OPTIMIZING A CRISPR GENE THERAPY FOR ANIRIDIA EMPLOYING A HUMANIZED CELLULAR MODEL

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Abstract

Aniridia is a rare, panocular disorder characterized by malformation or absence of the iris, and underdevelopment of other ocular tissues. At birth, patients have low vision, which eventually progresses to blindness by young adulthood. Aniridia is a dominant haploinsufficiency disorder caused by mutations in the transcription factor paired box 6 (*PAX6*) gene. Current interventions aim to slow the progression of the disease, but none exist to correct the underlying causal variant. One exciting approach is to utilize the gene-editing capabilities of CRISPR/Cas9 to correct the variant and restore gene function. Here, I hypothesize that a CRISPR therapy developed and optimized in minimally humanized mouse embryonic stem cells (ESCs) will be a suitable strategy to differentiate between wild-type and patient variant chromosomes, in order for a CRISPR therapy for aniridia to be effective in humans. We have generated humanized mouse ESC lines and tested therapeutic conditions by transfection of CRISPR reagents to ESCs by electroporation. Characterization of cell lines and therapeutic correction are assayed by PCR, RFLP, and Sanger sequencing. To date, I have found the most successful therapeutic strategy corrected the variant at an average frequency of 30%. Beyond my contribution to this research, the optimized CRISPR strategy will be tested on a humanized mouse model of aniridia to determine if the strategy can restore expression of *Pax6* and prevent blindness in mice. Most importantly, the innovative humanized models will allow for the development of a CRISPR therapy on human DNA, making it directly translatable to human cells, and eventually patients.

Aniridia is a Rare, Congenital Disorder that Affects Development of Ocular Tissues

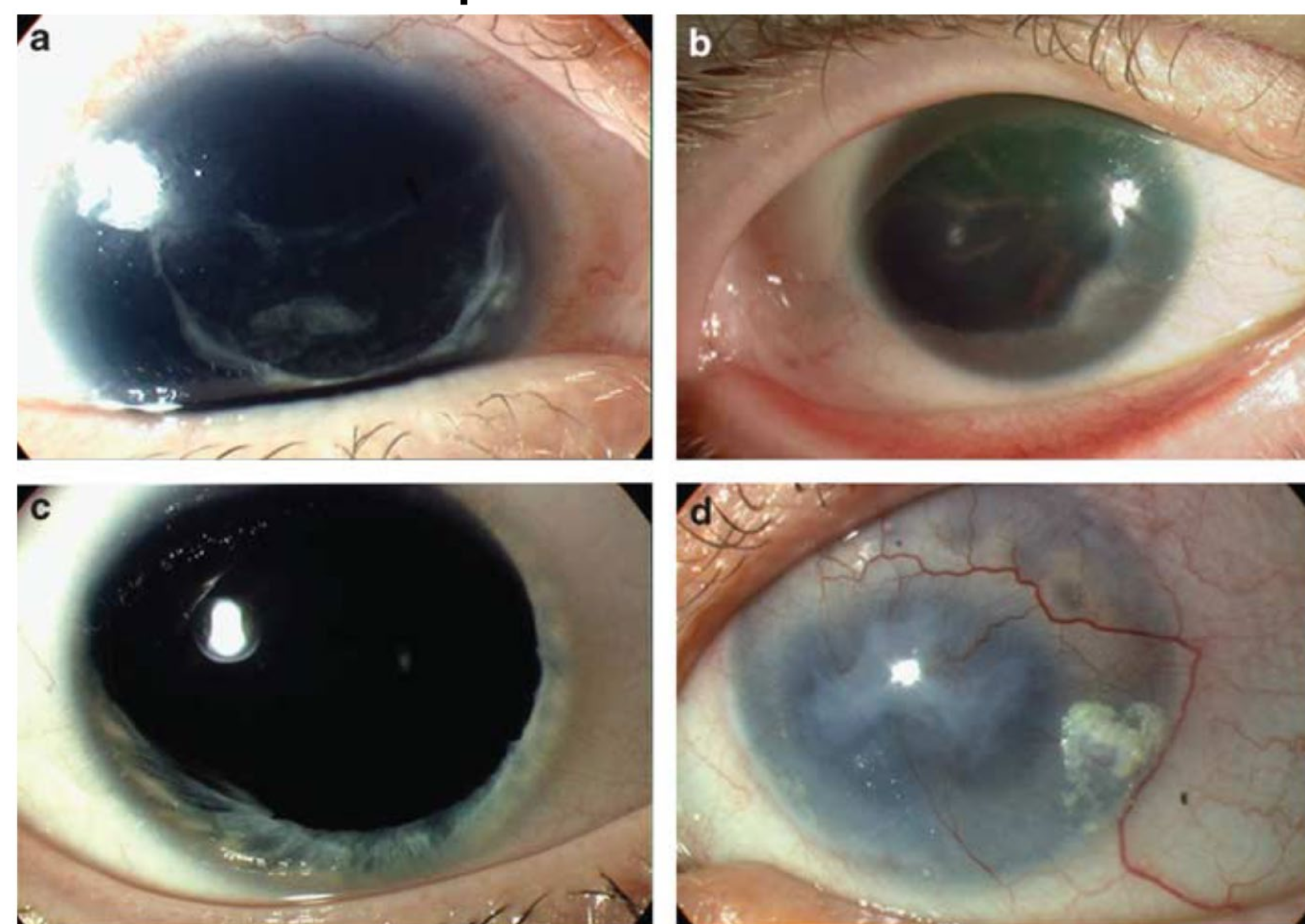


Figure 1. Clinical features of aniridia[1]. a) Complete absence of iris, b) Almost total absence of iris behind peripheral corneal neovascularization and opacification, c) Partial absence of iris, d) Severe opacification and neovascularization of cornea. Other reported clinical features include fovea hypoplasia, nystagmus, ptosis and glaucoma. Patients experience increased sensitivity to light and decreased visual acuity, which eventually progresses to blindness by young adulthood.

CRISPR/Cas9 Gene Editing Aims to Correct Pathogenic Variants at the Genomic Level

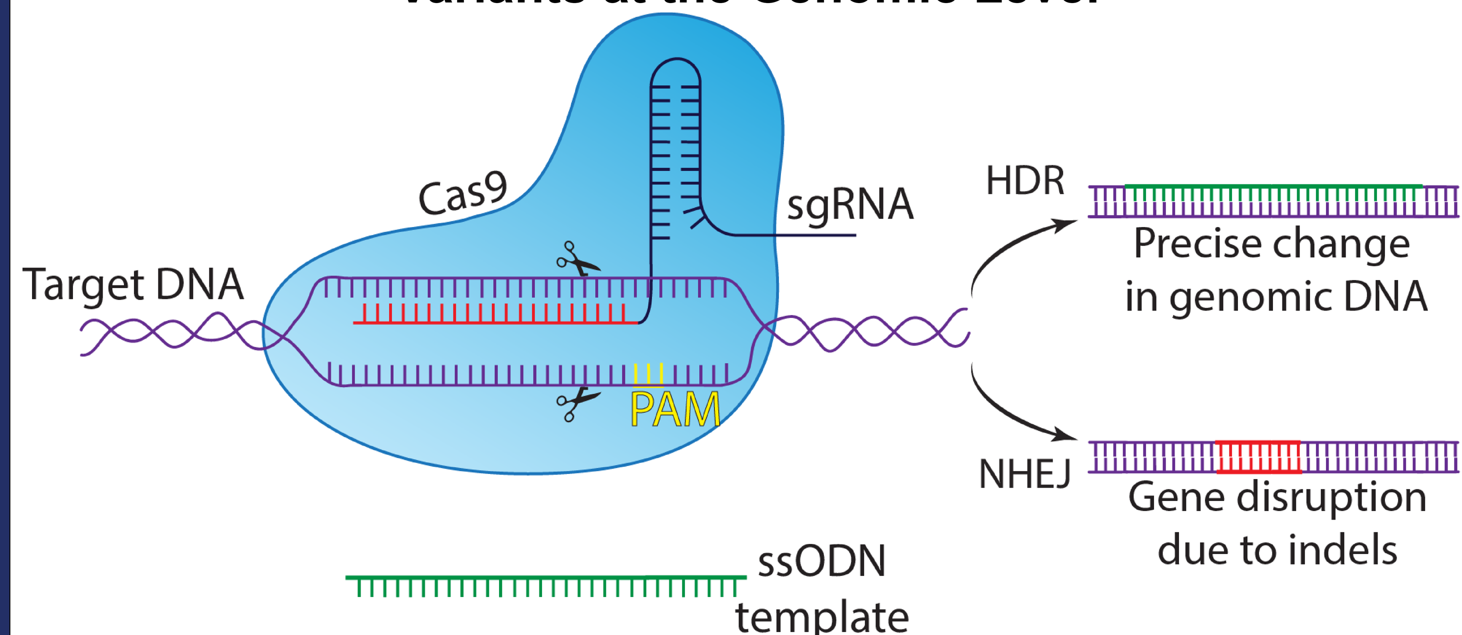


Figure 2. By introducing a single-stranded oligodeoxynucleotide (ssODN) template along with CRISPR/Cas9 into cells, the homology directed repair (HDR) pathway can be targeted to produce a precise change in genomic DNA.

Methodology to Minimally Humanize *Pax6* Using CRISPR

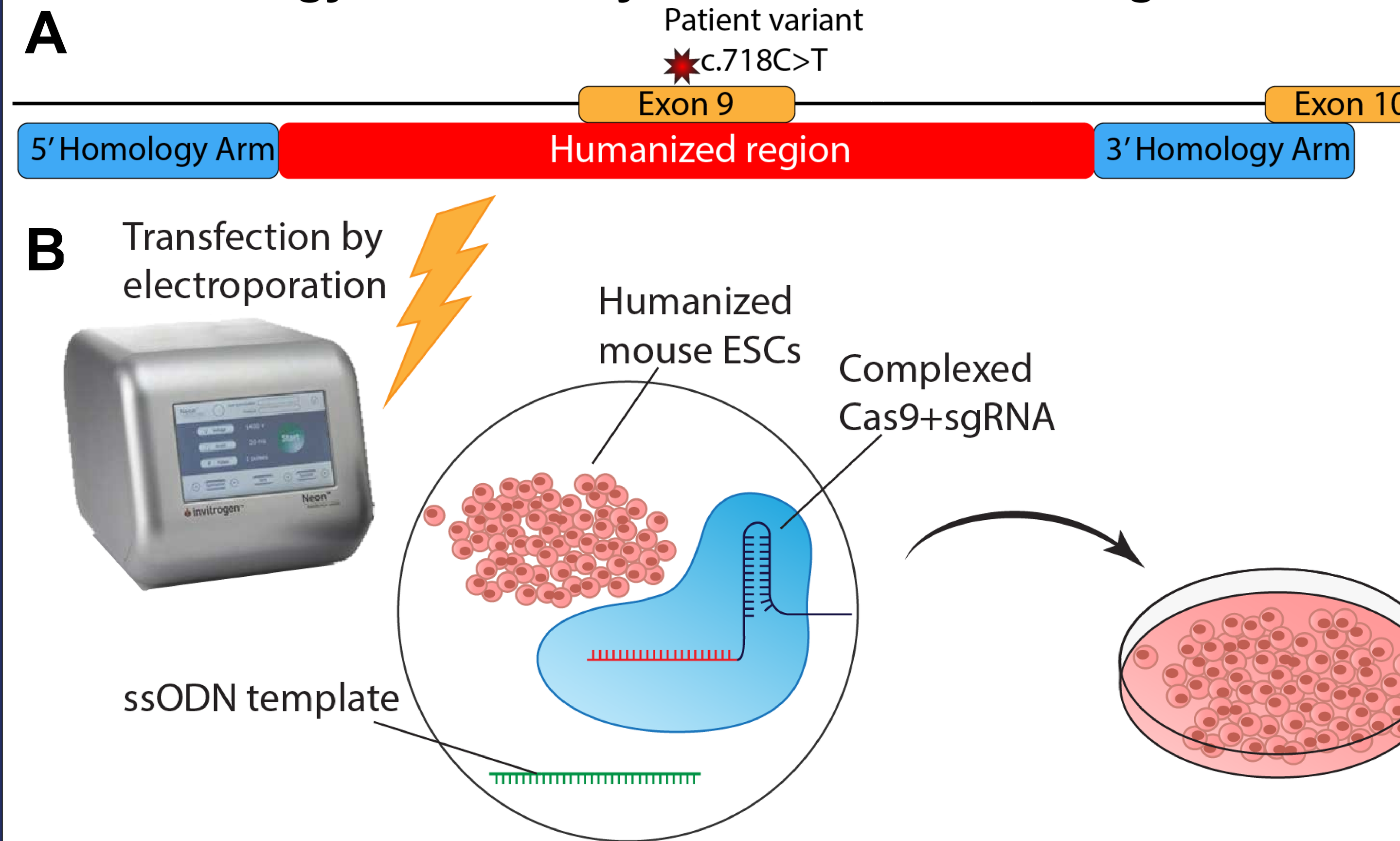
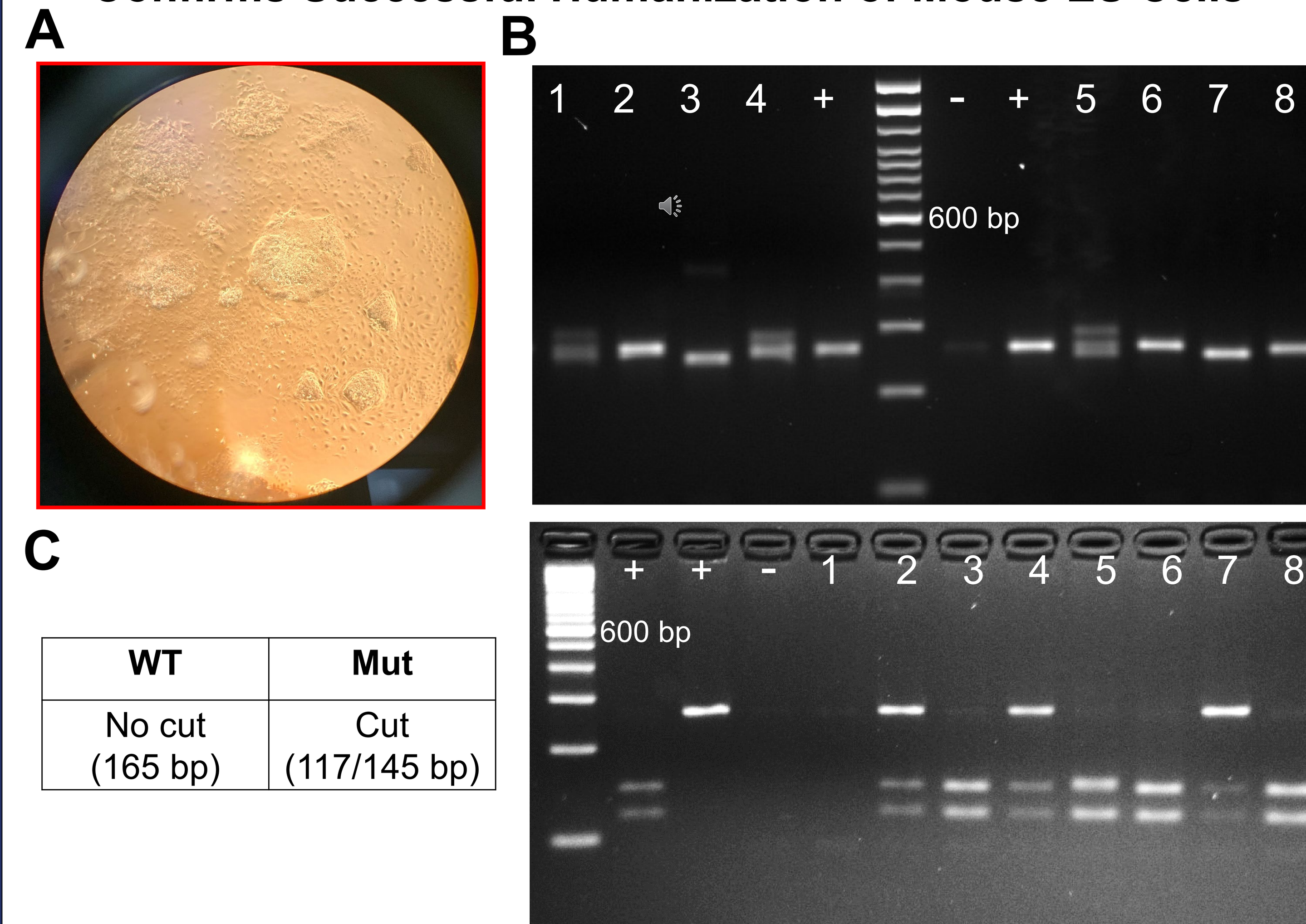


Figure 3. A) Schematic of the single stranded oligodeoxynucleotide (ssODN) template for CRISPR/Cas9-mediated exchange of the humanized sequence containing *PAX6* exon 9 and the patient variant, c.718C>T. B) Electroporation was used to transfect CRISPR reagents into ESCs.

Characterization of CRISPR-Edited Clones by PCR and RFLP Confirms Successful Humanization of Mouse ES Cells



WT	Mut
No cut (165 bp)	Cut (117/145 bp)

Figure 4. A) Humanized mouse ES cells under microscope. B) PCR assay confirming humanization of single clones (expected amplicon: 265 bp). C) RFLP screen demonstrating successful heterozygous correction (lane 2) or unsuccessful correction (lane 6) of patient variant in humanized clones.

Novel Humanized Mouse ES Cell Models of Aniridia Derived

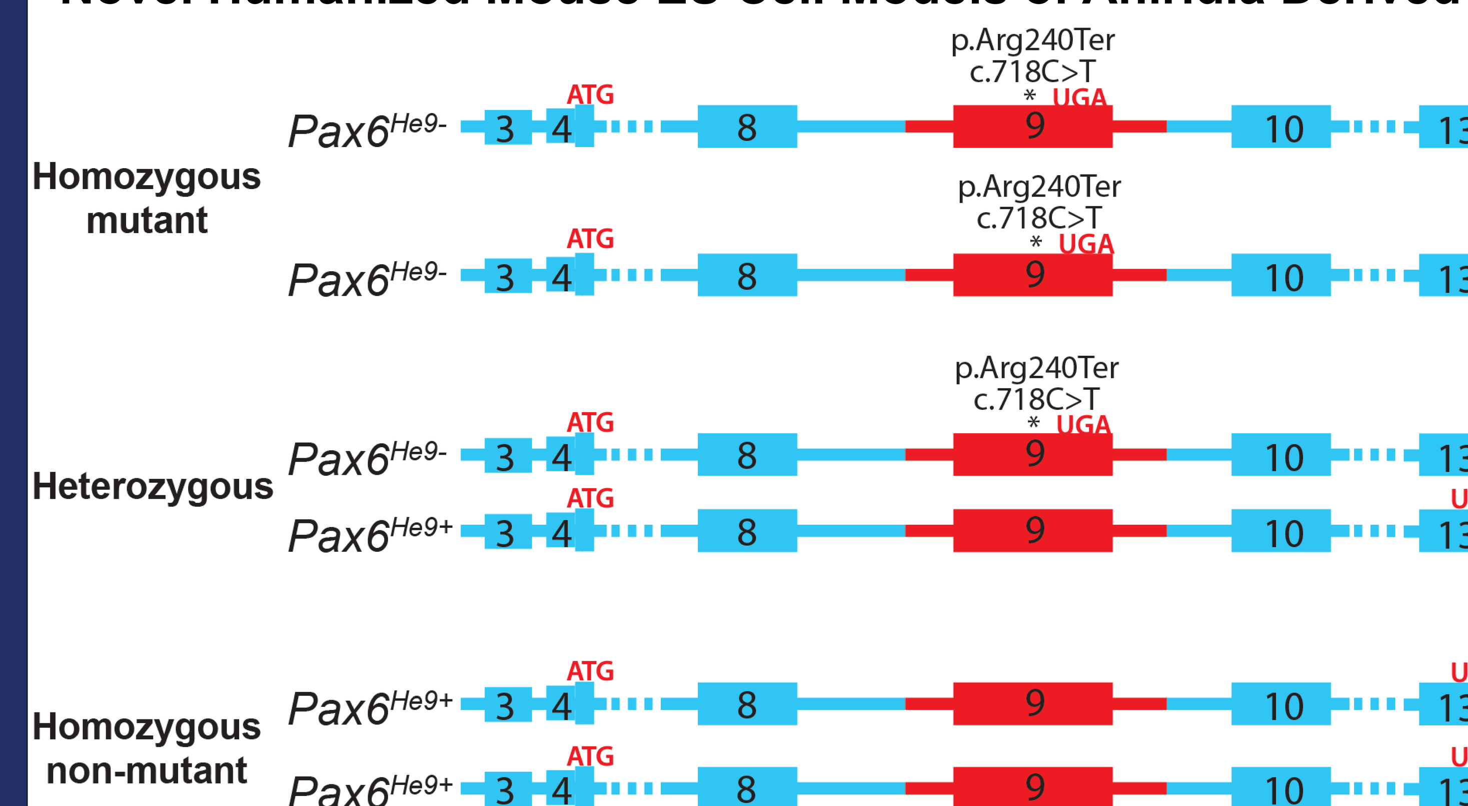


Figure 5. A correction strategy was derived using the CRISPR/Cas9 system to correct the patient variant in the humanized homozygous mutant cells, in order to obtain the humanized heterozygous and humanized homozygous non-mutant cell lines, to act as the control cell lines.

In vitro Gene Editing Corrected the Patient Variant with a 53.7% Success Rate

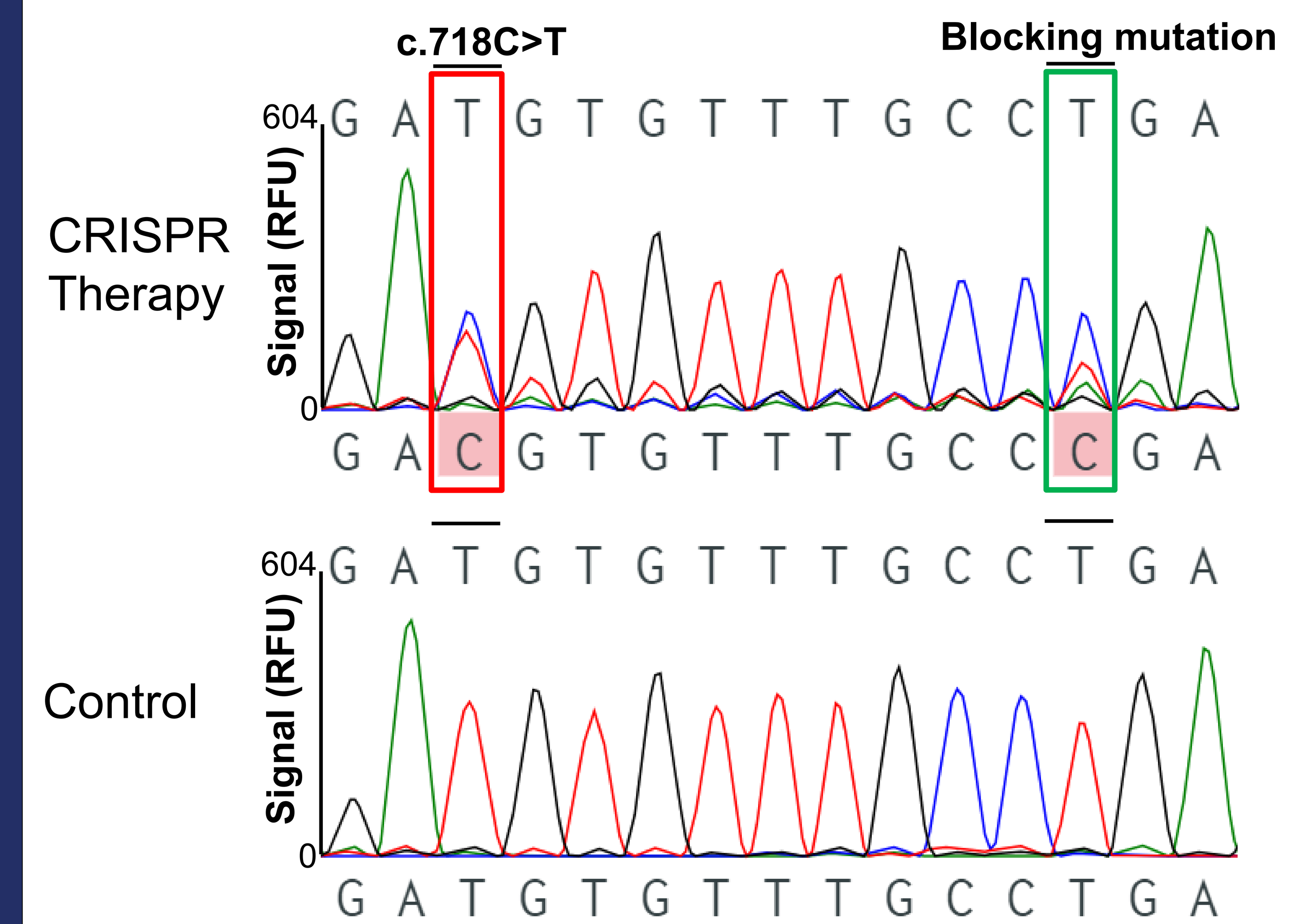


Figure 6. Multiple CRISPR-mediated therapeutic strategies to correct the patient variant were tested in our humanized cell lines, and the editing efficiencies were quantified by Sanger sequencing. Sanger sequencing chromatogram of the most successful CRISPR therapeutic strategy revealed a correction rate from T to C of 53.7% (red box). To prevent additional targeting of previously edited alleles, the single-stranded oligodeoxynucleotide (ssODN) also included a silent base change, referred to here as a blocking mutation, in the seed region of the gRNA, giving an alteration rate from T to C of 50.8% (green box). RFU, relative fluorescent units.

CRISPR Therapy Minimally Impacted Non-Mutant Chromosomes

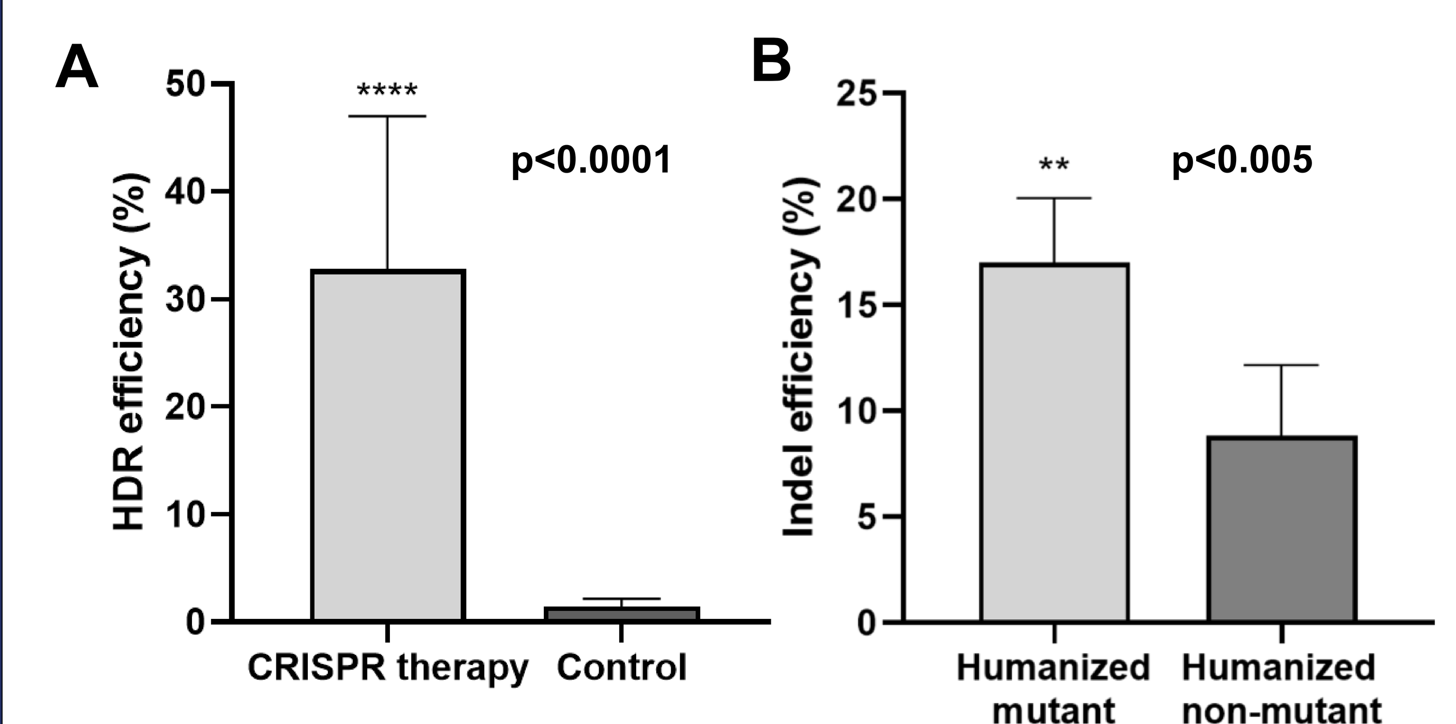


Figure 7. Average HDR and indel efficiencies of the best CRISPR strategy over multiple experimental trials were statistically significant compared to controls. A) The most successful strategy corrected the variant at a median frequency of 30.68% ± 5% (one-tailed t-test, $p<0.0001$). B) The ability of the CRISPR therapy to distinguish between mutant and non-mutant chromosomes was measured by the indel efficiency of the CRISPR therapy tested on the homozygous mutant and homozygous non-mutant chromosomes. The indel efficiency of the therapy tested in the mutant cell line was found to be 17% ± 2% (one-tailed t-test, $p<0.005$).

Future Optimization Will Include a CRISPR Base Editor Therapeutic Approach *in vitro*

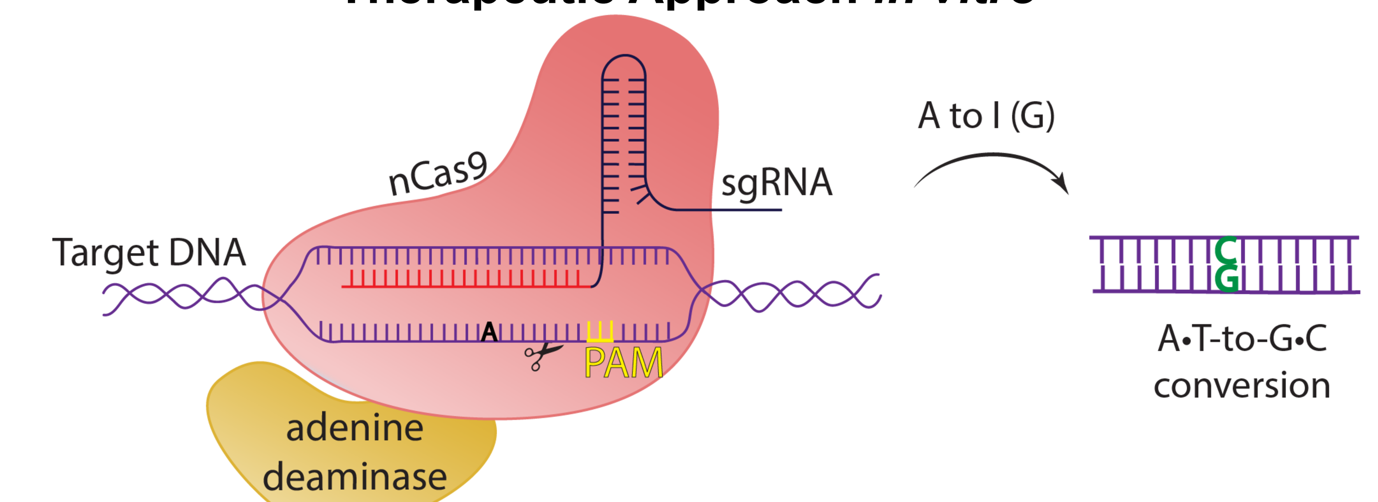


Figure 8. A mutated Cas9 nickase variant (nCas9) has been engineered to introduce single-stranded breaks into the DNA backbone. When fused with an adenine deaminase enzyme, the resulting CRISPR base editor is able to convert adenine bases to inosine, which are recognized by polymerases as guanine. Targeted edits by base editors prevent DSBs and unwanted indels.

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References

1. Hingorani, M., Hanson, I. & van Heyningen, V. Aniridia. *Eur J Hum Genet* **20**, 1011-1017 (2012).
2. Gaudelli, N.M. *et al.* Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. *Nature* **551**, 464-471 (2017).