Analyses of point mutation repair and allelic heterogeneity generated by CRISPR/Cas9 and single-stranded DNA oligonucleotides

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The repair of a point mutation can be facilitated by combined activity of a single-stranded oligonucleotide and a CRISPR/Cas9 system. While the mechanism of action of combinatorial gene editing remains to be elucidated, the regulatory circuitry of nucleotide exchange executed by oligonucleotides alone has been largely defined. The presence of the appropriate CRISPR/Cas9 system leads to an enhancement in the frequency of gene editing directed by single-stranded DNA oligonucleotides. While CRISPR/Cas9 executes double-stranded DNA cleavage efficiently, closure of the broken chromosomes is dynamic, as varying degrees of heterogeneity of the cleavage products appear to accompany the emergence of the corrected base pair. We provide a detailed analysis of allelic variance at and surrounding the target site. In one particular case, we report sequence alteration directed by a distinct member of the same gene family. Our data suggests that single-stranded DNA molecules may influence DNA junction heterogeneity created by CRISPR/Cas9.

Single-stranded DNA oligonucleotides (ssODNs) can be used to direct single base exchange in organisms ranging from yeast1–5, to plants6–9 to mammalian cells10–13. We have been studying the mechanism and regulation of single agent gene editing elucidating the regulatory pathways12,14,15. We have also utilized ssODNs in combination with programmable nucleases and RNA–guided engineered nucleases (RGENs) to execute point mutation repair in mammalian cells16–18. In this approach, the oligonucleotide is designed so that it invades the target duplex with a 3′ to 5′ polarity, consistent with traditional reaction mechanics of homologous pairing19–22. A critical reaction intermediate, known as a D-loop, is thus created and the resolution of this structure is the basis of single agent gene editing. This strategy directs base exchange to proceed through mismatch repair or through ssODN incorporation into a growing replication fork. In addition, we and others established that cells undergoing DNA replication, passing through S phase, are more amenable to gene editing activity23–28, consistent with this overall mechanism of action. For gene editing strategies, however, where the goal is to insert single-stranded DNA ultimately to execute gene replacement, the polarity with which the oligonucleotide interacts with the site is less stringent because it does not need to invade the duplex fully29–32. This pathway of gene editing utilizes the process of Homology Directed Repair (HDR).

In this study, we examine how successful single base gene editing correlates with CRISPR/Cas9 cleavage activity in a reaction where the objective is to correct a single base mutation in a well-established model gene editing system where both genotypic and phenotypic readout have been validated17. We sought to define the relationship between point mutation repair and DNA cleavage activity since the product of CRISPR/Cas9 activity is often a heterogeneous population of chromosomal ends created through DNA resection promoted by non-homologous end joining (NHEJ). DNA cleavage assays have often been used as a benchmark to measure the level of successful gene editing in mammalian cells, especially with regard to genetic knockout. While such correlations are likely

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to be valid for studies where the goal is to disable the gene, our experience led us to believe that gene editing of a single base mutation could be accompanied by variable DNA changes at the target site. Here, we examine the outcome of the combined activity of single-stranded oligonucleotides and a specifically designed CRISPR/Cas9 system at a specific target site with a particular emphasis on the evaluation of allelic mutagenesis.

Results and Discussion

The repair of a single point mutation by gene editing can be evaluated using a well-established reporter gene system consisting of a single copy of a mutant eGFP gene integrated into HCT116 cells. Repair of this mutation is executed by the combined action of a specifically designed ssODN, 72 bases in length, and the appropriate CRISPR/Cas9 system. The cells can be targeted as either an unsynchronized population or synchronized and released population, as shown in Fig. 1A. The reaction is initialized through strand invasion by the ssODN and subsequent alignment in homologous register with the target site (here, the mutant eGFP gene) except for a single base pair mismatch located in the center. Figure 1B illustrates the alignment of the ssODN with the appropriate strand of the mutant eGFP gene. Here, we evaluated gene editing activity by the combined action of the ssODN, and a CRISPR/Cas9 system previously designed for the same target and concurrently measured CRISPR/Cas9 cleavage activity using the well-known Surveyor endonuclease assay. Since this is a reporter gene, off-target effects are minimized, yet we include the ten top predicted off target sites in supplementary Table S1.

Next, DNA cleavage activity, generated through the action of the CRISPR/Cas9 system, was determined by the Surveyor endonuclease at dosages that support significant levels of gene editing. The data are presented in Fig. 3 and supplementary Figure S1. The Surveyor assay successfully detected CRISPR/Cas9 activity in the absence of the ssODN; a predictable rise in activity was observed as a function of the amount of expression vector present in the reaction. When cleavage activity is measured in complete reaction mixtures, i.e., in the presence of the oligonucleotide, Surveyor endonuclease activity is increased (orange line vs yellow line), particularly at the two doses where gene editing activity is near maximal. While this may appear to be counterintuitive, the Surveyor
endonuclease assay has a preference for DNA duplexes with small but definitive indels. DNA duplexes bear small insertions, deletions or single base changes which are more appropriate as substrates for cleavage34. This may suggest a significant degree of heterogeneity at the target site in conjunction with point mutation repair or perhaps independent of it. A similar observation was made by Schumann et al. using a T7 Endonuclease I (T7E1) recognition assay to measure CRISPR/Cas9 cleavage activity37. In that system, however, the objective was different; to insert a segment of DNA.

The correlation between the extent of single base gene editing and the degree of heterogeneity detected by the Surveyor assay prompted us to examine the DNA sequence both at and surrounding the target site, as a function of point mutation repair. A testable hypothesis is that point mutation repair could be accompanied by various

Figure 2. Gene editing dose curve using in synchronized and unsynchronized cells. Synchronized (blue) and unsynchronized (orange) HCT116-19 cells were electroporated with 0.1–10.0 μg of pX330 and 1.35 μg of 72mer. After a 48-hour recovery period, gene editing activity was measured using a Guava EasyCyte 5HT Flow Cytometer. Gene editing is displayed as correction efficiency (%), determined by the number of viable eGFP positive cells divided by the total number of viable cells in the population. Each treatment was performed in triplicate and standard error is illustrated with accompanying bars. Statistical analysis was performed using two-sample unequal variance students T-test distribution to compare the value of correction efficiency between synchronized and un-synchronized cells when treated with CRISPR/Cas9. *p < 0.05. Inset. Correction efficiency using varying lengths of ssODN at equimolar concentrations with 5 ug of pX330, 48 hours after electroporation.

Figure 3. Correlation between CRISPR/Cas9 cleavage and gene editing activity. Synchronized and released HCT116-19 cells were electroporated with 0.0–10.0 μg of pX330 and with (+ODN) or without (−ODN) 1.35 μg of 72-mer. CRISPR/Cas9 cleavage activity measured by Surveyor endonuclease assay (orange and yellow) as well as gene editing (dark green and light green) activity measured by FACS are shown. Standard error is represented by the bars on each data point.
degrees of onsite mutagenesis, resulting in a population of heterogeneous re-ligated DNA ends, the resection products. In order for our observations and data to have more relevance, we decided to do a detailed analysis of single base repair, as it may relate to onsite mutagenesis, on a native gene target. The beta globin gene in K562 cells is a well-established and well-known target for gene editing particularly with regard to Sickle Cell Disease\textsuperscript{38–40}. A mutation in the sixth codon of the beta globin gene is responsible for the onset of Sickle Cell Disease and, as such, a reversal of that point mutation through gene editing would have immediate translational applications. We utilized K562 cells which harbor 2–3 copies of the normal beta globin gene\textsuperscript{41,42}, bearing a wild type nucleotide (A), at position 16 in the coding region (GAG), as displayed in Fig. 4. We employed the same strategy used for eGFP gene editing; a CRISPR/Cas9 system and the appropriate single-stranded oligonucleotide to convert the A to a T nucleotide.

A CRISPR/Cas9 complex that induces DNA cleavage at the indicated site (Fig. 4) was chosen because it had the lowest potential for off-target activity yet would still induce a DNA break site closest to the target nucleotide in the beta globin gene. We also depict the 72-mer oligonucleotide that has been designed specifically to create a mismatch within the adenosine nucleotide (A, in bold) within the wild-type beta-globin (HBB) gene. Resolution of this point mutation through gene editing results in the creation of a T at the mismatch site and subsequently a T:A base pair at the target site (illustrated in Red). The CRISPR/Cas9 targeting system is harbored in an expression vector which also contains a wild-type eGFP gene so that K562 cells successfully transfected are discernible by FACS. The overall experimental flow, as illustrated in Fig. 5, was designed with the ultimate goal of full-scale analyses of sequence alterations. The cells exhibiting eGFP expression were sorted using a FACS Aria II and placed into individual wells for single cell clonal expansion. Cells were allowed to expand for approximately 3–5 weeks at which time DNA was extracted from the clonal expansions and prepared for sequence analysis. Figure 5 (lower panels) displays the results of the Sanger sequencing of 1 individual/expanded clonal population (Clone 22) as well as Sanger sequencing trace files analyzed by the software program, Tracking of Indels by decomposition (TIDE)\textsuperscript{43}. The TIDE program facilitates the examination of individual allelic sequences within complex Sanger sequencing data, which often displays a multiplicity of nucleotide peaks at or surrounding the position of DSB repair. An algorithm that isolates individual sub-sequences followed by an alignment mechanism relative to the control parental sequence to determine the indel structure of individual alleles is utilized. In addition, TIDE
affords the opportunity for qualitative analysis of clonally expanded populations, analyses that can provide unique insight into the cleavage profile of each allele in individual targeted cells. As an example of our analytical strategy, we display data from Clone 22 which harbors homozygosity for the biallelic A to T conversion, as indicated by the nucleotides presented in red. We know that while point mutation repair has occurred, each allele contains a different indel; the TIDE indel distribution displays a 2 base deletion (allele 1) and a 1 base insertion (allele 2).

Allelic analysis was performed on 26 individual clones and among them 23.08% (6) contained at least one allele with the repaired nucleotide. Of the 6 clones having some degree of conversion, 5 of the 6 had at least one allele corrected (partial conversion); the other one had both alleles corrected (full conversion). Figure 6 and Supplemental Figure S2 depict data from the four possible genotypic outcomes of the gene editing reaction and detailed clonal analyses. These include clones that have the precisely repaired point mutation (conversion) alone, conversion accompanied by indel formation, indel formation alone or clones with no genetic change. The data show that 100% of the 26 clones contained indels while 23% contained specific point mutation repair on at least one allele. Through Sanger sequencing and TIDE analyses, we found that a majority of the indels harbor between 1 and 3 base deletions. Additionally, Sanger sequencing and TIDE analyses reveal several distinct recombination patterns around the double-stranded break site. Of the 26 clones analyzed, only 14 distinct sequences were found surrounding the break site, and in several cases, relatively large groups of these clones were identical genetically. For example, 5 of the 26 clones displayed a 3 base pair deletion on one allele and a one base deletion on the other. A similar pattern was also observed with 5 additional clones, all of which had a 3 base pair deletion on both alleles. This interesting result is significant in that these 2 distinct groups comprise 38.46% of all the clones analyzed. Likewise, another 2 distinct groups emerge from our study. One group, making up 11.54% of analyzed clones, displayed a 1bp deletion directly downstream of the cleavage site in both alleles. The second group, which comprised another 11.54% of all analyzed clones, contained three HBB alleles, as determined by proportions of sub-sequence prevalence in the TIDE readout; one allele contained a 3bp deletion, and the other 2 alleles both

![Figure 6. Clonal analysis of the targeted HBB gene.](image-url)
Cells were released for 4 hours prior to transfection by washing with PBS. Following construction, clones were verified by DNA sequencing by Genewiz Incorporated (South Plainfield, NJ).

The HBB gene sequence was entered into the Zhang Lab's online generator (http://crispr.mit.edu/) and the appropriate CRISPR guide sequence which binds in close proximity to target (2bp downstream of the sickle mutation site) was chosen. The gRNA was cloned into the pX458 backbone vector (Addgene plasmid 4238), a human codon-optimized SpCas9 and chimeric guide RNA expression plasmid. The HBB gene sequence was entered into the Zhang Lab's online generator (http://crispr.mit.edu/) and the appropriate CRISPR guide sequence which binds in close proximity to target (+2bp downstream of the sickle mutation site) was chosen. The gRNA was cloned into the pX458 backbone vector (Addgene plasmid 4238), a human codon-optimized SpCas9 and chimeric guide RNA expression plasmid with a 2A-eGFP. pX330 and pX458 were purchased through Addgene (https://www.addgene.org).

The guide RNA and CRISPR/Cas9 for the eGFP system used for gene editing in this system was described previously. The guide RNA and CRISPR/Cas9 were constructed using standard cloning methods following the latest oligo annealing and backbone cloning protocol with single-step digestion-ligation. The eGFP target gRNA was cloned into pX330 backbone vector (Addgene plasmid 42230), a human codon-optimized SpCas9 and chimeric guide RNA expression plasmid. The HBB gene sequence was entered into the Zhang Lab's online generator (http://crispr.mit.edu/) and the appropriate CRISPR guide sequence which binds in close proximity to target (+2bp downstream of the sickle mutation site) was chosen. The gRNA was cloned into the pX458 backbone vector (Addgene plasmid 48138), a human codon optimized pSpCas9 and chimeric guide RNA expression plasmid with a 2A-eGFP. pX330 and pX458 were purchased through Addgene (https://www.addgene.org). Following construction, clones were verified by DNA sequencing by Genewiz Incorporated (South Plainfield, NJ).

Methods and Materials

Cell Line and Culture Conditions. HCT116 cells and K562 cells were acquired from ATCC (American Type Cell Culture, Manassas, VA). The HCT116-19 was created by integrating a pEGFP-N3 vector (Clontech, Palo Alto, CA) containing a mutated eGFP gene. The mutated eGFP gene has a nonsense mutation at position +67 resulting in a nonfunctional eGFP protein. For these experiments, HCT116-19 cells were cultured in McCoy's 5A Modified medium (Thermo Scientific, Pittsburgh, PA) supplemented with 10% fetal bovine serum, 2 mM L-Glutamine, and 1% Penicillin/Streptomycin. Cells were maintained at 37°C and 5% CO2. K562 cells were cultured in Iscove's Modified Dulbecco's Medium (ATCC, Manassas, VA), supplemented with 10% fetal calf serum and 1% Penicillin/Streptomycin. Cells were maintained at 37°C and 5% CO2. The eGFP targeting custom designed 72-mer oligonucleotide was synthesized by IDT (Integrated DNA Technologies, Coralville, IA). The HBB custom 72-mer single-stranded oligonucleotide used (5′-CCTTGCCCACAGGGCTCCTGAAGAGGTATGGTTGGTTGAGGTT-3′), was obtained from TriLink Biotechnologies, San Diego, CA.

CRISPR Design and Construction. The guide RNA and CRISPR/Cas9 for the eGFP system used for gene editing in this system was described previously. CRISPR/Cas9 were constructed using standard cloning methods following the latest oligo annealing and backbone cloning protocol with single-step digestion-ligation. The eGFP target gRNA was cloned into pX330 backbone vector (Addgene plasmid 42230), a human codon-optimized SpCas9 and chimeric guide RNA expression plasmid. The HBB gene sequence was entered into the Zhang Lab's online generator (http://crispr.mit.edu/) and the appropriate CRISPR guide sequence which binds in close proximity to target (+2bp downstream of the sickle mutation site) was chosen. The gRNA was cloned into the pX458 backbone vector (Addgene plasmid 48138), a human codon optimized pSpCas9 and chimeric guide RNA expression plasmid with a 2A-eGFP. pX330 and pX458 were purchased through Addgene (https://www.addgene.org). Following construction, clones were verified by DNA sequencing by Genewiz Incorporated (South Plainfield, NJ).

Experimental Strategy. For experiments utilizing synchronized cells, HCT116-19 cells and K562 cells were seeded at 2.5 × 10^6 cells in a 100 mm dish and synchronized with 6 μM aphidicolin for 24 hours prior to targeting. Cells were released for 4 hours prior to transfection by washing with PBS (−/−) and adding complete growth medium.
media. Synchronized and unsynchronized HCT116-19 cells were simultaneously transfected at a concentration of $5 \times 10^5$ cells/100 μl in 4 mm gap cuvette (BioExpress, Kaysville, UT). 0.6 μM of single-stranded oligonucleotide and/or the appropriate dose of pX330 constructs were electroporated (250 V, LV, 13 ms pulse length, 2 pulses, 1 s interval) using a Bio-Rad Gene Pulser XCell™ Electroporation System (Bio-Rad Laboratories, Hercules, CA). Cells were then recovered in 6-well plates with complete growth media at 37 °C for 24–48 hours prior to analysis. For K562 cell transfections, synchronized and unsynchronized cells were seeded in 6-well plates at a density of $5 \times 10^5$ cells/well in 1.5 mL complete media. Transfection complexes of 2.5 μg PX458 containing the targeting gRNA and 0.6 μM 72-mer ssODN were formed at room temperature in 500 μl Opti-MEM according to the optimized LTX protocol (Thermo-Fisher, Waltham, MA) and added drop-wise to K562 cells, followed by a 72-hour incubation. After incubation, individual cells were sorted into each well of a 96-well plate with a FACSAria II flow cytometer (BD Biosciences, San Jose, CA). Clones were expanded into larger plates as the individual clones reached confluence, with DNA isolation occurring when cells reached confluence in a 6-well plate ($\sim 1 \times 10^6$ cells/ml).

**Analysis of Gene Edited Cells.** HCT116-19 cell fluorescence (eGFP+) was measured by a Guava EasyCyte 5HT Flow Cytometer (Millipore, Temecula, CA). Cells were harvested by trypsinization, washed once with 1x PBS (−/−) and resuspended in buffer (0.5% BSA, 2 mM EDTA, 2 μg/ml Propidium Iodide (PI) in PBS (−/−)). Propidium iodide was used to measure cell viability as such, viable cells stain negative for PI (up/t). Correction efficiency was calculated as the percentage of the total live eGFP positive cells over the total live cells in each sample. Cellular gDNA was isolated from pellets of 1–2 × 10^6 K562 cells using the Qiagen DNeasy Blood and Tissue Kit (Cat. ID 69506, Valencia, CA). PCR was performed using Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo-Scientific, Waltham, MA) on isolated gDNA, with amplification parameters optimized for an amplicon size of 345 bp (FWD Primer: 5′-TCTTAAGCCAGTGCCAGAAGAG-3′, REV Primer: 5′-CTATTGGTTCTCCTTAAACCT-3′, obtained from Integrated DNA Technologies, Coralville, IA). Amplicon size was verified on 1% agarose gel, and PCR products were verified by DNA sequencing by Eton Bio Incorporated (Union, NJ).

Individual K562 cell clones were analyzed by the software program, Tracking of Indels by Decomposition (TIDE), to determine the individual sub-sequences within the multi-peaked breakdown product after CRISPR/Cas9 activity (Netherlands Cancer Institute, https://tide.nki.nl/). The TIDE analyses provide a visual of the sequence decompositions, the indel patterns of the clone, as well as relative ratios of each clonal indel pattern, serving as an intermediate step in determining each allelic profile. By utilizing the indel patterns and their relative ratios provided by TIDE, the control trace sequence and a clonal trace sequence were manually aligned, allowing for the visualization of the indel patterns of each allele of a particular clone.

**SURVEYOR Analysis of CRISPR/Cas9 Cleavage Activity.** HCT116-19 cells were electroporated at a concentration of $5 \times 10^5$ cells/100 μl in 4 mm gap cuvette (BioExpress, Kaysville, UT) with pX330 or pX330 and 1.35 μg of the 72 mer ssODN. Cells were then recovered in 6-well plates with complete growth media at 37 °C for 72 hours. DNA was isolated using the Blood and Tissue DNeasy kit (Qiagen, Hilden, Germany). The Surveyor assay was performed on 605 bp amplicons that were created using forward primer, 5′CTGGACGGCGACGTAAACGGC and reverse primer, 5′ACCATGTGATCGCGCTTCTCG. PCR samples were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). 200 ng of each PCR product was mixed with 200 ng of PCR product from the untreated sample and subjected to a heteroduplex formation: 95 °C for 10 minutes, 95°C to 85°C with a ramp rate of −2 °C/s, 85 °C for 1 minute to 75 °C at −0.1 °C/s, 75 °C for 1 minute to 65 °C at −0.1 °C/s, 65 °C for 1 minute to 55 °C at −0.1 °C/s, 55 °C for 1 minute to 45 °C at −0.1 °C/s, 45 °C for 1 minute to 35°C to 25°C at −0.1 °C/s, 25°C for 1 minute. After duplex formation products were treated with SURVEYOR Nuclease S and SURVEYOR Enhancer S (IDT Technologies) for 30 minutes at 42 °C, gel electrophoresed and stained with SYBR Safe DNA stain (Life Technologies). Gels were imaged with a Gel Doc EZ Imager (Bio-Rad) and densitometry was performed by measuring the area under the curves of each band, using the Image Lab software (Bio-Rad). Calculations were based on the following formulas:

\[
\text{Indel} \% = 100 \times \left(1 - \frac{1}{(a + b + c)^{1/2}}\right)
\]

\[
a = \text{Intensity of the undigested product (parent)}
\]

\[
b + c = \text{Intensity of cleavage products}
\]

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Author Contributions

E.B.K. and P.B. conceived, designed and acquired data; N.R.-T. carried out the gene editing reactions; B.S. carried out the HBB targeting in K562; E.B.K. did the primary writing of the manuscript. E.B.K., P.B., B.S., N.R.-T., K.B. and D.M. did the revisions of the article and interpretation of the results.

Acknowledgements

We thank Dr. Lynn Odenaker (CTCR-CCHS) for outstanding assistance with the FACS analysis. We also acknowledge the valuable advice and input from all of the members of the Kmiec laboratory. Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number P20GM109021, and by the Leukemia Research Foundation of Delaware (EBK). Plasmids pX330 and pX458 (Addgene 42230 and 48138) were a gift from Dr. Feng Zhang (MIT). We thank Hailey Weiner for her assistance in manuscript preparation.

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Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Bialk, P. et al. Analyses of point mutation repair and allelic heterogeneity generated by CRISPR/Cas9 and single-stranded DNA oligonucleotides. Sci. Rep. 6, 32681; doi: 10.1038/srep32681 (2016).

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