

Multiplexed CRISPR/Cas9 genome editing increases the efficacy of homologous-dependent repair of donor sequences in mammalian cells

AUTHORS:

Ezio T. Fok¹
Clement B. Penny¹
Musa M. Mhlanga^{2,3}
Marc S. Weinberg^{4,5,6}

AFFILIATIONS:

¹Medical Oncology Research Unit, Department of Internal Medicine, School of Clinical Medicine, University of the Witwatersrand, Johannesburg, South Africa

²Gene Expression and Biophysics Group, Synthetic Biology–Emerging Research Area, Council for Scientific and Industrial Research, Pretoria, South Africa

³Unit of Biophysics and Gene Expression, Institute of Molecular Medicine, Faculty of Medicine, University of Lisbon, Lisbon, Portugal

⁴Antiviral Gene Therapy Research Unit, Department of Molecular Medicine and Haematology, School of Pathology, University of the Witwatersrand, Johannesburg, South Africa

⁵HIV Pathogenesis Research Unit, Department of Molecular Medicine and Haematology, School of Pathology, University of the Witwatersrand, Johannesburg, South Africa

⁶Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California, USA

CORRESPONDENCE TO:

Marc Weinberg

EMAIL:

marc.weinberg@wits.ac.za

POSTAL ADDRESS:

Antiviral Gene Therapy Research Unit, Department of Molecular Medicine and Haematology, School of Pathology, University of the Witwatersrand Medical School, 7 York Road, Parktown 2193, South Africa

DATES:

Received: 05 Jan. 2015

Revised: 04 Mar. 2015

Accepted: 23 Mar. 2015

KEYWORDS:

genome repair; genome targeting; sgRNA; HDR; T7 endonuclease I

Efficient and robust genome editing tools and strategies allow for specific and exact genetic changes to be captured in model systems, thereby accelerating both forward and reverse genetics studies. The development of CRISPR/Cas9 as a facile designer nuclease toolset has allowed for defined genetic modifications to be efficiently made through homology-directed repair of targeted DNA double-stranded breaks (DSBs) using exogenous repair templates. However, traditional single DSB strategies are still relatively inefficient as the short gene conversion tracts of mammalian cell systems limit the extent of achievable gene alteration from the DSB site. In order to improve on the inefficiency, we devised a dual cut strategy, which relies on reconstituting entire deleted gene fragments to precisely modify extensive gene regions of interest. Using the CRISPR/Cas9 system, we were able to introduce targeted deletions and repair of the endogenous *KRAS* gene locus in cell culture. The use of two simultaneous DSBs can be employed for efficient application of homology-directed repair with a large dsDNA donor sequence, thereby improving the efficacy of deriving cells with a desired gene editing outcome. In conclusion, a multiplexed CRISPR/Cas9 editing strategy represents an efficient tool for the editing of complex, heterologous sequence tracts.

Introduction

Precise genome editing represents a powerful new paradigm for both forward and reverse genetics studies in model systems. Sequence-specific nucleases have been used to expand our ability for precision engineering of the genome, and can be programmed to introduce targeted chromosomal double-stranded breaks (DSBs) to trigger endogenous DNA repair pathways.¹⁻³ The error-prone non-homologous end joining (NHEJ) repair pathway involves the re-ligation of the broken DNA ends, and in the process introduces small mutagenic insertions and deletions (indels). The homology-dependent repair (HDR) pathway seamlessly repairs the damaged site by utilising a homologous template, a process which can be exploited for targeted gene modifications.⁴⁻⁶ These tools have enabled functional studies based on the systematic knockout and knockin of genes⁷, the modelling of human diseases in cell- or animal-based systems⁸⁻¹⁰, and the generation of isogenic cell lines for stable transgene expression^{11,12}.

The clustered regularly interspaced short palindromic repeats (CRISPR) and the CRISPR-associated (Cas) protein system constitute an adaptive immune system found in prokaryotes, which functions to silence foreign DNA by RNA-guided nucleolytic digestion.¹³⁻¹⁵ CRISPR/Cas was adapted to target DNA by using short chimeric single-guide RNA (sgRNA) and a Cas9 nuclease reconstituted to function in cultured mammalian cells.¹⁶⁻¹⁹ The Cas9 nuclease can be directed to a specific cognate DNA target via a ~20-nucleotide 'guide' sequence within the sgRNA. The ease at which the nuclease can be reprogrammed by one or many sgRNAs has made this system highly effective for multiplexed genome editing applications in different cells²⁰⁻²³ and eukaryotic model systems²²⁻³⁵.

Conventional genome editing strategies make use of a single cleavage event to stimulate gene conversion with either ssDNA or dsDNA homologous repair templates.³⁶ However, donor DNA sequences that are more distant from the DSB site, or which include heterologous sequences, are less efficiently repaired.³⁷⁻³⁹ This situation can be partially resolved by the simultaneous introduction of two separate DSBs at a targeted locus to induce deletion and replacement events over large genomic regions.^{26,27,40} However, the efficiency of this approach has not been determined and, to date, the HDR frequencies among approaches that apply single or multiplexed DSBs remain unknown. Using the CRISPR/Cas9 adopted from *Streptococcus pyogenes*, we exploited the capabilities of this system to induce DSBs to introduce an oncogenic *KRAS* (c.35G>T) mutant variant in a selectable human cell-based model system. We demonstrate that the facile introduction of two simultaneous DSBs improves the frequency, relative to a single DSB, of reconstituting dsDNA homologous donor sequences, but not ssDNA ODNs (oligodeoxynucleotides), at the desired locus. These results point to an improved approach for efficient HDR-mediated repair using the facile CRISPR/Cas9 editing system.

HOW TO CITE:

Fok ET, Penny CB, Mhlanga MM, Weinberg MS. Multiplexed CRISPR/Cas9 genome editing increases the efficacy of homologous-dependent repair of donor sequences in mammalian cells. *S Afr J Sci.* 2015;111(7/8), Art. #2015-0002, 7 pages. <http://dx.doi.org/10.17159/sajs.2015/20150002>

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Materials and methods

Cell culture

HEK 293 cells were maintained in Dulbecco's Modified Eagle Medium: F12 mixture containing glucose (3.15 g/L), HEPES buffer (15 mM) and L-glutamine (2.50 mM) (Lonza, USA), supplemented with 10% heat-inactivated foetal bovine serum (Hyclone, USA) and 0.2% penicillin–streptomycin antibiotic mix (10 000 U/mL) (Lonza, USA). The cells were grown in a 75-cm³ flask and incubated in a 37 °C humidified incubator with 5% CO₂.

sgRNA assembly

The sgRNA expression plasmid, pcDNA.H1.sgRNA, was generated by first using a H1.sgRNA gBlock sequence (Integrated DNA Technologies (IDT), Coralville, IA, USA) comprising two *BsmBI* sites for facile guide sequence cloning using annealed oligos. The H1.sgRNA gBlock was Gibson Assembly cloned into the standard vector pcDNA3.1(+) (Life Technologies, CA, USA). For each of the five CRISPR/Cas9 target sequences, a pair of complementary oligonucleotides was chemically synthesised by IDT (USA):

sgRNA F1 224 forward: 5' - GATCCGAGTTGTATTAAGGTTAC - 3',
sgRNA F1 224 reverse: 5' - AAACGTACCTTTAATACAACACTCG - 3',
sgRNA F2 225 forward: 5' - GATCCATGTGTGACATGTTCTAATA - 3',
sgRNA F2 225 reverse: 5' - AAACATTAGAACATGTCACACATG - 3',
sgRNA F3 226 forward: 5' - GATCCGTTTGTATTAAGGTTACTGG - 3',
sgRNA F3 226 reverse: 5' - AAACCCAGTACCTTTAATACAACG - 3',
sgRNA R1 227 forward: 5' - GATCCGTAATTAGCTGTATCGTCA - 3',
sgRNA R1 227 reverse: 5' - AAAGTACGATACAGCTAATTCACG - 3',
sgRNA R2 228 forward: 5' - GATCCACAAGATTTACCTCTATTGT - 3',
sgRNA R2 228 reverse: 5' - AAACACAATAGAGGTAATCTTGTG - 3'.

Each pair of oligonucleotides was composed of a 20-nucleotide guide sequence for their target and appropriate 5' overhangs that allowed for cohesive-end ligation into the *BsmBI* (Thermo Scientific, USA) digested pcDNA.H1.sgRNA expression plasmid. Sanger sequencing was performed to validate the insertion of the guide sequence into the sgRNA expression plasmid.

T7 Endonuclease I assay

At 24 h prior to transfection, HEK 293 cells were seeded at a density of 250 000 cells/well in a six-well plate. The cells were transfected with 2 µg of the sgRNA expression plasmid and 2 µg of the Cas9 expression plasmid (pX330) (Addgene plasmid #42230)¹⁶ using 12 µL of branched polyethylenimine (Sigma Aldrich, USA). At 48 h post-transfection, the cells were harvested and the gDNA was extracted from these cells using the QIAamp DNA Mini Kit (Qiagen, Germany). Genomic regions surrounding the CRISPR/Cas9 target sites were amplified from this gDNA with primers for the 5' cleavage region (5' *KRAS* forward: 5' - CGCAGAACAGCAGTCTGGC - 3' and 5' *KRAS* reverse: 5' - CTACGCCACGCTCCAAC - 3'), and the 3' cleavage region (3' *KRAS* forward: 5' - AAGGCCTGTGAAATGACTG - 3' and 3' *KRAS* reverse: 5' - GCACAGAGAGTGAACATCATGG - 3'). The T7 Endonuclease I (T7E1) assay was performed as previously described.^{36,41} Briefly, 600 ng of DNA amplified from the genomic target region was denatured and annealed to form heteroduplexes. Amplicons (150 ng) were digested with 3 U of T7E1 (New England Biolabs, Germany) and resolved on an 8% polyacrylamide gel stained with SYBR Green I Nucleic Acid Gel Stain (Invitrogen, USA).

Micro-deletion detection

HEK 293 cells were transfected using polyethylenimine with 2 µg of the 5' cleavage sgRNA expression plasmid, 2 µg of the 3' sgRNA expression plasmid and 2 µg of pX330. gDNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany) 48 h post-transfection. Polymerase chain reaction (PCR) amplification of the deletion junction was performed using Phusion Flash High-Fidelity PCR Mix (Thermo Scientific, USA) with an initial denaturation at 98 °C for 10 s, followed by 35 cycles of

denaturation at 98 °C for 1 s, annealing at 65.4 °C for 5 s, extension at 72 °C for 10 s and a final extension at 72 °C for 1 min using the 5' *KRAS* forward and 3' *KRAS* reverse primers described in the T7E1 assay. The PCR products were then visualised on an ethidium bromide (0.5 µg/mL) stained TAE agarose gel (2% (w/v)).

Restriction fragment length polymorphism analysis for detection of HDR

HEK 293 cells were transfected with 2 µg of sgRNA expression plasmid, 2 µg of pX330 and 2 µL of the ssODN repair template (100 µM) for the single cut strategy. For the dual cut strategy, 2 µg of each sgRNA expression plasmid, 2 µg of pX330 and 2 µL of the ssODN repair template (100 µM) were used. After 72 h, the gDNA was extracted from these transfected cells and the repaired region was amplified using PCR with the 5' *KRAS* forward and 3' *KRAS* reverse primers (described for the T7E1 assay). The PCR products were purified and 300 ng of the DNA was digested with *EcoRI* (Thermo Scientific, USA). The products were resolved and visualised on a 8% polyacrylamide gel which was stained with SYBR Green I Nucleic Acid Gel Stain (Invitrogen, USA).

dsDNA donor sequences

The ssDNA ODN sequences used were chemically synthesised (IDT, USA) (*EcoRI* site underlined):

F1 ssODN: 5'-
TTGAAATAATTTTCATATAAAGGTGAGTTTGTATTAAGGGGAATCTACTGG
TGGAGTATTGATAGTGTATTAACCTTATGTGTGAC
R2 ssODN: 5'-CAGCTAATTCAGAATCATTGTGTGGACGAATATGATCCAACA
GAATTCATAGAGGTAATCTTGTTTAATATGCATATTACTGGTGCAG
F1 + R2 ssODN: 5'-TTGAAATAATTTTCATATAAAGGTGAGTTTGTATTA^{AA}
GGGAATTCATAGAGGTAATCTTGTTTAATATGCATATTACTGGTGCAG.

The dsDNA donor construct was assembled by fusing five PCR products in an overlap extension PCR:

eGFP F: 5' -
TCAAGTTGGCGGGAGACGTCGAGTCCAACCCCTGGGCCCATGGTGAAGCAAG
GGCGAGGAGC - 3'; eGFP R: 5' -
CACACAAAAACCAACACACAGATGTAATGAAATAAAGATATTTTATTCTAG
AGTATACGGACCGTTACTTG - 3'; 5' HA F 5' -
GTTTGTATTAAGGTTACTGGTGGAGTATTGATAGTGTATTAACCTTATCACA
CAAAAAACCAACACACAGATG - 3'; NeoR F 5' -
TAATGAAATGTGACTATATTAGAATGTCACACGGCC
AGCACCATGGCCT GAAATAAC - 3'; NeoR 5' -
ACGTCTCCGCCAAC
TTGAGAAGGTCAAATTCAAAGTCTGTTTCACGAAG AACTCGTCAAGAAGG
CGATAGAAG - 3'; *KRAS* F: 5' -
GTGTGACATGTTCTAATATAGTCACATTTTCATTATTTTATAAGGCCTGCT
GAAAATGACTGAATATAAAGTGTGGTAGTTGGAG - 3'; *KRAS* R1: 5' -
GTCTACAAAATGATTCTGAATTAGCTGTATCGTCAAGGCACTCTTGCCTACGC
CAACAGTCCAACACCA CAAGTTT - 3'; *KRAS* R2: 5' -
GCATATTAAAACAAGATTTACCTCTATTG TTGGATCATATTCTGCTACAAAATG
ATTCTG - 3'; 5' HA F2: 5' -
TAGCTGTTGCATATTGACTTCTAACACTTAGAGG - 3'; 5' HA R2: 5' -
ATAAGGTTAATACACTATCAATACTCCACCAGTACC - 3'; 3' HA F2: 5' -
AATCATTTTGTGGACGAATATGATCCACAAT - 3'; 3' HA R2: 5' -
CCATCAACAATTATATTTCACTAGTACAATTAATCTAACCTTT - 3'.

The plasmid pCI-NeoR-EGFP was used as a template for SV40 driven NeoR. Amplicons comprising the 5' and 3' homology arms, *KRAS* sequence and SV40-Neo-2A-eGFP sequences were assembled by overlap-extension PCR. The dsDNA donor was ligated into the pJet1.2/blunt Cloning Vector from the CloneJet PCR Cloning Kit (Thermo Scientific, USA) and subsequently sequenced. The dsDNA donor was linearised by PCR amplification using Phusion Flash High-Fidelity PCR Mix (Thermo Scientific, USA) and the 5' HA F2 and 3' HA R2 primers, with an initial denaturation step at 98 °C for 10 s, followed by 35 cycles of denaturation at 98 °C for 1 s, annealing at 62.7 °C for 5 s, extension at 72 °C for 45 s and a final extension at 72 °C for 1 min. This linear dsDNA

donor construct was column purified with the GeneJet PCR Purification Kit (Thermo Scientific, Lithuania).

Quantitative real-time PCR

HEK 293 cells were transfected with 2 μ g of each sgRNA plasmid, 2 μ g of pX330 and 1 μ g of the linear dsDNA donor PCR product. Half of the transfected cells in each well were harvested for gDNA extraction 72 h post-transfection, and the remaining cells were maintained for another 14 days. Quantitative real-time PCR (qPCR) was performed using SYBR Green PCR Mastermix (Applied Biosystems, USA) on the Applied Biosystems 7500 RT PCR System, which was programmed for an initial hold stage at 50 °C for 2 min, followed by denaturation at 95 °C for 10 min and 50 cycles of denaturation at 95 °C for 15 s and annealing and extending either at 55 °C (for primer set A, B and β -actin) or at 60 °C (for primer set C) for 1 min. An inter-plate calibrator reaction was included for each primer set to control for any technical variations between the 72-h and 14-day samples. The inter-plate calibrator template mixture was made up by adding 10 μ L of each gDNA sample (16 ng/ μ L) obtained 72 h post-transfection. The primer sets were:

Primer set A F: 5' - CCAAGAGAACTACTGCCATGATGC - 3'

Primer set A R: 5' - GCATGGACGAGCTGTACAAGT - 3'

Primer set B F: 5' - TGATATTCCGGAAGCAGGCA - 3'

Primer set B R: 5' - GACCACCAAGCGAAACATCG - 3'

Primer set C F: 5' - TTATTTGGGCGGAAGGCTGA - 3'

Primer set C R: 5' - GTCAGGGACCGTCAGTTTCA - 3'

β -actin F: 5' - ACCAACTGGGACGACATGGAGAAA - 3'

β -actin R: 5' - TAGCACAGCCTGGATAGCAACGTA - 3'

The data of each primer set were mathematically corrected according to the changes seen in the threshold (C_q) values as recommended by the TATAA Interplate Calibrator SYBR protocol (TATAA Biocenter). The corrected data are represented as a fold change relative to β -actin.

Results

CRISPR/Cas9 can facilitate KRAS mutagenesis

The *KRAS* proto-oncogene is frequently found activated and facilitates a variety of cancers by acquiring point mutations at codons 12, 13 and 61, which code for a constitutively active Ras protein.⁴²⁻⁴⁴ In order to model an oncogenic c.35G>T variation in codon 12 of *KRAS*, we identified suitable sgRNA target sites within a 250-bp region around the point mutation.⁴⁵ The sgRNA guide sequences were chosen on the basis of the best returned score of each target site to minimise off-target DSBs. Two of the target sites were upstream of the c.35G>T mutation (5' cleavage sites) and two were downstream of the mutation (3' cleavage sites) (Figure 1a). A fifth sgRNA that made use of a degenerate NAG protospacer adjacent motif (PAM) was also included (sgRNA F2 225). The H1 Pol-III promoter drove the expression of the inserted guide sequence and the RNA scaffold, as a single chimeric sgRNA molecule comprising an optimised sgRNA architecture²² (Figure 1b). The distribution of sgRNAs allowed for potential gene deletions to span the mutation site. To determine the functionality of each sgRNA, we used a T7E1 assay to detect the indel frequency at the targeted *KRAS* locus. All the sgRNAs introduced indels at the DSB repair sites at varying efficiencies (1.28–6.01%) (Figure 1c). Guide RNAs sgRNA F1 224 and sgRNA R2 228 were the most effective, and ensured that effective targeting occurred at positions 5' and 3' of the *KRAS* mutation site. These sgRNAs were applied simultaneously, resulting in the deletion of 199 bp from the *KRAS* locus as determined by PCR amplification with primers that flank the deletion junction (Figure 1a and 1d).

Micro-deletions can be repaired precisely by HDR

In order to precisely modify genes, the CRISPR/Cas9 system needs to be able to stimulate the HDR pathway for the repair of DSBs according to specific exogenous repair templates. ssODNs have been shown to be able to seamlessly repair and modify targeted single DSB sites via HDR.⁴⁶ These are convenient repair templates that allow for small, targeted gene modifications to be made without the need for constructing large

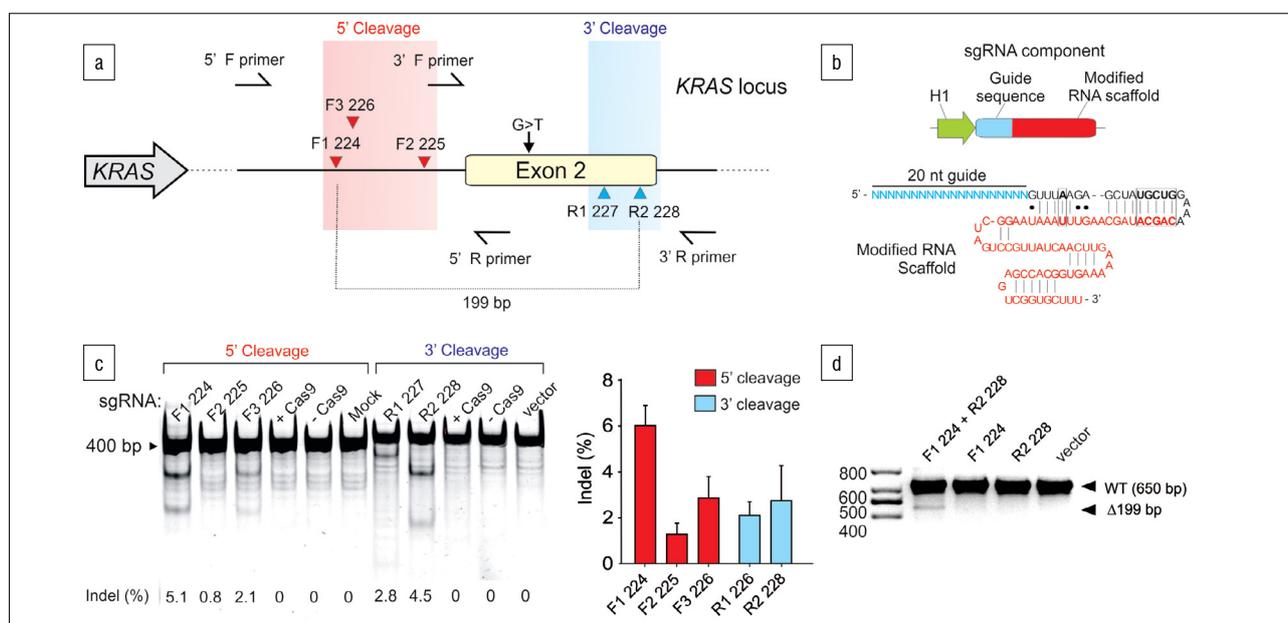


Figure 1: CRISPR/Cas9-mediated targeting of the human *KRAS* gene. (a) A schematic of the *KRAS* gene locus chosen for CRISPR/Cas9 targeting. The sgRNA target sites were designed around an oncogenic c.35G>T mutation in the *KRAS* gene, with the cleavage sites depicted as arrowheads. Two primer sets were used to amplify cleavage regions that were 5' and 3' of the point mutation. (b) The sgRNA construct was driven by an H1 Pol-III promoter for the expression of the guide sequence and the RNA scaffold as a single chimeric molecule. The sgRNA architecture used was previously optimised to improve sgRNA expression and Cas9 loading.²² (c) A T7E1 assay showed the performance of each sgRNA at generating indels ($n=3$; mean \pm SD, * $p<0.05$, ** $p<0.01$, unpaired Student's t -test). (d) The simultaneous application of sgRNA F1 224 and sgRNA R2 228 led to the deletion of 199 bp in the *KRAS* gene, which was detected with PCR.

dsDNA donor for HDR. The dilution of the episomal DNA was apparent after 14 days, with a decrease of approximately 99% in each sample (primer set B; Figure 3b and 3c). Despite this dilution, readily detectable amounts of episomal DNA were still present in the transfected cells after 14 days of sub-culture (600–1000-fold relative to β -actin). The amount of detected dsDNA donor integrated into the *KRAS* gene (data from primer set A; taken as a percentage of the total *KRAS* DNA detected (data from primer set C; Figure 3b and 3c), was used as a metric for site-specific donor integration efficiency (Figure 3d). The simultaneous use of the two sgRNAs proved to be the most efficient at facilitating the integration of the heterologous donor DNA sequences, and occurred at a frequency of 0.028% and 0.123% in transfected cells, after 3 and 14 days of selection-free culture, respectively. The c.35G>T *KRAS* mutation confers a slight growth advantage in the modified cells.⁴³ We observed an enrichment of modified *KRAS* mutants as the amount of

integrated donor DNA increased after 14 days of selection-free culture of the transfected cell population (Figure 3c and 3d).

Discussion

We used the CRISPR/Cas9 system for the improved efficacy of donor-led HDR using a multiple editing approach which allows for extensive and wide-ranging gene modifications to be achieved while maintaining gene function. As an example, the introduction of a putative oncogenic point mutation in the *KRAS* gene in a cell-based model was adopted in this study. We initially demonstrated the efficiency of introducing single cleavage events in the *KRAS* gene sequence using RNA-guided CRISPR/Cas9. These single cleavage events were repaired either by the NHEJ pathway, resulting in the introduction of mutagenic indels, or by the HDR pathway, which seamlessly repaired the DSB by using homologous ssODN templates. The NHEJ and HDR activity induced by

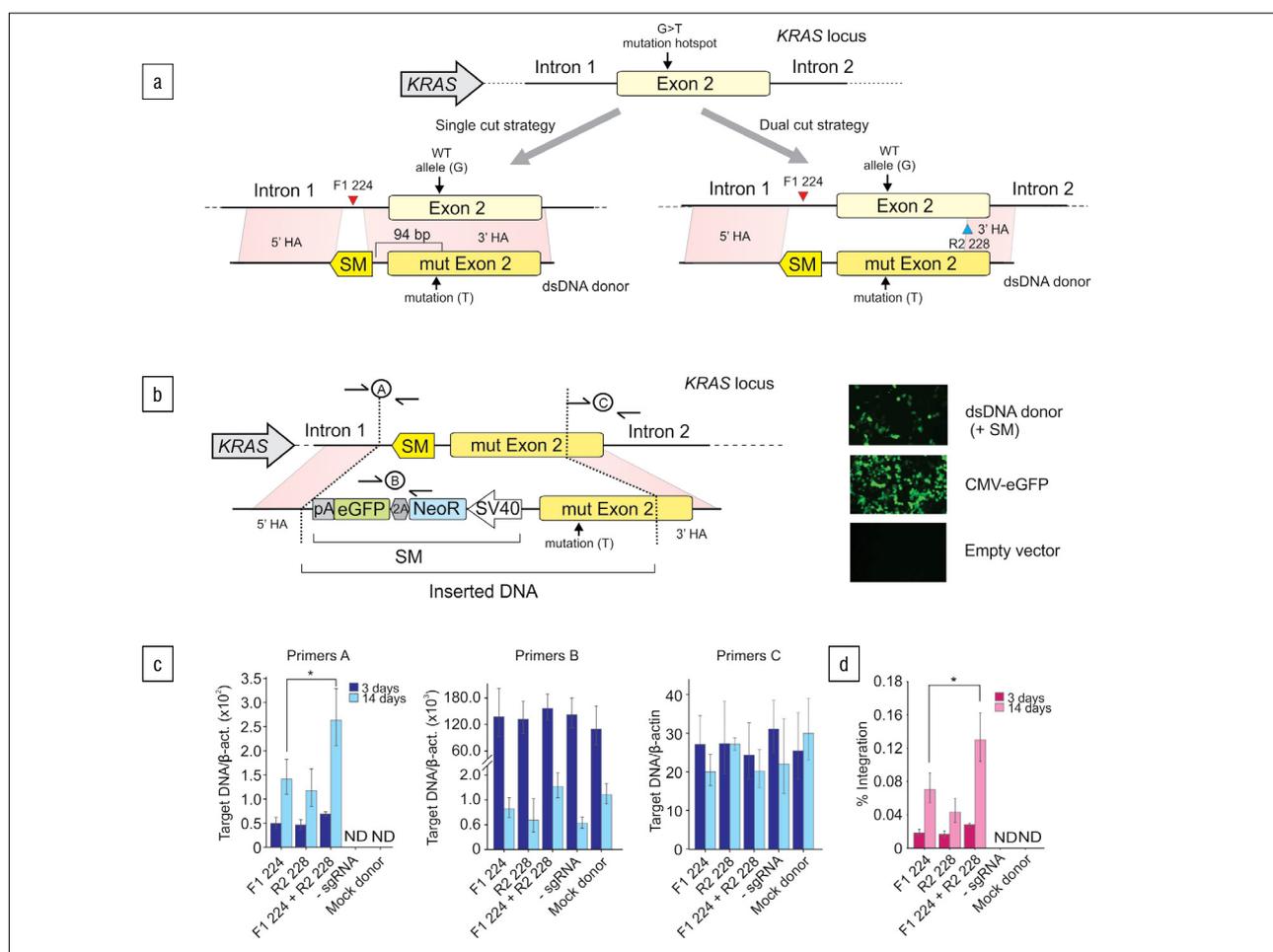


Figure 3: The envisioned dual cut strategy versus the traditional single cut strategy to modify the *KRAS* gene. (a) The extent of gene conversion is limited with the traditional single cut strategy, which uses a dsDNA donor that has two homology arms (HAs) that directly flank the DSB site. The successful co-transfer of both the gene mutation and the selection marker cassette (SM) into sites that are 94 bp apart will be very inefficient with a single cut, as a result of short gene conversion tracts. The dual cut strategy should overcome this inefficiency by deleting the entire region between the two sites and then reconstituting this region with a modified gene sequence that contains the mutation and the selection marker. (b) The dsDNA donor was designed to functionally separate the intended gene modification elements (the selection cassette and the point mutation) from the homology arms. This separation ensured that the homology arms maintained complete homology with the target sequence and functioned solely to direct the construct to the target region. The selection cassette was designed to be expressed in the opposite direction of *KRAS* gene transcription to produce a chimeric eGFP-NeoR protein that is cleaved by an embedded 2A self-cleaving peptide, allowing for a fluorescence or antibiotic-based approach for selection. Fluorescence micrographs showed the expression of eGFP in HEK293 cells that were transfected with this construct (right). A qPCR strategy was used to determine the integration efficiency of the dsDNA donor into the *KRAS* gene by measuring the amount of various DNA components (left). Quantification was done on extracted gDNA 3 and 14 days post-transfection. (c) Relative amounts of detected target DNA of each primer set, normalised to β -actin. (d) The percentage of *KRAS* alleles that successfully underwent integration of the selection cassette at the *KRAS* gene locus, as calculated by taking the data from primer set A (integration events) as a percentage of primer set C (total *KRAS* DNA) (mean \pm SD, ND denotes no detected amplification).

sgRNA F1 224 showed similar levels of stimulation of each pathway. However, DSBs mediated by sgRNA R2 228 clearly favoured the HDR pathway for DSB resolution. Furthermore, HDR of the micro-deletion, with a ssODN, occurred at relatively low levels compared to the deletion events detected, indicating that NHEJ repair was more favourable. These repair pathway preferences of DNA damage could be related to cellular mechanisms that prioritise the repair of certain genetic elements.⁴⁷ Nevertheless, such repair preferences could potentially alter the efficiency of the desired gene editing outcome. Further observation of gene editing at different genomic sites, on a more high-throughput scale, may provide further insight.

We demonstrated that the CRISPR/Cas9 system can be used in multiplex to improve the efficiency of HDR for precise modifications in the *KRAS* gene. We predicted that by applying two cleavage events, the gene region of interest could be deleted and efficiently reconstituted with our mutant-containing dsDNA donor, which would allow for a more expansive genomic region to be precisely modified, without being limited by the short gene conversion tracts that branch out from a single break site. Furthermore, the design of the dsDNA donor for this strategy removes the point mutation from the homology arms and relocates it into the reconstitution sequence, allowing for complete homology with the target sequence to be maintained. This strategy should improve the efficiency of gene conversion for single, as well as multiple, point mutations, as small levels of sequence divergence in the homology arms have been shown to greatly decrease the levels of recombination.³⁷ To illustrate this, we targeted two distal sites within the *KRAS* gene locus for concurrent modification, using a single donor. This approach allowed for the concurrent insertion of a selection cassette into a nearby intron of the oncogenic point mutation site in the *KRAS* protein-coding sequence, preserving the integrity and function of the gene and eliminating the need for any post-modification gene restoration to rescue function.

Conclusions

A dual cut gene editing strategy allows for distally located sites to be targeted simultaneously, enabling for extensive and expansive gene modifications to be achieved efficiently. We have shown that this method is particularly useful for maintaining the native function of the gene, in which selection cassettes used to identify cells with the modifications of interest can be inserted in neighbouring introns. We significantly improved on current HDR-based gene editing strategies, which can only modify regions within close proximity to the DSB site. Furthermore, the CRISPR/Cas9 system is well suited for this dual cleavage approach, as it is simple and cost effective to employ in a multiplex fashion. This method should improve the success rate of producing recombinant cell lines, and thus expedite efforts to gain a better understanding of causal genetic elements in biological systems.

Acknowledgements

Research reported in this publication was supported by the Strategic Health Innovation Partnerships (SHIP) Unit of the South African Medical Research Council with funds received from the South African Department of Science and Technology. Funding from the National Research Foundation is also acknowledged.

Authors' contributions

E.T.F. and M.W.S. were the project leaders; E.T.F., M.S.W., C.B.P., and M.M.M. made conceptual contributions and helped design the study; E.T.F. and M.S.W. performed the experiments and wrote the manuscript.

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