Gene Editing Proteins with Nickase Functionality Enable Scarless Targeted Gene Insertion in Primary Human Cells

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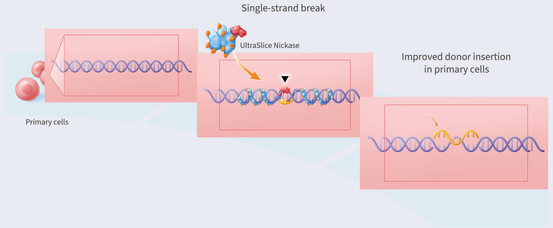
Introduction

Many gene editing strategies involve the use of nucleases to generate targeted double-strand breaks (DSBs) in genomic DNA, which is often associated with cytotoxicity and off-target effects that can prevent clinical translation. Such undesirable outcomes have led to the development of gene-editing nickases, which instead create targeted single-strand breaks (SSBs) that favor high-fidelity repair through the homology-directed repair (HDR) pathway rather than the more error-prone non-homologous end joining (NHEJ) or microhomology-mediated end joining (MMEJ) pathways. Here, we explore the use of UltraSlice (US) gene editing proteins containing cleavage domain variants with nickase functionality for targeted insertion of donor sequences into a defined genomic locus. Using UltraSlice gene editing proteins targeting exon 73 in COL7A1 (mutations in which cause dystrophic epidermolysis bullosa), we tested combinations of 3 mutations previously reported to confer nickase functionality to FokI, a Type IIS restriction endonuclease. Each mutation is a single base substitution that results in a single amino acid change; for D450A and D467A the change is from Asp to Ala, while D450N changes Asp to Asn.Our data demonstrate that gene-editing nickases enable scarless insertion of donor sequences into defined genomic loci, and thus may have the potential to improve the safety of in vivo gene insertion by reducing off-target effects.



Conclusions

- UltraSlice Nickase gene-editing protein pairs enable scarless insertion of donor DNA into both primary human cells and human stem cell lines.
- UltraSlice Nickases have the potential to improve the safety of *in vivo* gene insertion by reducing off-target effects.
- UltraSlice Nickase scarless gene editing may increase the efficiency of *in vivo* gene insertion by allowing for repeat dosing.

1 Designing UltraSlice Nickases

To reduce NHEJ/MMEJ activity, we explored a previously reported method of rendering the FokI domain inactive through targeted point mutations. These mutations to the domain still permit binding to the DNA and dimerization with the other US in the pair but prevent full cleavage of the DNA, producing an SSB. We created variants of our COL7A1_e73 UltraSlices with each of these mutations to the right pair and tested them when paired with a normal left UltraSlice to compare both NHEJ/MMEJ activity and insertion efficiency.

Figure 1. Illustration of COL7A1_e73 UltraSlice pair binding to target DNA.

Figure 2. Illustration of COL7A1_e73 UltraSlice Nickase pair binding to target DNA. Mutated FokI domain highlighted.

Table 1. Explanation of UltraSlice nomenclature and point mutations to the FokI domain sequence.

Pair Name	Left Protein	Right Protein	Right Protein DNA Sequence
US22	US2L	US2R	AAACCG**G**ACGGAGCAATT (450)
			ATCGTGG**C**TACTAAGCTT (467)
USN1	US2L	US2R(*D450A*)	AAACCGG**C**CGGAGGAATT (450)
USN2	US2L	US2R(*D450N*)	AAACCG**A**ACGGAGGAATT (450)
USN3	US2L	US2R(*D467A*)	ATCGTGG**A**TACTAAGCTT (467)

2 Scarless Gene Editing

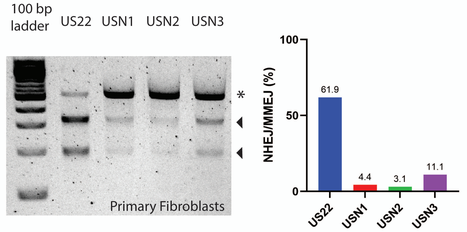


Figure 3. Primary human fibroblast electroporation results comparing all 4 US pairs. 2% agarose gel shows T7E1 assay with asterisk pointing to wildtype bands and arrows pointing to cut bands below as evidence of NHEJ or MMEJ activity. Graph quantifying relative intensity of cut bands, showing reduction of NHEJ/MMEJ in all US Nickase samples with USN2 exhibiting the lowest level.

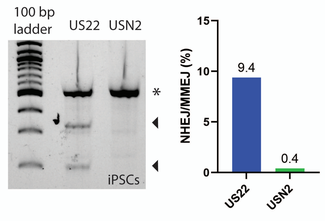
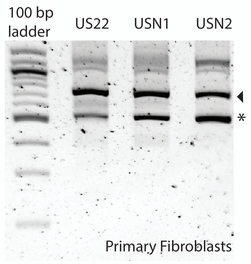
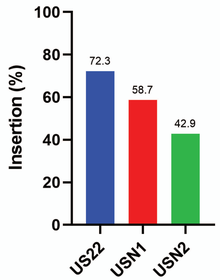


Figure 4. iPSC electroporation results comparing US22 and USN2. 2% agarose gel shows T7E1 assay with asterisk pointing to wildtype bands and arrows pointing to cut bands below as evidence of NHEJ or MMEJ activity. Graph quantifying relative intensity of cut bands, showing reduction of NHEJ/MMEJ in USN2

3 Characterization of Transgene Insertion





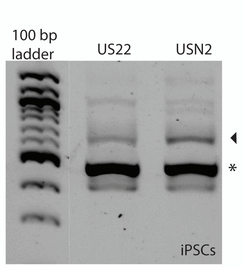
US22: Cut site

Ref- gccaccagcattctctcttccactcctgcagGGCCCCATCGGCTTTCCTGGAGAACGCGGGCTGAAGGGCGACCGTGGAGAC
F- GCCACCAGCATTCTCTCTTCCACTCCTGCGGGCCCCATCGGTTTTCGGGGAAGCGGGGGAGAGGGACACCGGGACCC
R- CCCCCCCCACTTTTTTTTCCTCCTGCAGGGCCCCATCGGCTTTCCTGGAGAACGCGGGCTGAAGGGCGACCGTGGAGAC

USN2: Cut site

Ref- gccaccagcattctctcttccactcctgcagGGCCCCATCGGCTTTCCTGGAGAACGCGGGCTGAAGGGCGACCGTGGAGAC
F- GCCACCAGCATTCTCTCTTCCACTCCTGCAGGGCCCCATCGGCTTTCCTGGAGAACGCGGGCTGAAGGGCGACCGTGGAGAC
R- GCCACCAGCATTCTCTCTTCCACTCCTGCAGGGCCCCATCGGCTTTCCTGGAGAACGCGGGCTGAAGGGCGACCGTGGAGAC

Figure 5. Insertion of 300 bp dsDNA donor sequence into primary human fibroblasts following electroporation. 2% agarose gel showing PCR-amplified gDNA with arrow pointing to insertion bands and asterisk pointing to wildtype bands below. Graph illustrating quantified relative insertion levels. Sanger sequencing of wildtype bands (535 bp) from US22 and USN2 fibroblast electroporation. Red letters indicate NHEJ/MMEJ.



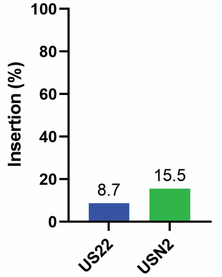
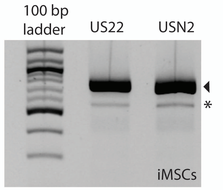


Figure 6. Insertion of 300 bp dsDNA donor sequence into human iPSCs following electroporation. 2% agarose gel showing PCR-amplified gDNA with arrow pointing to insertion bands and asterisk pointing to wildtype bands below. Graph illustrating quantified relative insertion levels where USN2 has greater insertion than US22. Overall reduced insertion is due to use of dsDNA when ssDNA would have been optimal.



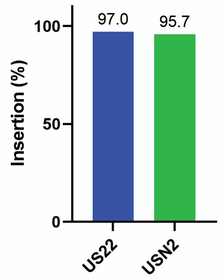


Figure 7. Insertion of 300 nt ssDNA donor sequence into human iMSCs following electroporation. 2% agarose gel showing PCR-amplified gDNA with arrow pointing to insertion bands and asterisk pointing to wildtype bands below. Graph illustrating quantified relative insertion levels which are similar between US22 and USN2.