Modulation of DNA repair pathways by HDR-CRISPR promotes seamless genome editing in primary human hematopoietic cells

Antonio Carusillo1, Raul Schäfer1, Manuel Rhiel1, Daniel Turk1, Tatjana Cornu1, Toni Cathomen1 and Claudio Mussolino1

[1]Institute for Transfusion Medicine and Gene Therapy, Medical Center - University of Freiburg, Freiburg, Germany

Email: claudio.mussolino(at)uniklinik-freiburg.de; antonio.carusillo(at)uniklinik-freiburg.de

Abstract

CRISPR-Cas system is a robust platform for genome editing application. The introduction of a DNA double-strand break (DSB) in precise gene locations can be exploited to achieve targeted gene knockout by harnessing the error-prone Non-Homologous End-Joining (NHEJ) DNA repair mechanism. However, using this technology for precise genome editing remains challenging. This relies on the homology-directed repair (HDR) pathway that uses a properly designed DNA donor as a blueprint to install the desired modification. NHEJ predominance during DSB repair together with restriction of HDR to certain phases of the cell cycle often results in HDR frequencies far below clinically relevant frequencies. Common strategies to increase HDR-mediated DSB repair include the use of chemicals to either arrest the cells in those cell cycle phases when HDR is most active or to inhibit NHEJ. However, the global effects of these drugs may pose safety concerns for clinical applications. To address this issue, we devised a strategy to recruit HDR-promoting factors or NHEJ-inhibiting proteins at the DSB site. This is achieved via their direct fusion to the Cas9 nuclease to promote the engagement of HDR during DSB repair and increase the frequency of precise genome editing. We generated 16 different Cas9-fusion proteins (referred to as HDR-CRISPR) and extensively investigated their impact on DNA repair choice by using two reporter systems, the traffic light reporter (TLR) and the BFP-to-GFP (B2G) assay. These two assays allowed us to investigate the outcome of DNA repair mediated by a DNA donor supplied either as plasmid or oligodeoxynucleotides (ODN) respectively. Our results indicated that HDR-CRISPRs can affect DSB repair leading to a 3-fold increase in HDR frequency over baseline levels. The simultaneous reduction of NHEJ-mediated repair led to a 5-fold increase in the HDR: NHEJ ratio using our best performing HDR-CRISPR. Next, we evaluated the capability of HDR-CRISPR to precisely integrate a large GFP expression cassette into the endogenous AAVS1 safe harbor locus of K562 and Jurkat cell lines. Independently by the cell type used, HDR-CRISPR increased 2.5-fold the precise integration events. The most efficient HDR-CRISPR fusion was then delivered as mRNA in T lymphocytes and Hematopoietic Stem Cells (HSCs). Using an appropriate ODN as a repair template, we aimed at introducing a stop codon within the exon 3 of the CCR5 gene to generate immune cells resistant to HIV infection. Our results show that HDR-CRISPR is capable to alter the normal resolution of a DSB, leading to a 2-fold increase in precise genome editing events as compared to the standard Cas9. Our data support the hypothesis that DSB repair choice can be altered through the local recruitment of key factors capable of either promoting HDR or inhibit NHEJ. We envision this technology may contribute to increase precise editing yield in clinically relevant settings.

Strategy

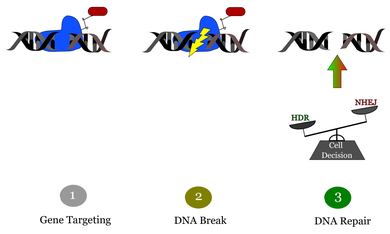

Fig. 1 Hijacking DNA repair strategy

In order to affect DNA repair decision after CRISPR/Cas9-mediated DSB introduction,DNA modulating factors are fused directly to the SpCas9 nuclease. The hyphotesis is that having one or more than one factor already available at the site of the DSB will force the cell to engage HDR over NHEJ

The Platform

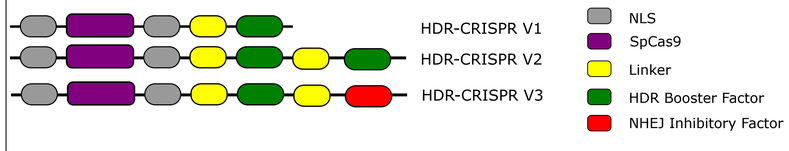

Fig. 2 HDR-CRISPR design

To bias cell decision 3 different versions of HDR-CRISPR were generated: *V1)* a single factor promoting HDR (HDR Booster) was fused to the SpCas9 *V2)* two factors promoting HDR (HDR Booster) were fused in tandem to the SpCas9 *V3)* one HDR Booster and one NHEJ inhibitory factor were fused in tandem to the SpCas9

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DNA Repair outcome assays

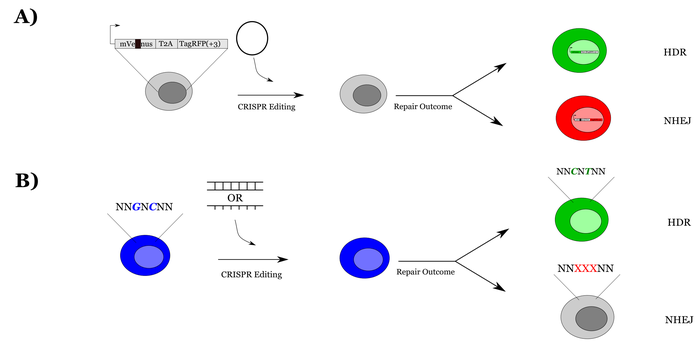

Fig.3 Reporter systems for DSB repair outcomes assessment

A) TLR is based on a non functional mVenus protein and an out-of-frame TagRFP protein. When DSB is induced, if HDR is engaged and the donor (plasmid) to restore mVenus is used the cells turn green. Contrary if indels are introduced via NHEJ, the TagRFP shifts in the correct frame and the cells turn Red.

B)B2G takes advantage of the similarity beween BFP and GFP. When DSB is induced, if HDR is engaged and donor provided (ssODN or dsDNA) is used, the nucleotide change causes a shift from BFP to GFP. Conversely, introduction of indels within the BFP coding sequnece, results in loss of cell fluorescence.

Reporters Screening

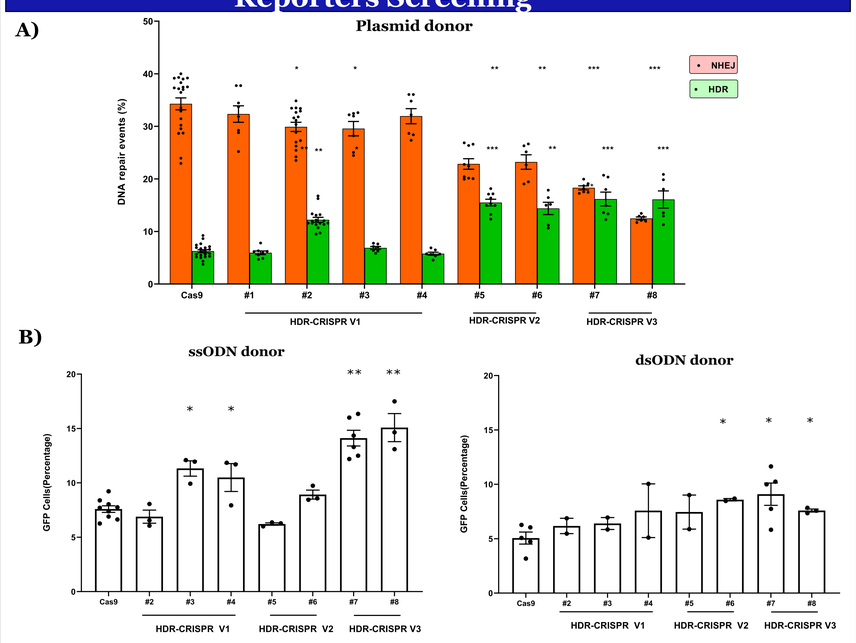

Fig. 4 Testing HDR-CRISPR functionality via Reporter systems

A)The TLR assays shows among the single fusions, #2 provided significant increase in HDR and a slight reduction in NHEJ. However the double fusion combining an HDR boosting factor and an NHEJ inhibitor (V3) yielded the best effect.

B)The BFP to GFP assay shows that HDR-CRISPR can perform better than standard Cas9 when the DNA template is delivered as ssODN or dsDNA. Interestingly in this assay the #2 (V1) did not show any advanateg. While the V3 (#7 and #8) showed the best performance in particular in case of an ssODN being used as donor

Statistics: Each dot represents the mean of a single experiment performed in technical duplicate; 1-Way ANOVA the stars indicate the significance, *p< 0.05, **p< 0.005 and ***p< 0.0005

HDR-CRISPR improves targted integration

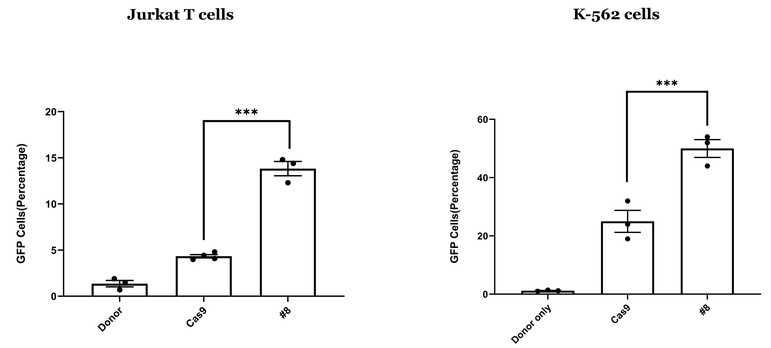

Fig. 5 HDR-CRISPR improves targeted integration

The HDR-CRISPR V3 #8 was select for testing its abilty to increase targeted integration of a SA-T2A-GFP at endogenous *AAVSI* locus in Jurkat T cells and K-562 cells.

The results indicate that the double fusion can outperform standard Cas9 in both cell lines.

Statistics: Each dot represents the mean of a single experiment performed in technical duplicate; two-tails t-Test the stars indicate the significance, *p< 0.05, **p< 0.005 and ***p< 0.0005

HDR-CRISPR increases precise editing in HSCs

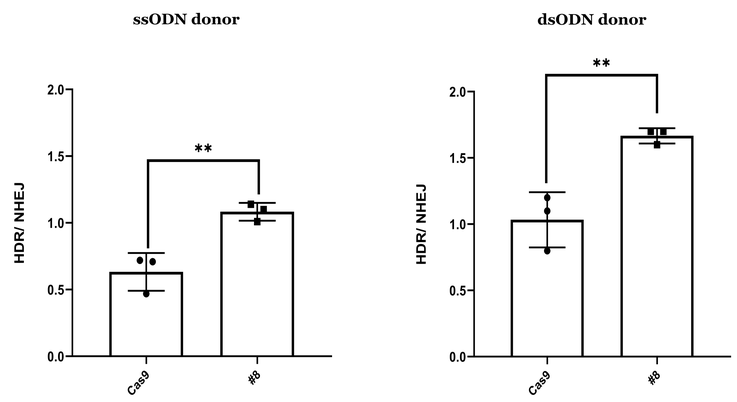

Fig. 6 HDR-CRISPR improves HDR/NHEJ ratio in HSCs

The HDR-CRISPR V3 #8 was delivered as mRNA into Hematopoietic Stem Cells (HSCs) to introduce a SNP within the *CCR5* exon 3 using an ssODN or a dsDNA. Editing outcome was evaluated via TIDER and the results shows that HDR-CRISPR can improve HDR/NHEJ ratio compared to the standard Cas9

Statistics: Each dot represents the mean of a single experiment and a different HSCs donor; t-test the stars indicate the significance, *p< 0.05, **p< 0.005 and ***p< 0.0005

Conclusions

We could prove that local recruitment of DNA modulating factors may alter the cell decision toward which DNA repair pathway commit upon introudction of a target DSB via CRISPR. In particular the combinaed action of a HDR-promoting protein and an NHEJ-inhibitory factor results in improved integration event in cell lines and it also improves the overall HDR/ NHEJ ratio in HSCs up to 2-fold in respective of standard spCas9.