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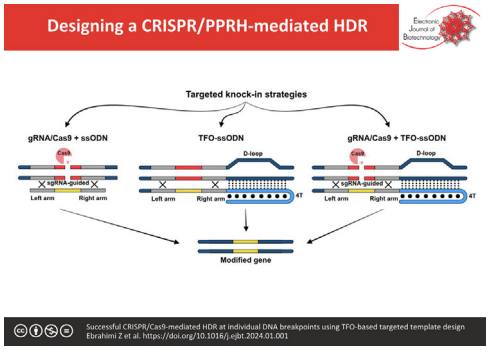
Research article

Successful CRISPR/Cas9-mediated HDR at individual DNA breakpoints using TFO-based targeted template design ☆

Zahra Ebrahimi [a], Bahram Kazemi [a,b], Mohammad Salehi [a,b], Vahid Jajarmi [a,b,*]

[a] Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[b] Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

GRAPHICAL ABSTRACT



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Targeted Template Design
Triplex-forming oligonucleotide (TFO)

ABSTRACT

Background: Targeted insertion of the repair template into the genome is a common strategy for high-precision base replacements; however, the main challenge likely remains regarding the limited efficiency of homologous-directed repair (HDR). A precise genome cut achieved by CRISPR-Cas9 system combining with a single-stranded oligodeoxynucleotide (ssODN), as the donor template, improves significantly the rate of HDR. It is well-established that the spatial availability of the donor template to the repair system effectively enhances knock-in events in CRISPR-Cas9. PolyPurine Reverse Hoogsteen hairpins (PPRHs), as an alternative repairing strategy, benefits from a Triplex-forming oligonucleotide (TFO) for the repair template providing the ease of access. The main objective of the study was to evaluate the HDR frequency as a result of improvement of the spatial accessibility of the donor template adjacent to the cutting site. Hence, a flanking purine-rich hairpin complementary to the genomic DNA adjacent to the repairing site was fused to the ssODN with the incorporated bases for the alteration of EGFP to EBFP.
Results: Results from the comparison between the donor templates, ssODN and TFO-tailed ssODN, demonstrated an increased rate of knock-in from 18.2% ± 1.09 to 38.3% ± 4.54, respectively. From another perspective, findings indicated that the targeted Cas9-mediated DNA cleavage improves the efficiency of the repair-PPRH approach four-fold, as well.
Conclusions: The present study provides a viewpoint that highlights the significance of the designing of the donor template in terms of the structural features and positional access for the HDR-based repairing CRISPR-Cas9 systems.

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* Corresponding author.
E-mail address: v.jajarmi@gmail.com (V. Jajarmi).

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1. Introduction

The clustered regulatory interspaced short palindromic repeat (CRISPR)-associated nucleases (Cas)-9, as an emerging technology, offers several routes to conduct manipulating nucleic acid sequences, these include insertion and deletion mutations (indels) to disrupt genes or base editing and sequence replacement predominantly for gene recovery [1]. Its prominent impact, however, lies in promoting the HDR-mediated gene repair supplied with an exogenous DNA template resulting in the precise incorporation of desired nucleotide(s) into the target sequence [2]. Following DNA strand breakage, the preference of the repair systems for non-homologous end joining (NHEJ) over HDR is the main challenge for efficient gene editing [3]. To enhance the HDR rate, a multitude of strategies have been suggested including inhibition/overexpression of the repair complex components, cell cycle S/G2 phases-timed delivery and structurally designed DNA donor templates [4,5,6]. Recent studies have revealed that localizing the donor template to the target locus leads to an increased access to the template for the repair machinery, temporally and spatially, contributing to HDR enhancement [7,8]. Accordingly, tethering the single-stranded oligodeoxynucleotides to the cas9 protein or construction and expression of a chimeric RNA consisting of a guide and scaffold RNA flanking with the edited RNA template at the 3′ end are the rational choice approaches [9].

In recent years, an alternative strategy termed PolyPurine Reverse Hoogsteen hairpin (PPRH) has been developed for gene editing [10]. A repair-PPRH consists of a ∼40 nucleotide-single stranded DNA of homology arms as the donor template and an antiparallel purine hairpin (linked by a thymidine loop) complementary to a polypyrimidine stretch adjacent to the repair loci. Following attachment of the hairpin, the ssODN promotes HDR-mediated replacement; however, due to the lack of DNA strand breakage, it leads to poor performance [11].

Based on this scenario, a PPRH-based design of donor template provides distinctive features resulting in template stability, D-loop formation and DNA donor localization close the nuclease-induced DNA break [12,13].

The current study provided a proof-of-concept that the combination of CRISPR-Cas9 and PPRH could induce a dramatic improvement of HDR-mediated gene correction and it is also a straightforward method that can be applied widely for other genetic sequences.

2. Materials and methods

2.1. Guide RNA and donor template designing for CRISPR-Cas9

An antisense pairing guide RNA (gRNA) was constructed for the eSpCas9 endonuclease (1.1 version) for induction DNA double-strand breaks (DSBs) precisely at the editing point, three nucleotides upstream of the PAM sequence. The oligonucleotides of the sgRNA were synthesized by Metabion International AG (Germany). The guide RNA was cloned into the vector carrying the ampicillin resistance gene. The BbsI restriction site was used to insert the guide under the U6 promoter's control.

It is well established that for creating of minor nucleotide changes, ssODN as a HDR donor template is more efficient than linear dsDNA or plasmid [14]. However, a double-stranded template PCR product was obtained that incorporated the desired nucleotide and was compared with the ssODN template. Consequently, we designed an ssODN with a minimal length of homology arms (20-nt each) incorporated with corresponding base substitutions. Briefly, to obtain EGFP-derived enhanced blue fluorescent protein (EBFP), as already reported in the literature, an essential $Y_{66}H$ substitution (196 T > C) and a reversal $T_{65}S$ mutation (194 C > G) (to increase EBFP fluorescence intensity) was incorporated in the donor template [15].

To avoid re-cutting the knocked-in ssODN by the cas9 riboendonuclease, a blocking silent mutation was included in the PAM sequence of the template. Furthermore, an antisense-ssDNA was employed to maximize the knock-in efficiency.

2.2. Repair-PPRH designing

Generally, a repair-PPRH consists of a polypurine hairpin core to bind a complementary polypyrimidine sequence (triplex formation) located near the mutation site and a flanking ssODN as the donor template was designed for HDR-mediated repair. To design the triplex-forming oligonucleotide, we used the Triplex-Forming Oligonucleotides searching tool to find the local polypurine stretches (available at https://utw10685.utweb.utexas.edu/tfo//) and BLASTN tool for the identification of the potential off-targets. The antisense-binding mirror repeats linked by a tetra-thymidine loop were designed to form an antiparallel triplex domain on the target sequence located in upstream of the mutation site. Subsequently, the repairing oligonucleotide with incorporated point mutations was included at the 3′ end of the hairpin.

2.3. Gene modification frequency

2.3.1. Quantification of HDR and NHEJ

To generate a reporter cell, an EGFP-expressing stable cell line of HEK 293 T was established and maintained in a low concentration of antibiotic (600 µg/ml of Geneticin (G418 disulfate salt)) during the downstream processes. In this study, a high throughput screening system based on the traffic light approach was designated for the evaluation of HDR and NHEJ rates. Briefly, following HDR-mediated sequence alteration of EGFP to EBFP, distinct and non-overlapping fluorescence signals, shifting from green (484 nm and 507 nm) to blue (382 nm and 459 nm) spectrum, were detected by the fluorescence microscopy. Alternatively, the NHEJ repair pathway results in indel formations (frameshift mutations) and loss of fluorescence.

After 24 h incubation of the transfection mixture, GFP and BFP fluorescence cells were analyzed using fluorescent microscopy. The BFP-positive (blue) and non-fluorescent and total cells of all experimental groups were counted to measure the percentage of the HDR and NHEJ events. It should be noted that GFP-positive cells (green) were considered as non-transfected cells or the lack of the gRNA efficiency. The HDR rate percentage was assessed using the following equation:

$$HDR\ ratios = \frac{number\ of\ BFP - positive\ cells}{total\ number\ of\ cells} \times 100$$

2.4. Evaluation of the ssODNs performance

To evaluate the effect of a TFO structure on the repairing template efficiency, two structurally different ssODNs, a template flanked by a PPRH-based hairpin (TFO-ssODN) and a standard repairing template consisting of the only two homology arms (CRISPR-ssODN), were employed in the following experiments:

1. CRISPR-Cas9/ssODN

The ssODN donor was co-delivered with the CRISPR vector containing the EGFP targeting sgRNA into the reporter cell lines. To achieve the optimal performance of CRISPR-mediated knock-in, different ratios of the plasmid and ssODNs were examined.

2. TFO-ssODN

The hairpin structure of the PPRH promotes a template localization and HDR as a consequence of a DNA triplex domain formation adjacent to the repair site. The efficiency of knock-in was evaluated using the repair template with various concentrations transferred into the EGFP-expressing cell lines. It is worth noting that this study is in keeping with other studies displaying decreased gene correction rate in repair-PPRHs, but we acquired the maximum frequency value when transfection was done with lower cell seeding confluency [16].

3. CRISPR-Cas9/TFO-ssODN

To determine the synergistic effects of the two approaches (targeted cleavage of the DNA via CRISPR-Cas9 system and localization of the repairing template to the cutting site through PPRH-based ssODN, in an experiment, the CRISPR-ssODN was replaced by the TFO-ssODN and the EGFP expressing cell lines were transfected with gRNA-cas9/TFO-ssODN.

2.5. Transfection

Some studies have demonstrated that the multiple transfection (particularly triple transfection) may improve CRISPR-Cas9-mediated HDR rates. To examine the effect of the successive transfection, cells were subjected to the transfection mixture including Turbofect transfection reagent (Thermo Fisher Scientific, USA) and nucleic acid contents (plasmid and templates). 24 h after transfection, the cells were monitored for the fluorescence and were transfected again with mixture for the second and third times during the following days (72 h) and finally were evaluated for fluorescence (blue and green) (All experiments were performed independently in triplicate).

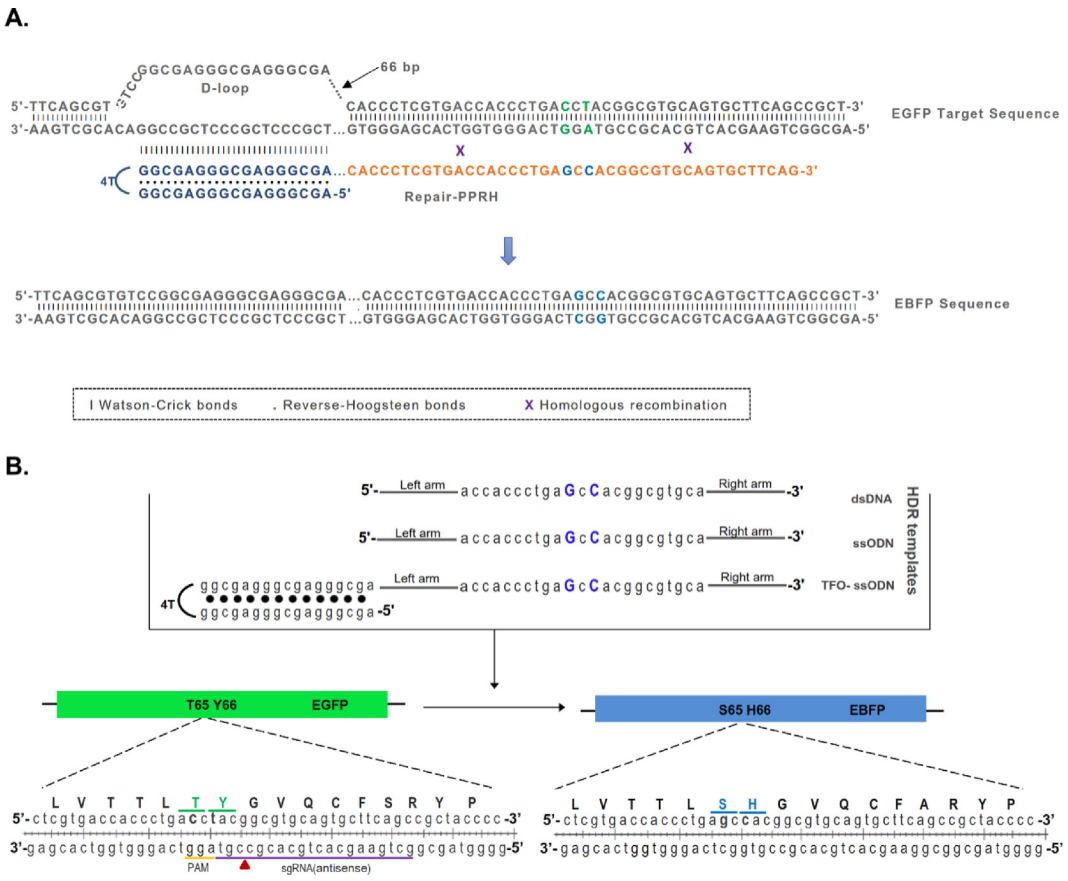

Fig. 1. Designs for the EGFP to EBFP conversion. (A) Scheme describes the mechanism of action of a repair-PPRH. A repair-PPRH consisting of a TFO core and the extension sequence (ssODN) (with the incorporating intended bases (blue)) homologous to the target sequence. The polypurine-hairpin core of the PPRH binds to its polypyrimidine target sequence of the EGFP gene by Watson-crick bonds, creating a D-loop structure and exciting the repair process. The repairing oligonucleotide at the 3′ end of the hairpin serves as a donor to put the corrected nucleotide in the mutation site by homologous recombination-mediated repair. (B) Designs for the HDR templates and the sequence alteration process. The view illustrates the sgRNA (underlined purple), PAM sequence (underlined orange), cutting site (red arrow) and the bases to be modified shown in bold black.

2.6. Statistical analysis

For statistical comparisons between groups, statistical analyses were calculated using ordinary one-way ANOVA and t-test. The data analyses were performed using GraphPad Prism version 8.0 software. Data were represented as the ±SD of the mean of two experiments, and three replicates in each experiment were performed for each of the experimental groups.

3. Results

3.1. Efficiency of specific RNA guides for DSB induction and correcting with exogenous donor templates

We utilized a human-codon optimized SpCas9 and a chimeric sgRNA expression vector to direct a DSB induction at the site-specific gene editing, efficiently. The sgRNA cleavage efficiency was evaluated using gRNA-Cas9 containing plasmids (3 ug/ml) delivered into the EGFP-positive cells by quantification of non-fluorescence cells (NHEJ rate) that obtained 72% ± 4 of the guide-Cas9 efficiency. After generating at an embedded nuclease breakage site and editing of the break produced distinct fluorescent signals through HDR with exogenous donor templates of dsDNA and ssDNA that acquired 11.6% ± 0.65 and 18.2% ± 1.09 of the HDR rate, respectively (Figs. 1B, 2B).

3.2. HDR efficiency was increased by targeted localization of the donor template

In our experiments, a comparison between the two structurally different repair templates served as a proof-of-concept for evaluating the donor localization effects on the HDR frequency. Accordingly, a repair ssODN with 20/20-nt of homology arms (CRISPR-ssODN) was compared with a Short-Distance repair PPRH (SDR-PPRH) consisting of a PPRH core and the 20/20 nt-repair tail (TFO-ssODN) (Fig. 1A). Findings indicated that presence of PPRH structure at the 5′ end of the donor template improves HDR efficiency 38.3% ± 4.54 comparing to the commonly used ssODN structure which was 18.2% ± 1.09 (Figs. 2A, 2B**)**.

3.3. Improvement of repair-PPRH frequency via DNA strand breakage

Sensing of DSB results in the repair machinery recruitment, including HDR, to the site of damage. From this point of view, in

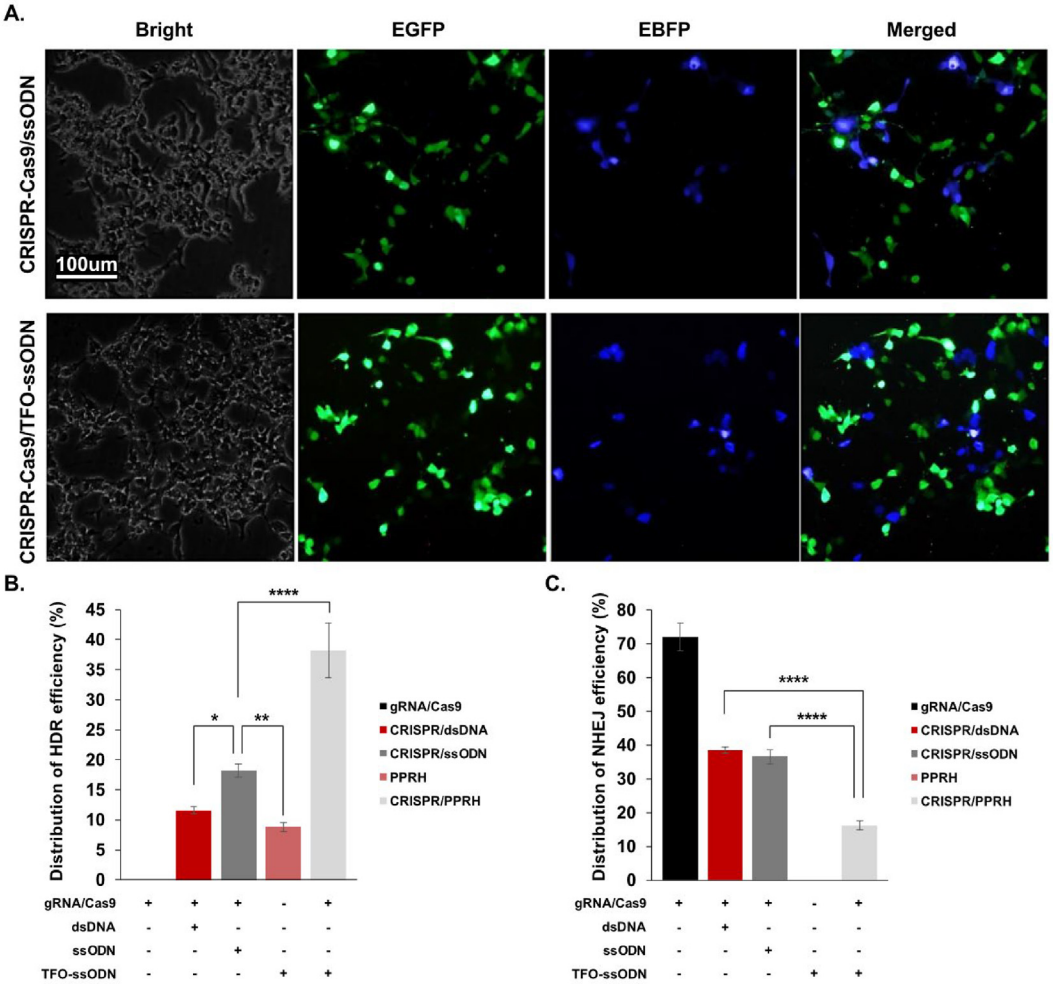

Fig. 2. Evaluation of HDR and NHEJ events. (A) Representative fluorescent microscopy images of HEK293T expressing EGFP and EBFP. Cells were co-transfected with gRNA-Cas9 and ssODN template (top row) / TFO-ssODN template (bottom row). (B and C) Graphs represent the HDR and NHEJ efficiency and ±SD of the mean of two experiments, respectively (all experiments were performed independently in triplicate). Results showed a relatively higher efficiency of HDR rate with the TFO-ssODN template coupled with CRISPR-Cas9 (38.3% ± 4.54). Furthermore, it was indicated that TFO-ssODN decreases the rate of NHEJ (16.2% ± 1.30) comparing to the ssODN (36.6% ± 2.07). The levels of statistical significance were meant as follows: $p < 0.05^{*}$, $p < 0.01^{**}$, $p < 0.001^{***}$ and $p < 0.0001^{****}$.

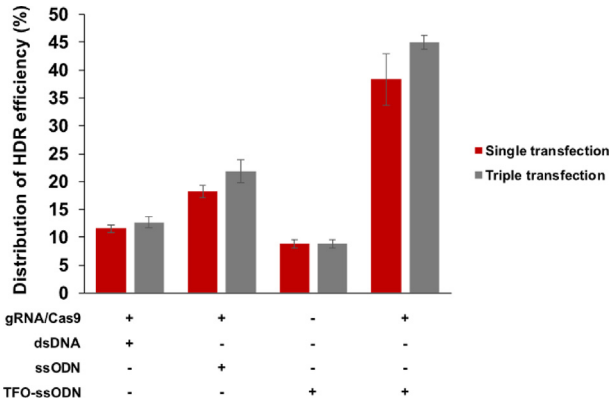

Fig. 3. Triple transfection effect on the HDR efficiency. Triple transfection was performed with the ± SD of the mean of two experiments and three replicated in each experiment. As it shows, in the CRISP-Cas9/TFO-ssODN group, a comparison between two methods of transfections indicated a relative increase from 38.3% ± 4.54 to 45.4% ± 5.30. Results from all groups showed no significant changes following triple transfection.

principle, the frequency of HDR is relatively poor in the repair-PPRH method. In this study, it was demonstrated that the Cas9-mediated DSB in a synergistic manner enhanced the HDR frequency from 8.8% ± 0.75 (HDR rate of repair-PPRH oligonucleotides) to 38.3% ± 4.54 using CRISPR-Cas9/PPRH strategy (Fig. 2B).

3.4. Reduced NHEJ in CRISPR-Cas9/PPRH

In constant competition with HDR, Cas9-induced NHEJ is the preferential pathway to repair DSB. In the current study, we provided evidence that a repair template consisting of a PPRH core and a PAM-disrupted ssODN results in NHEJ rate reduction. We speculate that this may be as a result of further PPRH-mediated strand displacement of indels (Fig. 2C).

3.5. HDR efficiency with consecutive transfection

CRISPR-mediated HDR efficiency is a complex interplay of factors including experimental conditions. To achieve an optimal performance, the reporting cells were subjected to a sequential transfection. Comparing the single and triple transfection showed a relative increase in the HDR rate in most experiments, except for the PPRH. However, it was not significant even for the CRISPR-Cas9/TFO-ssODN group (38.3% ± 4.54 to 45.4% ± 5.30, respectively) (Fig. 3).

4. Discussion

Targeted knock-in is considered as the leading approach in gene manipulation experiments. However, despite improvements, certain challenges remain to the new solutions. Precise CRISPR/Cas9-mediated genome cuts trigger various repair machinery recruitments resulting in improvement of gene editing in the presence of a ssODN as the repair template [17,18]. Over recent years, several studies have shown that enhanced HDR-mediated knock-in can be achieved through the hairpin-structured repair template localization to the mutation site [19]. Triplex formation of the template with the target DNA promotes a D-loop-induced recombination between the homology arms which is the basic concept of the PPRH-mediated repair system [13].

Principally, our current strategy exploits both the DNA cleavage and proximal binding of the template to enhance the HDR rate. From other point of view, in line with our findings, we assume that a CRISPR-mediated DNA break enhances the PPRH-based repair frequency through the co-localization of the recombinational repair machinery. As such, we speculate that the hairpin structure of the ssODN may improve the stability of the template or DNA conformational changes near the target site resulting in higher HDR efficiency [20,21].

In this study, we designed a functional hairpin core with a four-thymidine linker for the two polypurine mirror repeats (regularly, in the repair-PPRH designing, it is considered as five-thymidine and without the spacer for the repair sequence) [22]. Moreover, the TFO-ssODN was designed as a Short-Distance repair PPRH (SDR-PPRH) (86-nt), although, Long-Distance (LDR-PPRH) with hundreds of nucleotides away from the repair site may be designed and evaluated [23].

In line with other experiments regarding the repair-PPRH, our findings showed a limited efficiency of HDR based on using TFO-ssODN in the absence of CRISPR-Cas9 [24]. This lies on the fact that TFO-mediated D-loop formation leads to the limited DNA repair machinery recruitments compared to the DSB via CRISPR-Cas9 which triggers various repairing systems [13]. Consequently, to improve the efficiency of the repair-PPRH, we recommend providing a parallel DNA cleavage system, although, by which the simplicity of the method becomes complicated.

Several studies have revealed that the optimal length of homology arms for the CRISPR-Cas9 system is in the range of 30-60-nt [14]. Our findings indicated that shorter lengths of 20-nt may be sufficient for efficient repair. However, further investigations are required for enlighten the effect of size and the proportion of arm's length on HDR rate in the TFO-ssODN.

In this study, all experiments were conducted using a CRISPR-Cas9 system which generates a DSB. It is well-established that the mechanisms of DNA repair in single-stranded break (DNA nicks) are different from DSB. Some studies have obtained higher efficiencies of knock-ins with DNA nicks [25]. Therefore, comparing the HDR frequency of the two strategies of the CRISPR-Cas9 (wild type and nickase) would be recommended. Moreover, for more conclusive validation, conducting the relevant experiments in other cell types and alternative sequences of genes and genomes would be required.

5. Conclusions

The current study represents an integrating strategy from the CRISPR-Cas9 and PPRH systems to improve HDR frequency. It indicated that in addition to the targeted DNA cleavage, the structural features of the donor template with potential positive effects including stability improvements, genome conformational changes and more substantially, spatial availability to the cutting site contribute greatly in the knock-in efficiency.

Ethical approval

This study was approved by the ethical committee of Shahid Beheshti University of Medical Sciences (Ethical code IR.SBMU.RETECH.REC.1402.005SSS).

Author contribution

- Study conception and design: V Jajarmi
- Data collection: Z Ebrahimi
- Analysis and interpretation of results: V Jajarmi, Z Ebrahimi

- Draft manuscript preparation: V Jajarmi, B Kazemi, M Salehi, Z Ebrahimi
- Revision of the results and approval of the final version of the manuscript: V Jajarmi, B Kazemi, M Salehi.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary material

https://doi.org/10.1016/j.ejbt.2024.01.001.

Data availability

Data will be available upon request to the corresponding author.

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