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Systematic analysis of factors that improve HDR efficiency in CRISPR/Cas9 technique

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Abstract

The bacterial CRISPR/Cas9 system has proven to be an efficient tool for genetic manipulation in various organisms, but the efficiency of sequence replacement by homologous direct repair (HDR) is substantially lower than random creation of indels.

Many studies focused on improving the efficiency of HDR using double sgRNA, cell synchronization cycle and the delivery of ssODN with a rational design.

In the present study, we tested and compared the combination of these three methods to improve HDR efficiency. To our tests, we chosen the *TNFα* gene (NM_000594) for its crucial role in a variety of biological processes and diseases.

Our results showed a dramatic increase of HDR efficiency from undetectable HDR event to 39% of HDR efficiency and provide a new strategy to facilitate CRISPR/Cas9-mediated human genome targeting.

Furthermore, we showed that *TNFα* gene could be edited with CRISPR/Cas9 methodology, an opportunity to safely correct, in the future, the specific mutations of each patient.

Introduction

In the last decade, the use of the novel CRISPR-associated endonuclease Cas9 protein has been implemented for analytical and therapeutic approaches, in a broad spectrum of cell types and model organisms (1)(2). After the introduction of this technique, the creation of a knock-in and knock-out gene has become as simple, rapid, and economical

as never before.

The CRISPR/Cas9 system takes advantage of the ability of the bacterial Cas9 nuclease to induces DNA double-strand breaks (DBS) in a trinucleotide region repeated in the genome (the proto-spacer adjacent motifs or PAM), directed by a 20–22 bp synthetic RNA sequence (gRNA) located next to PAM (1).

Then, DBS stimulates the cells repair mechanisms including non-homologous recombination end joining (NHEJ) and homologous direct recombination (HDR) to repair the DNA strands (3)(4)(5)(6)(7)(8)(9).

NHEJ creates insertions and deletions in the target DNA sequence, thus producing a frameshift that abolishes the correct protein production, while HDR-based targeting has been widely used to manipulate coding or non-coding regions of DNA by introducing selected DNA sequences in the correct context.

In the fast-evolving field of gene editing using CRISPR/Cas9 important efforts to improve the efficiency of the generation of targeted variants have been made; however, the reported success rates remain very low in many cells type and *in vivo* studies (10)(11)(5).

So far, many protocols have been published that describe how to improve the efficiency of HDR. Acosta et al. described a highly efficient HDR targeting approach based on the use of two sgRNAs flanking the targeted region, in mouse ESC lines in three different loci by a new method called “two gRNA-driven homozygous HR” (1). Zhou et al. reported that the use of dual sgRNAs increases the endogenous gene targeting efficiency in mouse cells (1,12).

Other authors tried to improve the HDR by blocking the cells cycle at different phases, showing that treatment with nocodazole, increases the efficiency of HDR. Nocodazole blocks cells at G2/M phase when DNA is completely replicated and the nuclear membrane is broken, allowing Cas9 to easily access DNA and enhancing the HDR (13).

Richardson et al. focused on understanding how Cas9 enzyme interacts, cuts and dissociates from the target DNA in physiological condition to improve the genome editing by HDR event. They showed that the enzyme locally releases the PAM-distal non-target strand after cleavage but before complete dissociation, making this strand available for complementary annealing of ssODN. For this reason, the ssODN with asymmetric arms with the arm of 36bp on the PAM-distal side and the 91bp arm on the PAM-proximal site of the break, complementary to non-target DNA strand, seems to have the highest efficiency of HDR (14).

70 These results inspired us to investigate the effect on HDR combining the three
71 approaches described. We wondered if the three protocols synergically increase the
72 efficiency of HDR.

73 For this aim, we have chosen the *TNF α* gene (NM_000594) for its crucial role in a
74 variety of biological processes and diseases. *TNF α* encodes a multifunctional
75 proinflammatory cytokine that belongs to the tumor necrosis factor superfamily. This
76 cytokine is involved in the regulation of a wide spectrum of biological events, such as
77 immune cell regulation, cell proliferation, differentiation, apoptosis, lipid metabolism,
78 coagulation and thus is implicated in a variety of diseases (15)(16)(17). *TNF α* is also
79 involved in rheumatoid arthritis (RA), a complex autoimmune disease, with a relatively
80 constant prevalence of 0,5-1% in the world's populations that affect many organs;
81 including kidney, eyes, spleen, heart, and lungs (18). In addition, *TNF α* can alters the
82 regulation of insulin response, and its expression plays a role in the pathophysiology
83 of insulin resistance (19). Recent studies focused their attention on the role of the
84 proinflammatory cytokine tumor necrosis factor in the development of heart failure
85 showing a direct relationship between the level of *TNF α* expression and the severity of
86 heart disease (20). Furthermore, this cytokine has a relevance in tumor immune
87 surveillance, and play crucial roles in tumor development and progression (15).

88 Based on this scenario, the possibility of modifying this gene became a fascinating
89 and intriguing objective to safely correct, in the future, the specific mutations of each
90 patient. Here we report a simple and robust approach to edit this gene.

92 **Material and methods**

93 **Design of the sgRNAs and Plasmid Constructions**

94 Two different sgRNAs were designed according to the following criteria: close location
95 to the mutation(s); following PAM at the 3' end; and 20-nt length. The sgRNA were
96 purchased by IDT (Integrated DNA Technologies, IDT, USA). The sgRNA consists of
97 two RNA domains: crispr RNA (crRNA), which is specifically designed to be
98 complementary to the target locus, and the constant trans-activating crRNA
99 (tracrRNA), which is required for coupling with the Cas9 nuclease. The crRNAs were
100 already cloned in pX333-U6-Chimeric_BB-CBh-hSpCas9 (Addgene, plasmid ID
101 #42230). The two opposite BbsI and BsaI restriction sites were used to insert the guides
102 under the control of a U6 promoter. For this purpose, self-complementary

oligonucleotides (Integrated DNA Technologies, IDT, USA) were annealed by gradual cooling with prior denaturalization at 94° C. The duplex oligonucleotides also presented cohesive ends with the 3' overhangs left after pX333 incubation with the BbsI and BsaI restriction enzymes, serving for the ligation of the insert-plasmid with T4 DNA ligase (EL0014; Thermo Fisher Scientific, Waltham, MA, USA). Moreover, the sgRNA1 and sgRNA2 were cloned in the plasmid pX459 (Addgene, plasmid ID #62988), which shows the puromycin antibiotic resistance. DH5α competent cells were transformed with each of the plasmid constructs for Ampicillin selection and amplification in liquid culture. The vectors were purified using a HiSpeed Plasmid Midi Kit (QIAGEN, Hilden, Germany) and were Sanger sequenced to verify the correct cloning of the specific crRNA inserts.

HEK293 Cell Culture and Transfections

HEK 293 cells were maintained in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C under 5% CO₂. 5x10⁵ cells were transfected with 3ug of plasmids using Fugene transfection reagent (Promega). For the directed editing, 3ug of plasmid was delivered to 5x10⁵ HEK293 cells with 1uL of the pertinent ssODN at 10 µM (10 pmol). The nocodazole was added after transfection at concentration of 100 ng/mL for 24 hours. Each Cas9-gRNA vector was co-transfected in HEK293 cells with an empty CRISPR vector coding for a green fluorescent protein (pX458 Addgene, plasmid ID #48138), in order to monitor the transfection efficiency. All experiments were assayed in triplicate.

PCR amplification of target region

A 1286 nt region of *TNFα* locus, containing the target site, were PCR amplified using the following primer sets. The target locus was amplified for 35 cycles with specific forward (TNF-X-Fw:5'-CGCCACCACGCTCTTCTG-3') and reverse (TNF-Alw-Rv:5'-CGGTCCAGCCACTGGAGC-3') primers targeting exon 1 and exon 4 of the *TNFα* gene. The PCR reaction was performed using 200 ng of genomic DNA and Kapa Hot start high-fidelity polymerase (Kapa Biosystems, Wilmington, MA) in high GC buffer according to the manufacturer's protocol. The thermocycler setting consisted of

one cycle of 95°C for 5 min, 35 cycles of 98°C for 20 s, 61°C for 15 s and 72°C for 30 s, and one cycle of 72°C for 1 min. The PCR products were analyzed on 1,3% agarose gel containing Midori Green Xtra (NIPPON Genetics Europe, Dueren, Germany). The concentration of PCR DNA was quantitated based on the band intensity relative to a DNA standard using the software Image Lab (Bio-Rad, Hercules, CA). About 200 ng of PCR DNA was used for T7 endonuclease I and SmaI analyses.

T7-Endonuclease I Assay

The widely used T7-endonuclease I assay targets and digests hetero-duplexes formed by hybridization of mutant WT strands resulting in two smaller fragments, and this method was performed to assess sgRNA-specific activity. After transfection, cells were incubated for 48 hr. The cells were then pelleted, and the lysis performed using QIAamp DNA Mini Kit (Qiagen). The PCR products were denatured and then reannealed using the following program: 95° C for 5 min, ramp down to 85°C at 2°C/s, and ramp down to 25°C at 0.1 C/s. Immediately after the reannealing step, and the consequent heteroduplex formation, 5 units of T7 endonuclease I (New England Biolabs, Ipswich, MA) was added to the mix and incubated for 1 hr at 37°C. The product was resolved on 1,3% agarose gel containing Midori Green Xtra (NIPPON Genetics Europe, Dueren, Germany).

Clonal Amplicon Sanger Sequencing

A PCR product obtained from the target locus was cloned using a kit for sequencing purposes (TOPO TA Cloning Kit, Thermo Fisher Scientific) and introduced in *E. coli*. Sanger sequencing was used to sequence 10 individual colonies to reveal the clonal genotype and thus the general indel and HDR frequency.

Design of the ssODNs

Single-stranded donor oligonucleotide (ssODN) for the HDR were designed with symmetric (60nt long) and non-symmetric (90nt, 36nt long) homology arms, in both orientation (5'-3';3'-5'), complementary to target and non-target DNA strand. The ssODNs sequence includes a new and unique SmaI restriction site, juxtapose to the homology arms. These ssODNs were synthesized as Ultramer Oligonucleotides (Integrated DNA Technologies, IDT). Since the edited sequence contained a newly acquired SmaI restriction site, PCR products of both amplifications were restricted

immediately after with SmaI enzyme (NEB, Ipswich, MA).

Analysis of HDR by SmaI restriction digestion

The reaction consisted of 1.6 µg of PCR DNA and 20 units of SmaI enzyme in CutSmart Buffer (NEB, Ipswich, MA). After 1hr of incubation at 37°C, the reaction was arrested with heat inactivation at 65°C for 20 min. The product was resolved on 1.3% agarose. The band intensity was quantitated using Image Lab. The percentage of HDR was calculated using the following equation $(b + c / a + b + c) \times 100$ for the single cut strategy, $(b + c / a + b + c + d) \times 100$ for the dual cut strategy. In the first equation, 'a' is the band intensity of DNA substrate and 'b' and 'c' are the cleavage products. In the second equation, 'a' is the band intensity of DNA substrate wild type, 'b' and 'c' are the cleavage products, and 'd' is the deleted fragment in which both sgRNA worked properly but there wasn't a KI event. To further confirm the presence of the edited sequence, conventional Sanger sequencing was performed (**Fig. S3A**).

Off-target analysis

To predict the most likely off-target sites for the sgRNAs used to knock-down the *TNFA* gene in this study, we used a public webserver: (https://eu.idtdna.com/site/order/designtool/index/CRISPR_PREDESIGN) able to assess and prioritize potential CRISPR/Cas9 activity at off-target loci based on predicted positional bias of a given mismatch in the sgRNA protospacer sequence and the total number of mismatches to the intended target site. The CRISPR design tool (IDT) scored a total of 202 (101 for sgRNA1 and 101 for sgRNA2) potential off-target sites in the human genome. The off-targets are scored between 0 to 100, where a major number indicate a lower possibility that the off-target occurs. The top three potential off-target sites ($11 \leq \text{score} \leq 26$) for each sgRNA, and the first genomic locus independently by its position on the off-target list, were assessed by T7 endonuclease assay in HEK 293 cells.

Results

Genome editing of the human *TNFA* gene

With the aim of improving the efficiency of homologous direct recombination (HDR), we tested and further combined three strategies already reported in literature able to increase the HDR efficiency, but never used together. We wondered if these protocols

(use of double sgRNA, rational design of ssODNs and cells synchronization), could work in synergy to improve the HDR efficiency. In parallel, we added a fourth condition in which the cells were transfected three consecutive times.

For this aim we proceeded in two steps:

1) First, to increase the efficiency of excision of the Cas9 nuclease we explored and compared the use of one sgRNA (who induces one DSB) and dual sgRNAs (who induces two DSB) located in the region of *TNF α* gene.

2) Next, after choosing the best sgRNA strategy, we compared the use of various donor ssODNs in synchronized and unsynchronized cells with single and multiple transfection events.

Selection of specific RNA guides for DSB induction

For this purpose, we used the previously described *Staphylococcus pyogenes* nuclease, that utilizes a human-codon optimized SpCas9 and a chimeric sgRNA expression vector to direct efficient site-specific gene editing (21)(22). We designed two 20-nt long sgRNA with a wide-spam of 473 nucleotide to guide Cas9 to introns 1 and 3 of the *TNF α* gene (sgRNA1 and sgRNA2) (**Fig. 1**). Without the presence of donor DNA containing the homology arms, cells repair the DNA primarily by NHEJ, leaving insertion and/or deletion (indels). Considering the small introns size ($\approx$ 300-600bp) in *TNF α* locus, indels can involve adjacent exons and may generate frameshift mutations that knocking-out the *TNF α* gene.

In detail, in pX333 plasmid the two sgRNAs were cloned as single guide (pX333-sgRNA1; pX333-sgRNA2), in tandem combination (pX333-2sgRNA1/1; pX333-2sgRNA2/2) and the two different guides were cloned in the same vector (pX333-2sgRNA1/2). To asses if the plasmid with puromycin can helps for positive clone selection, we cloned the two sgRNAs also in pX459 vector (pX459-sgRNA1 and pX459-sgRNA2), a plasmid that carry the puromycin resistance. The seven constructs thus obtained were further transfected into HEK293 cells and the puromycin antibiotic was added for 48h only in cells transfected with pX459 vector.

To evaluate the efficiency of DNA cutting and NHJE repair after the use of sgRNA guides, the genomic DNA was isolated from HEK293 cells and screened for the presence of site-specific gene modification by PCR amplification and T7E1 endonuclease assay, of region around the target sites (**Fig.2A**).

The results showed detectable bands in pX333-sgRNA2, pX333-sgRNA2/2, pX459-sgRNA2. The use of sgRNAs cloned in single or in tandem when co-expressed with the SpCas9 nuclease was able to mediate gene modification with comparable level of efficiency. No differences were finally detected between pX333 or pX459 plasmids after puromycin selection, maybe due to high efficiency of transfection obtained in HEK293 cells. Notably, HEK293 cells transfected with the 2sgRNA plasmid (2sgRNA1/2) and not treated with T7E1 nuclease resulted in a full-length (FL) and in short-edited (SE) amplicons confirming the expected deletion of the region between the two selected protospacers (**Fig. 2A**).

Moreover, the frequency of Indels in the cells transfected with all sgRNAs, were measured by sequencing 10 PCR amplicons encompassing the target sites. As reported in the figure 2B, the highest editing frequencies achieved were 50% and 75% using sgRNA2 and 2sgRNA1/2 guides, respectively (**Fig. 2B**). The types of insertions and deletions at this locus presented variable patterns of rearrangements of the coding sequence, insertion from 1 to 10 nucleotides and deletion from 5 to 930 nucleotides. Deletion of region between the 2 PAMs was observed in the cells transfected with 2sgRNA plasmid (2sgRNA1/2) (~480nts).

Furthermore, we observed the predominance of a precise junction between the two DSBs when 2sgRNA1/2 was transfected, a mechanism already described (1)(12)(23).

These data further confirmed that the dual sgRNA (2sgRNA) is the most efficient method for DNA excision in the endogenous locus. Based on these genomic results, we selected the sgRNA2 and the 2sgRNA1/2 guides for the following HDR editing.

Homologous direct recombination efficiency (HDR)

With the aim to improve the HDR efficiency we chose the plasmids pX333-sgRNA2 and the pX333-2sgRNA1/2 that showed a highest degree of DSB in HEK293 transfection. Traditional HDR gene editing requires long homology arms to allow proper and high-specificity recombination. The use of Cas9-gRNAs directed recombination allows the use of much smaller homology arms (~90 bp to 700 bp) with higher recombination rates than conventional HDR (1).

We decided to assess the efficiency of HDR by transfection of single (pX333-sgRNA2) and double sgRNA (pX333-2sgRNA1/2) coupled with a rational design of ssODNs. The structure of ssODNs with asymmetric arms complementary to a non-target locus with long arm on the PAM-proximal side and short arm on the PAM-distal

side of the break, has been previously reported to induce highest HDR efficiency (14). However, we decided to test this asymmetric donor for the *TNF α* locus by comparing it with other possible ssODN structures on HDR efficiency.

Thus, we generated twelve ssODN molecules having different sequences overlap on the 5' and 3' side of the break, specific for pX333-sgRNA2 and pX333-2sgRNA1/2 guides, and complementary to either target or non-target DNA strand (**Fig.3A,5A**). We co-delivered these ssODNs in combination with single sgRNA and for the first time with a couple sgRNAs (2sgRNA1/2).

To facilitate the selection of HDR events, we inserted a restriction site for the enzyme SmaI in all the ssODNs. The pX333-sgRNA2 and pX333-2sgRNA1/2 plasmids and the respectively six ssODNs were then co-transfected in HEK293 cells. In addition, we introduced a nocodazole cells synchronization, described to improve the HDR (5). Then, we included a fourth condition in which the cells were transfected three consecutive times with the same donor and plasmid.

Since the edited sequences contain a newly acquired SmaI restriction site, the HDR efficiency, can be easily detected using the digestion on PCR products obtained with primers flanking the targeted locus.

Single cut and HDR efficiency

First, we determined the HDR efficiency with the co-delivery of pX333-sgRNA2 guide (who induces single DSB) and six ssODN molecules (A-F). We compared the HDR efficiency after SmaI digestion in single transfection events, triple transfection and with nocodazole cells treatment. Notably, among the six ssODNs, only the donors A, C and E showed HDR event (**Fig.3B,C,D,E**). The three donors are all complementary to the non-target DNA strand, and this observation is consistent with previous studies (14). The donors A and C are able to induce HDR but only after nocodazole treatment though with a low frequency of 5,4% and 3,9%, respectively. With triple transfections in unsynchronized cells, donor A increase the HDR efficiency from not detectable to 10,5%, and donor C from non detectable to 9,3%. Donor E, after triple transfection, increases the HDR efficiency of 1,4-fold. The highest HDR frequency achieved was 22.1% for donor E, with triple transfection and nocodazole cells treatment.

The graphic in **Fig. S1A,B** (supplementary figure) compares the HDR efficiency in synchronized and unsynchronized cells in one and triple cell transfections, respectively. The graphic in **Fig. S1C,D** instead, compares the HDR efficiency between single and

triple transfection in synchronized and unsynchronized cells. These data showed that no significant differences have been detectable with nocodazole treatment in our experimental condition, while the triple transfection increases the HDR efficiency, but only for the ssODNs A, C and E. While the other three donors (B, D, F) characterized to be complementary to target DNA strand didn't induce HDR event.

Notably, the donor E induces the highest HDR efficiency. It showed the same structure described by Richardson et al. to be the best for the HDR, with asymmetric arms complementary to a non-target locus with 90bp on the PAM-proximal side and 36bp extension arm on the PAM-distal side of the break (14). The HDR efficiency using donor E increased up to 22%, (more than two-fold) with triple transfection in synchronized cells (**Fig.4**).

Dual cut and DBS efficiency

Furthermore, we tested the HDR efficiency combining the use of dual 2sgRNA and rational design of ssODNs. HEK293 cells have been co-transfected with px333-2gRNA1/2 guide (who induces double DSB) and six ssODNs (G-N) (**Fig.5A**). Next, we determined systematically the effect on HDR efficiency in controls, in nocodazole synchronized cells and triple transfection.

To note, as already here described, a simple PCR amplification before the T7E1 assay, (that shows the DNA deletions caused from 2sgRNA1/2) is sufficient to assess the DSB efficiency in various experimental conditions. Interestingly, the DSB efficiency increases more than three-fold with triple transfection in synchronized cells (**Fig.6A**)

Dual cut and HDR efficiency

We further compared the HDR efficiency using px333sgRNA1/2 and the six ssODNs (G-N) with SmaI digestion (**Fig.5A**). With the use of 2sgRNA1/2 all ssODNs designed showed detectable SmaI digestion bands who indicates that HDR occurred in all experimental condition used. The graphic in **Fig.S2** (supplementary figure) showed the effect of nocodazole and triple transfection on HDR efficiency using double sgRNA guides. Again, the nocodazole doesn't have a significant effect on HDR; while the triple transfection increased the HDR for all donors of about 1,3-2,6 fold. The nocodazole diminished the HDR efficiency except for donor M. The donor ssODN M, has the same design of donor E (the most efficient donor in the single cut comparison) with a PAM of 90bp proximal arm and a 36bp arm with a distal PAM complementary to a non-

target DNA strand, and it is the most efficient ssODN, achieving a HDR efficiency up to 39% (**Fig.5B,C,D,E**). The graphic in figure 6B compares the HDR efficiency of donor M in all experimental conditions used. The HDR after triple transfection in sync cells, increases up to 1,8-fold. As already reported in literature, even in *TNF α* locus, the use of 2sgRNA dramatically increase the HDR compared to single sgRNA more than double (1).

Collectively, the results demonstrated that the combination of 2sgRNA, asymmetric donor and triple transfection, induce a dramatic increase of HDR, from undetectable to 39% HDR efficiency.

Off-targets analyses

To predict the most likely off-target sites for the sgRNAs used to edit the *TNF α* gene in this study, we used a public webserver:

(https://eu.idtdna.com/site/order/designtool/index/CRISPR_PREDESIGN) able to assess and prioritize potential CRISPR/Cas9 activity at off-target loci. The top three potential off-target sites for each sgRNA were assessed by the T7E1 assay. None of the loci analyzed showed detectable levels of off-target events (**Fig.S3B**).

Discussion

Here, we report a new and simple approach to enhancer genome engineering in human cells. We compared the use of single sgRNA with coupled 2sgRNA to edit the *TNF α* locus. According to data already reported, our results showed that the use of 2sgRNA increases dramatically the DSB efficiency. Furthermore, the use of 2sgRNA creates a precise DNA excision between each PAM sequence, a huge advantage over the randomly sized indels created by single sgRNA transfection (**Fig.2**). In addition, the deletions between the 2sgRNA can be easily identified by PCR amplification and agarose electrophoresis, thus avoiding the T7E1 assay. Further, the results showed that the DSB increases after triple transfection up to two-fold and, in combination with nocodazole treatment increases up to three-fold (**Fig.6**).

As already known, even in *TNF α* locus, the use of 2sgRNA increases the HDR efficiency more than double compared to transfection of single sgRNA. This could be explained by the capability of double 2sgRNA to create a precise cut on the target site, oscillating from time to time only for a few bases, without generating unpredictable indels. Otherwise, a single-cut approach, induces truly extensive deletions reducing a

lot the possibility of binding the arms of homology, thus leading to a drastic drop in HDR rates.

We then tested the HDR frequency by transfecting various ssODN molecules with different structure in combination with single sgRNA and 2sgRNA pair. In particular, with the use of single sgRNA, we observed that donor DNA complementary to the non-target strand are more effective than the ones complementary to target strand, and this is consistent with previous studies performed with various ssODNs structure in human cells (14), or using symmetric ssODNs to introduce mutations at the EMX1 and AAVS1 loci in human cell lines (5)(24) (**Fig.3**). With the 2sgRNA pair since all ssODNs have higher HDR efficiency, this effect is less marked (**Fig.5**).

Our results further showed, again consistent with Richardson et al., that the asymmetric ssODN donor complementary to non-target strand with the arm of 36bp on the PAM-distal side and the 90bp arm on the PAM-proximal site of the break showed the highest HDR efficiency using single sgRNA as well the couple 2sgRNA (donor E, M) (14). The asymmetric donor allows the shorter arm to bind to distal PAM early released strand, and the longer arm to bind to the PAM proximal portion of non-target strand, by strand intrusion and complementary strand displacement. This donor structure increases the HDR of about two-fold.

In contrast with literature data, we showed that the treatment with nocodazole doesn't increase the HDR efficiency (13). Lin et al. showed a systematically studies of nocodazole concentration on HDR efficiency. They transfected the cells after 17h of the nocodazole treatment at concentration of 200ng/ml. They showed that the nocodazole increases the HDR efficiency when is used in combination with low concentration of Cas9 (30pmol), while 100pmol diminished the enhancement. Is plausible to think that in our experimental condition, since we transfected a plasmid who induces Cas9 expression to high dosage, we didn't use the correct Cas9 concentration to induces an increase in HDR efficiency. Moreover, we used 100ng/ml of nocodazole that was added four hours after the transfection. In our experiments, the nocodazole effect was detected only on the donor E and M, the best donor structures, also capable of increasing HDR efficiency in cells synchronized with a wrong Cas9 concentration.

Interestingly the triple transfection increases the HDR from 1,3-2,6 fold. The triple transfection was assessed in order to enrich the HDR cells colony expansion. Starting by the evidence that any ssODNs, when properly integrated, destroy the sgRNA

recognition sequence; it has been decided to give a day of rest after every transfection event, in order to facilitate the HDR colony expansion. Due to the impossibility of re-modifying HDR colonies, the Cas9 protein is able to modify only non-recombinant cells.

In conclusion, our results showed that the highest HDR efficiency has been achieved when 2sgRNA, asymmetric donor (M) and triple transfection in synchronized cells were used. Using this system, we have maximized the efficiency of HDR, from undetectable HDR events (single guide, symmetric donor, unsynchronized cells, with single transfection) near to 40% HDR efficiency. We also proved that the *TNF α* gene can be edited with CRISPR/Cas9 methodology with high efficiency.

Finally, the results of our work can be used as a guideline to improve the efficiency (and the utility) of CRISPR/Cas9-mediated genome engineering using the most effective optimizations to date.

Author contributions

M.D.S., N.F. and A.P.D. conceived and designed the project. N.F. and M.D.S. performed the experiments. E.A. performed graphic analysis. M.D.S., A.P.D. and P.G. wrote the manuscript.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Conflict of Interest

There are not conflict of interest to declare

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Figure legends

Fig.1 CRISPR/Cas9 targeting of the human *TNF α* gene. Schematic representation of human *TNF α* gene. The magnified view illustrates the sgRNAs (in red) and the PAM sequences (in green).

Fig.2 NHEJ-mediated knock-out of human *TNF α* gene using the CRISPR/Cas9

system. (A) The T7EI nuclease assay on *TNFα* gene showed targeted cleavage of the digested PCR products in HEK293 cells transfected with pX333-sgRNA1, pX333-sgRNA1/1, pX459-sgRNA1, pX333-sgRNA 2, pX459-sgRNA2, pX333-sgRNA2/2 and pX333-sgRNA1/2 . Cells transfected with 2sgRNA shows the short edited PCR product. (not determined, ND; negative control, NC; full-length, FL; short-edited, SE). **(B)** Sequence analysis of PCR products surrounding the Cas9 target sites in the genome of HEK293 transfected with sgRNA1, pX333-sgRNA1/1, pX333-sgRNA2, pX333-sgRNA2/2 and pX333-2sgRNA1/2, (in bold) showed a wide variety of Indel mutations mediated by NHEJ. The top sequence in red is the unmodified sequence, in green are the PAMs. The mismatches/insertions are indicated in gray. The number of PCR amplicons for each sequence is indicated in parentheses and the modified length is indicated.

Fig.3 Systematic investigation of DNA templates for efficient HDR at the *TNFα* locus in HEK293T cells. (A) Segment of human *TNFα* shows the genome structure, the sgRNA2 guide site and the primer used for PCR amplification (in violet). +1: ATG. Six HDR templates (color coded) were tested for HDR efficiency, the PAM region (in green). Template ssODN contains SmaI restriction sites (in red) that are flanked by various lengths of homology arms. **(B)** HDR efficiency was tested in single and triple transfection, in combination with synchronized cells. The mean % HDR and standard deviation (error bar) was determined by SmaI digestion from three experiments. Representative gels from PCR and HDR analyses are shown for each cell condition.

Fig.4 Graphic comparison of HDR efficiency of donor E. The triple transfection and nocodazole treatment increase the HDR efficiency of about two-fold. StUns, Single transfection in unsynchronized cells; StSyn, Single transfection in synchronized cells; TtUns, triple transfection in unsynchronized cells; TtSyn, triple transfection in synchronized cells.

Fig.5 Systematic investigation of DNA templates, and two sgRNA, for efficient HDR at the *TNFα* locus in HEK293T cells. (A) Segment of human *TNFα* shows the genome structure, the two sgRNA 2 guides (sgRNA1, sgRNA2) sites and the primer used for PCR amplification (in violet). +1: ATG

Six HDR templates (color coded) were tested for HDR efficiency, the PAM region (in green). Template ssODNs contains SmaI restriction sites (in red) that are flanked by various lengths of homology arms. **(B)** HDR efficiency was tested in single and triple transfection, in combination with synchronized cells. The mean % HDR and standard deviation (error bar) was determined by SmaI digestion from three experiments. Representative gels from PCR and HDR analyses are shown for each cell condition.

Fig.6 DSB efficiency with double sgRNA guides. (A) Editing efficiency tested on four different conditions. StUns= Single transfection unsynchronized, StSyn= Single transfection nocodazole synchronization. **(B)** Single cut strategy and donor M, in single and triple transfection, in synchronized and unsynchronized cells. The triple transfection increase the HDR efficiency. StUns= Single transfection method, unsynchronized cells; StSyn= Single transfection in synchronized cells, TtUns= Triple transfection method in unsynchronized cells, TtSyn Triple transfection in synchronized cells.

Suppl. Fig.S1 Single cut strategy with sgRNA2 guide. (A, B) HDR efficiency between synchronized and non-synchronized cells, in single and triple transfection, respectively. **C,D** HDR efficiency between single and triple transfection in synchronized and unsynchronized cells.

Suppl. Fig.S2 Double cut strategy with 2sgRNA1/2 guide. (A, B) HDR efficiency between synchronized and non-synchronized cells in single and triple transfection, respectively. **C,D** HDR efficiency between single and triple transfection in synchronized and unsynchronized cells.

Suppl. Fig.S3. (A) Sanger Sequence who showed the correct HDR events. **(B)** Evaluation of CRISPR/Cas9 off-target effects for sgRNAs designed to knock-out the human *TNF α* gene. T7 assay analysis at the top three potential off-target sites and the first potential genic off-target site in HEK293T cells. OT: off-target locus

Fig.1

B

sgRNA1

Deletions

GTGGAGGAACAGCACAGGCTTAGTGGGATACTCAGAACGTCATGGCCAGGTGGGATGTGGGATGACAG (X8)
 -----TGGGATACTCAGAACGTCATGGCCAGGTGGGATGTGGGATGACAG **-267bp**

Insertion

GTGGAGGAACAGCACAGGCTTAG- TGGGATACTCAGAACGTCATGGCCAGGTGGGATGTGGGATGACA
 GTGGAGGAACAGCACAGGCCTTAGTTGGGATACTCAGAACGTCATGGCCAGGTGGGATGTGGGATGACA **+1bp**

Editing Efficiency 20%

sgRNA1-1

Deletions

GTGGAGGAACAGCACAGGCTTAGTGGGATACTCAGAACGTCATGGCCAGGTGGGATGTGGGATGACAG (X8)
 -----GGATACTCAGAACGTCATGGCCAGGTGGGATGTGGGATGACAG **-930bp**
 -----GCCAGGTGGGATGTGGGATGACAG **-209bp**

Editing Efficiency 20%

sgRNA2

Deletions	Lenght
CCAGACAGGCAGCCAGCTGTTCTCCTTTAAGGGTGACTCCCTCGATGTTAACCATTCTCCTTCTCCCC (x3)	
CC-----AAGGGTGACTCCCTCGATGTTAACCATTCTCCTTCTCCCC	-27bp
CCAGACAGGCAGCCAGCT-----	(X2) -378bp

Insertion

CCAGACAGGCAGCCAGCTGTTCTCCTTT- AAGGGTGACTCCCTCGATGTTAACCATTCTCCTTCTCCCC	
CCAGACAGGCAGCCAGCTGTTCTCCTTTTAAAGGGTGACTCCCTCGATGTTAACCATTCTCCTTCTCCCC	+1 bp

Editing Efficiency 50%

sgRNA2-2

Deletions

CCAGACAGGCAGCCAGCTGTTCTCCTTTAAGGGTGACTCCCTCGATGTTAACCATTCTCCTTCTCCCC (X5)	
CCAGACAGGCAGCCAGCTGTTCTCCTT-----	-181bp
CCAGACAGGCAGCCAGCTGTCC-----CTCGATGTTAACCATTCTCCTTCTCCCC (X2)	-19bp
CCAGACAGGCAGCCAGCTGTTT-----TTAAGGGTGACTCCCTCGATGTTAACCATTCTCCTTCTCCCC	-5bp

Insertion

CCAGACAGGCAGCCAGCTGTTCTCCTTTAA-----GG-----TGACTCCCTCGATGTTAACCATTCTC	
CCAGACAGGCAGCCAGCTGTTCTCCTTTAACAGCAGGAACAGTGACTCCCTCGATGTTAACCATTCTC	+10bp

Editing Efficiency 50%

sgRNA1-2

	Lenght
CCAGACAGGCAGCCAGCTGTTCTCCTTTAAGGGTG(446bp)GAACAGCACAGGCTTAGTGGGATACTCAGAACGT (X3)	
CCAGACAGGCAGCCAG----- (446bp)-----GGATACTCAGAACGT	-486bp
CCAGACAGGCAGCCAGCTGTTCTC----- (446bp)-----GATACTCAGAACGT	-478 bp
CCAGACAGGCAGCCAGCTGTTCTCT----- (446bp)-----TGGGATACTCAGAACGT (X2)	-474 bp
CCAGACAGGC----- (446bp)-----TGGGATACTCAGAACGT	-490bp
CCAGACAGGCAGCCAGCTGTTCTCT----- (446bp)-----ATACTCAGAACGT	-477 bp
CCAGACAGGCAGCCAGCTGTTCTCT----- (446bp)-----TGGGATACTCAGAACGT	-474 bp
CCAGACAGGC----- (446bp)-----ACTCAGAACGT (X2)	-497 bp

Editing Efficiency 75%

Fig.2

Fig.4

Fig.5

Fig.6

Fig.S1

Fig.S2

A

B

Fig.S3