considerably improved the prognosis, but gene therapy has turned to be less effective than expected. Capable of correcting mutations direct in the cells, genome editing, and specifically the CRISPR-Cas9 technology, raises hope of causal treatment for patients with cystic fibrosis. The aim of this work was to compare and improve the efficacy of F508del editing using different combinations of guide RNAs and Cas9. The study was carried out in HEK293T cells. The efficacy of editing was assessed for both plasmid and genomic sites by T7E1 analysis. The best effect was demonstrated by a combination of SaCas9 and sgRNA targeting F508del: 29% of alleles were successfully edited. A combination of SpCas9 and a similar sgRNA showed low efficacy due to the low expression of this guide RNA. All attempts to improve its expression failed. SgRNA stabilization by introducing a G-quadruplex into the sgRNA sequence and adding GG to the 5′-region also did not work. Perhaps, low performance of this guide RNA is determined by its nucleotide sequence, limiting its use.

Keywords: cystic fibrosis, CRISPR-Cas9, *CFTR*, genome editing, F508del mutation, guide RNA

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ЭКСПЕРИМЕНТАЛЬНЫЕ ПОДХОДЫ К ТАРГЕТНОМУ РЕДАКТИРОВАНИЮ ГЕНА *CFTR* С ПОМОЩЬЮ CRISPR-CAS9

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Муковисцидоз — тяжелое аутосомно-рецессивное заболевание, обусловленное мутациями в гене *CFTR*, основной из которых в европейской популяции является F508del. Патогенетическая терапия существенно улучшила прогноз для жизни у пациентов с муковисцидозом, однако генная терапия не оказалась такой эффективной, как ожидалось. Геномное редактирование, в том числе с помощью CRISPR-Cas9, открывает новые возможности для этиотропного лечения, так как позволяет исправить мутации в клетках. Целью исследования было сравнение эффективности коррекции мутации F508del с помощью различных комбинаций направляющих РНК и Cas9 и повышение эффективности редактирования. Работу проводили на культуре клеток HEK293T, эффективность редактирования генома оценивали с помощью анализа T7E1, как на геномном, так и на плазмидном сайтах. Наиболее эффективной оказалась комбинация SaCas9 вместе с РНК на мутацию F508del — произошло редактирование 29% аллелей. Комбинация аналогичной направляющей РНК на F508del для SpCas9 показала небольшую эффективность редактирования, что связано с низкой экспрессией направляющей РНК. Были предприняты попытки увеличения экспрессии данной РНК с помощью разных подходов, однако повышения эффективности ее работы получено не было. Стабилизация направляющей РНК путем добавления в последовательность G-квадруплекса, укорочения и добавления GG в 5′-область также не принесла результатов. Вероятно, низкая эффективность работы использованной направляющей РНК обусловлена ее нуклеотидной последовательностью, что ограничивает ее использование.

Ключевые слова: муковисцидоз, CRISPR-Cas9, *CFTR*, геномное редактирование, мутация F508del, направляющие РНК

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Cystic fibrosis (CF, OMIM#219700) is an autosomal recessive disease caused by mutations in the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene. These mutations result in the impaired transport of chloride and sodium ions across the cell membrane. CF is one of the most common hereditary diseases striking 1 in every 4,500 people. The carrier rate is as high as 1 in 25 [1]. Lung damage is the main clinical symptom of the disease and the major cause of death in patients with CF [2]. Other organs can also be affected, including the pancreas, the liver and the intestines. The most common *CFTR* mutation is F508del. It results in the premature degradation of the encoded protein and its total absence on the cell surface [3]. There have been tremendous advances in the management of CF in the recent decades aimed at blocking disease progression [4–7], but no cure has been found yet.

Genome editing, specifically CRISPR-Cas9, prompts us to take a fresh look at the potential of gene therapies for hereditary conditions [8]. It can be used to correct (or "edit") mutations and eliminate the causes of yet incurable diseases [9–12]. Earlier works describing the attempts of F508del correction by different genome editing techniques [13–20] stimulate discovery of novel approaches to F508del editing. However, a serious drawback of the techniques applied in those studies is their low success rate (<1% cells), which they share with other genome editing tools.

To improve the efficacy of F508del correction, we selected a few guide RNAs specific to the regions flanking the mutation site and introduced a few Cas9 orthologs that had not been used previously for such purposes to design a few combinations of Cas9 + sgRNA and to choose a combination that worked best.

The aim of our study was to compare and improve the efficacy of different combinations of guide RNA and Cas9 in editing the F508del mutation.

METHODS

The initial plasmids for CRISPR-Cas9 were gifts from Feng Zhang (Addgene #71814 and #61591) and Keith Joung (Addgene #72249). Guide RNAs (sgRNAs) for SpCas9, SpCas9(HF4) and SaCas9 were designed using the free-access software developed by Broad Institute (USA; http://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design). The cloned plasmids are shown in Fig.1. To test the performance of the obtained constructs, a 400-nucleotide-long sequence flanking the mutation site on both sides and containing the F508del mutation was cloned into the plasmid pGEM-TA-CFTR, which was then transfected into the cell together with the plasmid expressing Cas9 and sgRNA. HEK293T cells (a gift from Skoblov M.Yu., Laboratory of Functional Genomics, Research Centre for Medical Genetics, Moscow) were cultured in DMEM (PanEco, Russia) supplemented with 10% fetal bovine serum (PAA Laboratories GmbH, Austria), 100U/ml/100μg/ml penicillin/streptomycin and 4 mM L-glutamine (PanEco, Russia). To assess the role of temperature, a part of the transfected cells was cultured at standard 37 °C for 72 h; the rest were cultured in two steps: at 37 °C for 24 h followed by 48 h at 30 °C. Calcium-phosphate transfection of HEK293T cells was performed in 12-well plates at 50% confluence as described in [21]. It total, there were 1.5 μg or 5.5 μg of plasmids per well (1 μg or 5 μg, respectively, of the plasmid expressing Cas9 and sgRNA transfected into the cells together with 0.5 μg of the target plasmid). Six hours after transfection, the medium was replaced with a fresh growth medium supplemented with 10% fetal bovine serum. The pEGFP-C1 plasmid (Clontech, USA) served as a reporter. For DNA isolation we used the Genomic DNA-Tissue MiniPrep kit (ZymoResearch, USA) according to the manufacturer's

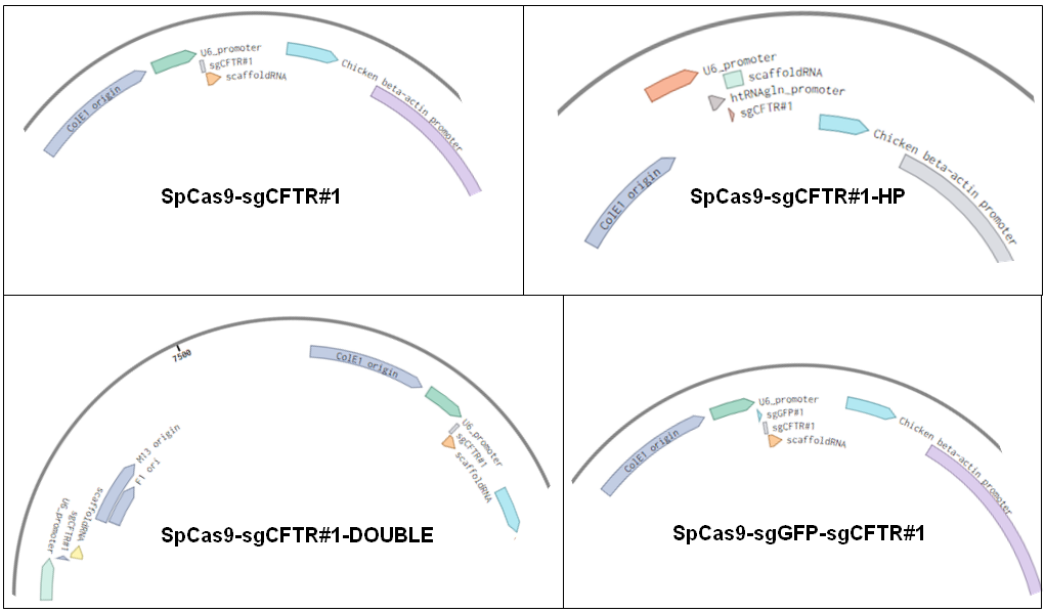

Fig. 1. Maps of synthetic plasmids used for F508del mutation editing in the *CFTR* gene

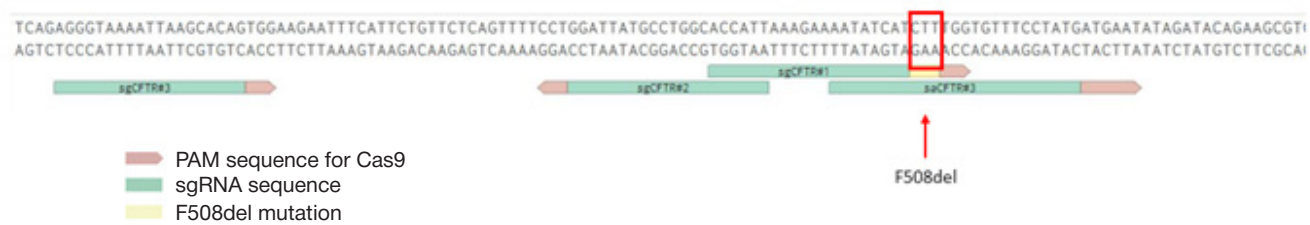

Fig. 2. sgRNAs for the *CFTR* locus used in this study

protocol. T7E1-analysis was carried out as described in [22]: PCR products with anticipated insertions and deletions at the site of a double strand break were heated and immediately cooled down, formation of heteroduplexes was inferred from the presence of extra bands in the electrophoretic gel after the heteroduplexes were treated with endonuclease T7E1.

RESULTS

Editing of the *CFTR* locus

In this work we attempted to compare the efficacy of genomic editing of F508del located in the *CFTR* gene using a few combinations of 2 mutant SpCas9 proteins (eSpCas9(1.1) [23] and SpCas9(HF4) [24]) or SaCas9 [25] and different sgRNAs. For SpCas9 three sgRNAs were selected targeting the sequence of *CFTR* exon 10 harboring F508del (Fig. 2). The first guide RNA sgCFTR#1 precisely targeted the mutation site (in the absence of F508del there was no PAM). The second sgRNA (sgCFTR#2) targeted a region near the mutation and could be used to edit both mutant and wild type alleles. The third sgRNA (sgCFTR#3) was selected for the sequence located 85 nucleotides upstream the mutation [13]. Because SaCas9 requires a different PAM, we selected a different sgRNA (saCFTR#3) for this nuclease, precisely targeting the mutation site. Since HEK293T cells do not have F508del in their genome and the structure of a genomic site presumably affects the efficacy of editing, sgRNAs were tested using a synthetic plasmid containing the *CFTR* locus with the F508del mutation. The synthetic construct was transfected into the HEK293T cells together with the plasmid expressing Cas9 and sgRNA.

The best editing effect was observed for the combination of SaCas9 and saCFTR#3: 29% of alleles were successfully edited (Fig.3). SgCFTR#1 combined with different SpCas9 proteins demonstrated an average success rate of 13%. For sgCFTR#2 the success rate was 18% (16% for the plasmid site and 22% for the genomic site), sgCFTR#3 demonstrated 12% efficacy (6% for the plasmid site and 14% for the genomic site). The editing activity of sgCFTR#1 was comparable to or lower than that exhibited by other guide RNAs, including the control sgGFP targeting the *EGFP* gene (Fig. 3).

Increasing the expression of guide RNAs

Our previous study revealed that low editing efficacy of sgCFTR#1-based systems correlates with its low expression [22]. To improve expression of sgCFTR#1, we inserted an extra cassette consisting of a promoter and sgCFTR#1 (SpCas9–sgCFTR#1–DOUBLE) into the plasmid, but it produced no significant effect on the performance of this guide RNA (Fig. 4). For positive control, we chose sgGFP targeting the *EGFP* gene. Because sgGFP was always known to be expressed vigorously and demonstrated high editing efficacy, we decided to combine it with sgCFTR#1 (SpCas9–sgGFP–sgCFTR#1). Unfortunately, the resulting synthetic sgRNA only negatively affected the efficacy of *CFTR* editing (Fig. 4).

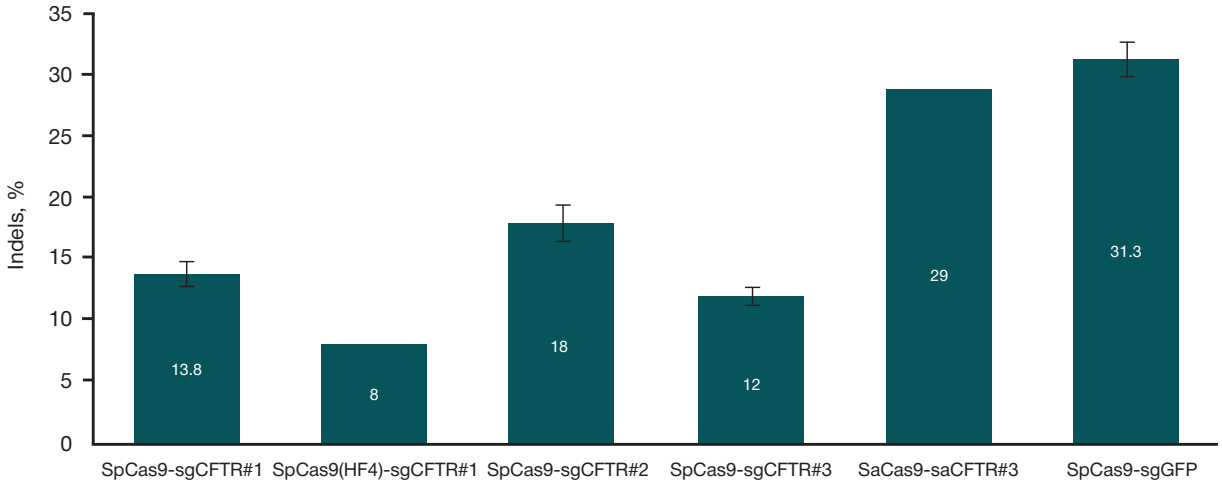

Fig. 3. Efficacy of *CFTR* and *EGFP* editing in HEK293T cells 48–72 hours after transfection. The results are represented as a mean and a standard error of the mean

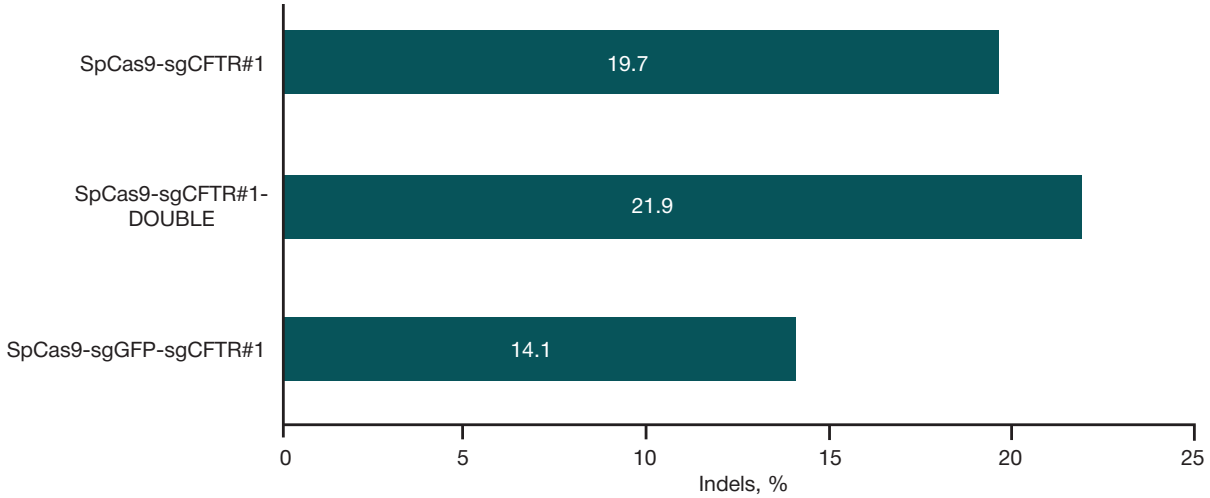

Fig. 4. Comparison of *CFTR* editing efficacy in HEK293T cells. The results are represented as a mean

A number of works have shown that RNA expression can be improved by a synthetic hybrid consisting of two promoters [26]. Perhaps, such effect is achieved because different promoters attract different transcription factors. In our plasmids sgRNA was expressed from U6, a standard promoter for CRISPR-Cas9. A few authors have demonstrated, though, that sgRNA is better expressed from the tRNAgln promoter [26, 27]. Therefore, we decided to clone into the plasmid a hybrid promoter consisting of U6 and tRNAgln (designated as the plasmid's name +HP in the pictures). As shown in Fig. 5, all sgRNAs, except sgCFTR#1, exhibited a poorer performance; sgCFTR#1's activity increased only slightly.

Improving the efficacy of *CFTR* locus editing

It is known that sgRNA molecules shorter than 20 nucleotides in length and starting with the G- or GG-nucleotide produce a better editing outcome [28]. Our sgCFTR#2 and sgCFTR#3 contained two GG nucleotides in their 5′-region, therefore, we shortened them from their 5′-ends down to 17 nucleotides (see SpCas9–sgCFTR#2(GG17) and SpCas9–sgCFTR#3(GG17), respectively). SgCFTR#1 did not have a GG sequence in its 5′-region, so we shortened it down to 19 nucleotides and replaced CC with GG (SpCas9–sgCFTR#1(gg19)). As a result, the activity of the modified sgCFTR#1 and sgCFTR#3 dropped from 20.3% and 11.8% to 8.7% and 0%, respectively (Fig. 6), whereas the modified sgCFTR#2 increased its performance from 10.5% to 22.1%.

Because guide RNA performance is presumably associated with its stability, we attached the sequence CACCGGGAGGGCGGGGAGGG to the 5′-ends of sgCFTR#1 and sgGFP in order to facilitate formation of G-quadruplexes (sgCFTR#1quad and sgGFPquad, respectively) that could improve sgRNA stability [29]. We found that the efficacy of target DNA cleavage using the modified guide RNAs was lower because of their 2- to 16-fold reduced expression, as compared to the unmodified sgRNAs [22].

Lastly, we attempted to stabilize the SpCas9 nuclease by transient hypothermia, i.e. culturing of the transfected cells at 30 °C [14, 30]. As a result, the success rate of *CFTR* editing plunged from 17.6 to 10.9% (Table).

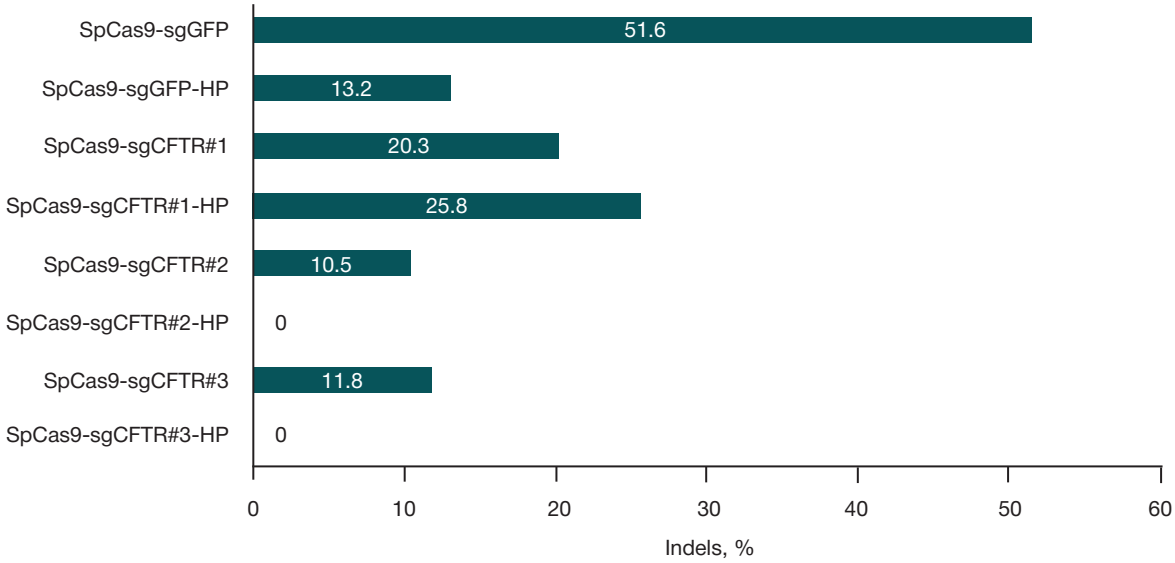

Fig. 5. Efficacy of *CFTR* and *EGFP* editing in HEK293T cells using sgRNA expressed from the standard U6 and the hybrid U6-tRNAgln (plasmid +HP) promoters. The results are represented as a mean

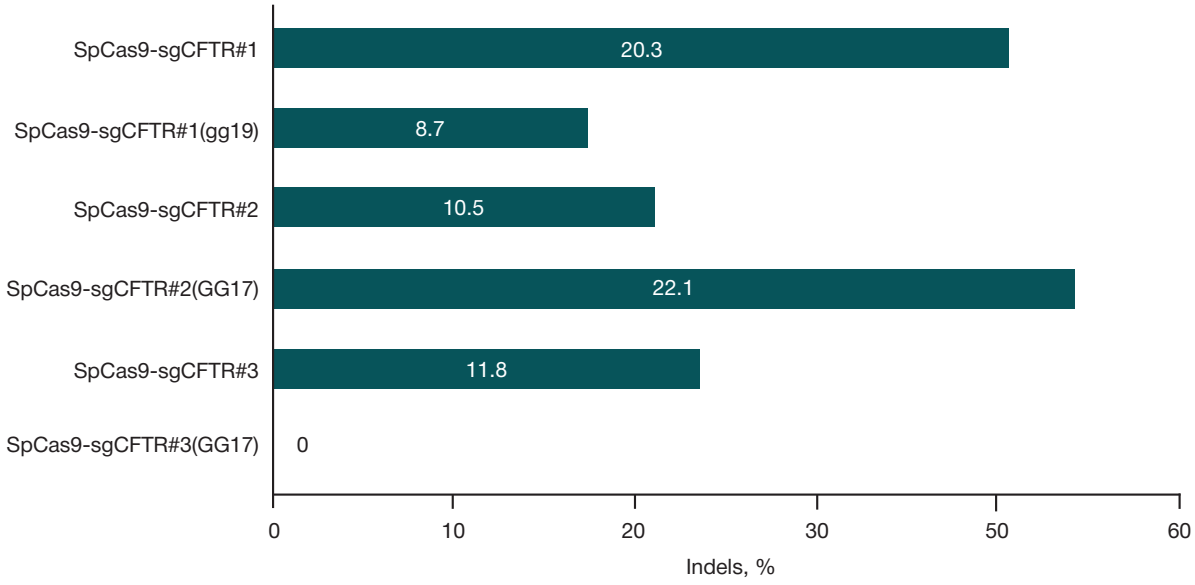

Fig. 6. Comparison of *CFTR* editing efficacy in HEK293T cells using modified sgRNAs. The results are represented as a mean

Table. Comparison of *CFTR* editing efficacy using transient hypothermia of HEK293T cells

Culture conditions	Transfected plasmids	Indels, mean %
72 h at 37 °C	SpCas9-sgCFTR#1+ pGEM-TA-CFTR	17.6
	pGEM-TA-CFTR	0
24 h at 37 °C, 48 h at 30 °C	SpCas9-sgCFTR#1+ pGEM-TA-CFTR	10.9
	pGEM-TA-CFTR	0

DISCUSSION

Attempts to correct *CFTR* mutations by genome editing tools started in 2012 [14], however no effective techniques are yet available. The proposed approaches demonstrate low efficacy, yielding only a small percentage of cells with corrected sequences, which necessitates cell selection [13, 16]. This incurs additional costs and makes the whole cell culture process longer. Besides, such treatment is not systemic.

Evolution of genome editing gives rise to more advanced CRISPR/Cas tools with better efficacy and specificity. The enzymes we use routinely for genome editing are highly specific [23, 24], which improves the safety of the method. Also, we are currently working on the technique that will correct only the mutant site using a guide RNA precisely targeting F508del. This might be a leap to a new level: we expect the technique to work not only in the isolated cells but also in the living organism, since only mutant alleles will be affected. We believe that this approach will prevent repeated cleavage of the already edited sites.

Still, in the course of this experiment we established that the efficacy of sgCFTR#1-based editing of F508del was lower than demonstrated by the majority of other sgRNA used in the study. The underlying reason is low expression of sgCFTR#1 in the cells [22]. We tried a few techniques to stimulate expression of this guide RNA: inserted an extra cassette (promoter + sgCFTR#1) into the plasmid, combined sgCFTR#1 with a more active sgGFP, used the hybrid promoter U6–tRNAgln, but none improved sgCFTR#1 performance.

It was shown previously that transcription from the U6 promoter is initiated in the presence of G or GG nucleotides on the 5′-end of guide RNA [28], therefore we tried shortening sgRNA down to the first G and replacing the initially present nucleotides with G/GG to upregulate sgRNA expression and increase its activity. But this approach did not work.

Given that initially our guide RNAs had one and the same promoter U6, we assumed that transcription of both sgRNAs would be the same. The actual difference in the expression levels may have been the result of a more rapid degradation of sgCFTR#1 in comparison with sgGFP. Screening of a huge guide RNA set [29] showed that sgRNA with G-enriched regions (>8 nucleotides) were more stable because of G-quadruplexes. But G-quadruplexes did not help to increase sgCFTR#1 activity [22].

Some authors report that *Fok*I nuclease has a more stable performance at 30 °C [14, 30]. We hypothesized that exposure of transfected cells to lower temperatures during culture would increase Cas9 activity, but that did not happen [22].

Experiments conducted *in vitro* show that up to 41% of guide RNAs are not active against the target site [31]. The main reason for that is sgRNA nucleotide composition: T- and TT-enriched sequences reduce editing efficacy [32], and the presence of certain nucleotides at certain positions in guide RNA sequences are reliably associated with different degrees of sgRNA activity [31]. Secondary structures formed by guide RNAs may also have a role here [31]. If the low performance of sgRNA is associated with its sequence, then the only solution is to choose a different sgRNA.

CONCLUSIONS

Our attempts to edit the *CFTR* locus in HEK293T cells demonstrate that the most effective combination is SaCas9 + sgRNA selected to precisely target the F508del mutation (the success rate here is 29%). Combinations of sgCFTR#1 targeting the F508del site with two different SpCas9 have the lowest efficacy: 13.8% for Cas9(1.1) and 8% for Cas9(HF4). Such poor outcome is associated with the low expression of the guide RNA. Attempts to improve sgCFTR#1 expression by inserting another expression cassette into the plasmid, fusing sgCFTR#1 with a more active sgGFP or using a hybrid promoter did not result in any significant increase in sgCFTR#1 activity. Stabilization of sgCFTR#1 by introducing a G-quadruplex into its sequence, shortening or adding GG to the 5′-region did not produce a desired effect. Transient hypothermia also did not improve the efficacy of editing. Therefore, the low performance of sgCFTR#1 is probably determined by its nucleotide sequence, and different guide RNAs, different Cas9, such as SaCas9, or different PAM expanding the choice of possible guide RNAs targeting F508del should be used instead.

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