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CRISPR-Mediated Gene Editing Tool Development for Patients with Usher Syndrome Type II

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Abstract

Usher syndrome, a rare genetic disease affecting hearing and vision, is an autosomal recessive condition impacting 4 to 17 per 100,000 people and accounting for about 50 percent of hereditary deaf-blindness cases. There are three distinct types of Usher syndrome. Usher syndrome type II is most commonly caused by mutations of the USH2A gene, a 15.7 kb gene on chromosome 1q41 encoding the protein usherin, which localizes to photoreceptor cilia in the eye and cochlear hair cells in the ear. This subtype of Usher syndrome involves hearing loss from birth and, during adolescence, the onset of progressive loss of vision, prompting retinitis pigmentosa (RP) that also causes night blindness. Prime editing is a viable option for addressing USH2A mutations and restoring usherin protein expression and function. To begin, a HEK293T cell line needs to be established for subsequent testing of prime editors. Since prime editing to make the cell line was unsuccessful, homology-directed repair was used to create a cell population with a single base-pair deletion (the mutation of interest). The objective of creating a cell population with the desired edit is for prime editors to be tested and optimized to address the mutation in the HEK293T cell line and, subsequently, patient-derived induced pluripotent stem cells that are differentiated into photoreceptor precursor cells.

Keywords: Usher syndrome, retinitis pigmentosa, CRISPR, prime editing, clinical trials, homology-directed repair, induced pluripotent stem cells, photoreceptor precursor cells

Introduction

Prime Editing to Induce Mutation c.14483del in HEK293T Cells

Usher syndrome is a rare genetic disease that affects both hearing and vision. This inherited autosomal recessive condition affects 4 to 17 per 100,000 people and accounts for about 50 percent of all hereditary deaf-blindness cases. There are three distinct types of Usher syndrome. Josara Wallber (2009), AuD, CCC-A, writes that patients with Usher syndrome type I experience significant hearing loss or deafness at birth and issues with balance. Usher syndrome type II

includes moderate to severe hearing loss, as well as decreased night vision and progressive loss of vision beginning in adolescence. Lastly, Usher syndrome type III is characterized by a progression in hearing loss and vision loss in adolescence (Wallber, 2009). Of these three types of Usher syndrome, Usher syndrome types I and II are the most common and are major recent focuses of scientific research on inherited retinal diseases ("Usher Syndrome," n.d., Fig.1A). Due to the progressive nature of the condition, Usher syndrome has received more attention recently in the hopes to find a cure in a timely manner and prevent as many patients as possible from losing significant vision, as this could potentially hinder the effectiveness of any potential treatments.

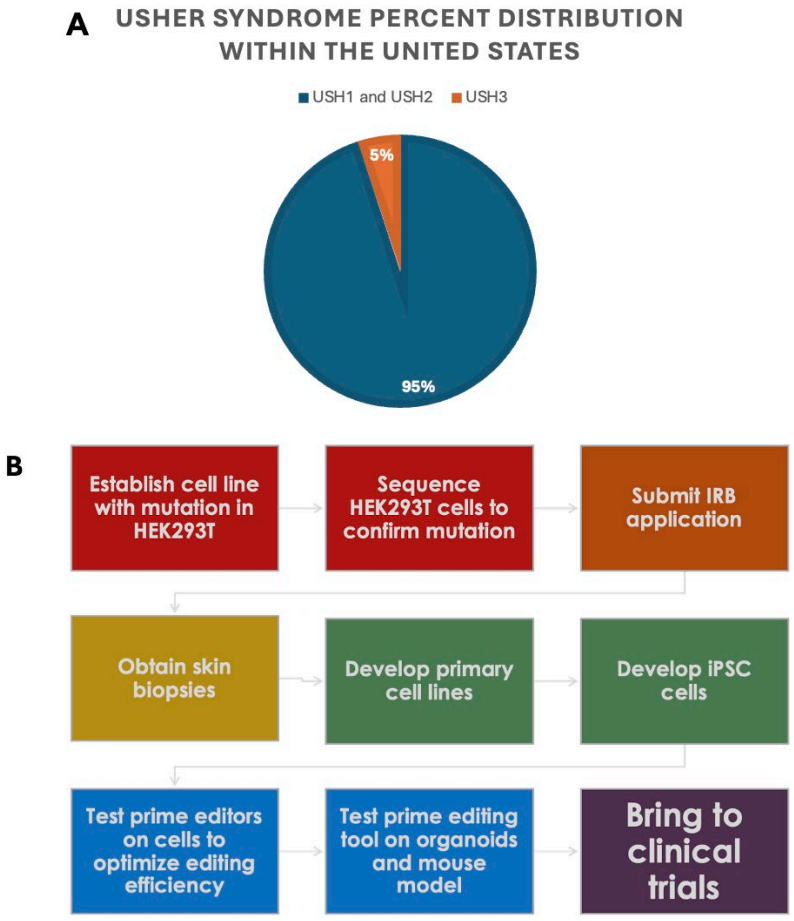

Figure 1. USH2A methods of addressing mutation of interest

(A) Percent distribution of Usher syndrome types 1, 2 and 3, with 1 and 2 being the most prevalent ("Usher Syndrome," n.d.).
(B) Overview of methodology for studying the efficacy of prime editors to address USH2A mutations.

Usher syndrome type II is caused by mutations in the USH2A gene, a 15.7 kb gene consisting of 72 exons that is located on chromosome 1q41 (Ocieczek, 2022). This gene encodes for the protein usherin, which localizes to photoreceptor cilia in the eye and cochlear hair cells in the ear. Mutations in the USH2A gene are the most common cause of Usher syndrome type II. USH2A mutations can lead to a frameshift and a resulting premature stop codon (PTC) that produce a truncated, non-functional protein. This is what prompts the hearing and vision loss associated with Usher syndrome type II.

Ordoñez-Labastida (2023) at the University of Mexico and her colleagues delineate that the lack of expression of the usherin protein triggers progressive deterioration of photoreceptor function, commencing with the more susceptible rod cells. This deterioration of photoreceptor function can result in hearing and vision loss associated with Usher syndrome type II. This subtype of Usher syndrome includes moderate to severe sensorineural hearing loss from birth and progressive loss of vision, prompting retinitis pigmentosa (RP). RP typically begins with nyctalopia, or night blindness. Additionally, in RP, the photoreceptors progressively lose function and patients experience decreased night vision by adolescence in addition to the previously mentioned peripheral vision loss that becomes more severe over time (Ordoñez-Labastida et al., 2023).

CRISPR-mediated prime editing is the major focus of this project and can be used to address specific mutations to remove PTCs and restore protein expression. It is not clear what percentage of transcripts need to be corrected to halt the progression of vision loss and potentially restore lost vision and hearing, but it is hypothesized to be less than 50 percent, since carriers of Usher syndrome only have about 50 percent of correct transcripts that are produced and do not exhibit symptoms of this condition.

Despite the varying severity of hearing loss, patients with Usher syndrome type II can benefit from hearing aids and cochlear implants. Because of the existence of these technologies, additional focus is given to treating the visual component to provide a more permanent fix due to the progressive nature of the condition. RP is usually diagnosed during late adolescence in people with Usher syndrome type II. USH2A mutations often involve nonsense mutations that induce a gene frameshift leading to introduction of PTCs. Such mutations in conjunction with large gene size limit options for therapeutic approaches for these patients. Additionally, little is

known about the exact molecular function of the usherin protein. This aspect is explored further in the discussion section of this study as a potential future avenue of research.

To date, there is no cure for this disease. Adeno-associated virus (AAV)-mediated gene therapy has been previously attempted to restore expression of the usherin protein for patients with this condition (Toualbi et al., 2020). AAV-mediated gene therapy is an approach in which a viral-based vector is utilized to deliver a new, correct copy of the gene to the affected cells. However, the large size of the gene of 15.7 kb makes AAV-mediated gene therapy a less feasible approach since an AAV has a size limit of 4.7 kb.

Exon skipping is an alternative method in which antisense oligonucleotides (ASOs) are used to sterically hinder the cell's splicing machinery to skip parts of a gene that may include a PTC or mutation, so they are not translated. Exon skipping can also be used to restore the reading frame. Eteplirsen is an exon skipping therapy that has been FDA approved for Duchenne Muscular Dystrophy patients carrying mutations in exon 51 due to its promising results (Agboola 2020). A limitation of this approach is that the ASOs are synthetic pieces of RNA, which are not permanently expressed. Due to the degradable nature of ASOs, these treatments may require repeated injections to prolong the therapeutic effect of exon skipping.

Homology-directed repair (HDR) is another approach to addressing Usher syndrome (Liu et al., 2021). HDR is a mechanism used by cells to repair double-strand DNA cuts through homologous recombination, in which genetic information is exchanged between two similar molecules. It is not currently a safe treatment since it requires double-strand DNA breaks that can lead to mutations and chromosomal rearrangements, which may lead to cancer.

Another approach for addressing USH2A mutations is base editing. You Jeong (2020) and her colleagues at Hanyang University and the University of Tokyo explain that base editing can only address substitutions, which limits its capacity to address a many other mutations patients may have like insertions and deletions.

Due to the limitations of these various approaches, CRISPR-mediated prime editing currently holds the most potential for addressing USH2A mutations. Precision genome editing in the eye could enable precise gene correction and potentially treat a wide range of inherited diseases, including genetic disorders of vision. Prime editing is the process of making edits to DNA through using a reverse transcriptase (RT) template containing the desired edit. The RT template is used to synthesize a new single-strand DNA sequence containing the edit and insert it into the

DNA. For this to occur, the prime editor must form a complex with the pegRNA containing the cas9 enzyme fused to a reverse transcriptase. The newly developed CRISPR tools represent an exciting prospect for treatment of USH2A mutations and prevention of currently untreatable blindness. Chenyu Lu (2022) and his colleagues at the National University of Defense Technology describe prime editing as a novel approach that can provide diverse edits without double-strand DNA breaks. Due to its promising potential to address a wide variety of mutations while avoiding ramifications of double-strand DNA breaks, CRISPR-mediated prime editing is currently the most promising method for therapeutic development for Usher syndrome and is the focus of this project.

A patient has been identified with the mutation of interest, which is c.14483del (p.Pro4828Hisfs*56). This is a single base-pair deletion in exon 65 of the USH2A gene causing a proline amino acid to be changed to a histidine and resulting in a PTC occurring 56 amino acids later. This PTC leads to the usherin protein not being fully translated, instead producing a truncated protein that is terminated in exon 66. This mutation is being used as a test case for prime editing for USH2A mutations, since optimization of a prime editing tool can be used to address a wide variety of USH2A mutations through simply changing the guide RNA. This project can be broken down into three major steps, which include inducing the c.14483del mutation in HEK293T cells, optimizing and testing prime editors on the HEK293T cells, and testing the optimized prime editor in differentiated induced pluripotent stem cells (iPSCs) as well as retinal organoids and mouse models (Fig. 1B).

The ultimate objective of this project is to develop a CRISPR-based gene editing technology directed towards addressing mutations in the USH2A gene causing Usher syndrome type II, to be used in clinical trials in the future. However, prior to the testing of this gene editing technology, a solid model exhibiting the USH2A mutation of interest needs to be developed, which is the sole focus of this paper and the first part of this project. Upon successful development of a mutated cell line, a variety of CRISPR/Cas-based prime editing systems will be designed and tested for correcting USH2A gene mutations to treat Usher syndrome in vitro and then in vivo.

Methods

Prime Editing to Induce Mutation in HEK293T Cells

PrimeDesign and PegFinder were both used to identify the highest ranked pegRNAs, with varying spacer and 3' extension sequences. The best candidate was chosen and used for nucleofection of HEK293T cells to create the single-base pair deletion. 3.5 uL PE2 prime editor protein, 6.5 uL of guide RNA, and 41 uL of SF nucleofection buffer were used per reaction. The concentration of the prime editor and pegRNA were 100 uM. The reaction was then complexed for about 25 minutes and 2 uL of electroporation enhancer were subsequently added. During the RNP incubation period, 200,000 cells were used in each reaction. HEK293T cells were trypsinized, resuspended, counted, and prepared. Cells were resuspended in nucleofection buffer and the cell mixture was added to the complexes following incubation. 100 uL of the final mixture was transferred to a SF Cell Line 4D-Nucleofector™ X Kit S (Lonza) cuvette. Nucleofection was initiated with code DS-150 and post-zap recovery was performed by allowing the cells to sit in nucleofection cuvettes for 10 minutes. 400 uL of pre-warmed DMEM medium (no FBS, no Pen Strep) was added to the cuvettes and transferred to 24 well plates. The medium was changed to D10 about six hours later.

Homology-directed repair

Since prime editing was unsuccessful in inducing the desired mutation, HDR was completed. HDR was completed with RNP nucleofection and an HDR donor using SpyCas9 NLS from New England Biolabs. Cas9 and guide RNA were incubated for 20 minutes at room temperature to allow for them to complex. HDR single stranded DNA donor template was then added. Next, cells were prepared for a total of five reactions and approximately 300,000 cells per reaction. The five reactions included sgRNA 1 with HDR donor template 1, sgRNA 1 with HDR donor template 3, and sgRNA 1 with donor template 5, HEK293T cells with sgRNA and no HDR donor template, and untreated cells. When preparing the cells, about 1.5x10^6 total cells was used and resuspended in 50 uL of SF buffer. 10 uL of solution containing cells with buffer was used per reaction.

For the reactions, 7.5 uL of SF buffer, 3 uL of Cas9 protein, 1.5 uL of guide RNA, and 3 uL of the corresponding HDR donor template were used. The HDR donor template was added after the cells were incubated for 20 minutes at room temperature after the addition of the buffer, Cas9 protein, and guide RNA for complexing. HDR successfully created a population of cells containing the single-base pair deletion mutation.

Serial Dilutions

The next objective was to isolate a single cell out of the population of cells with the mutation of interest to ensure the cell line that is developed only contains the desired edit and no other genotypes. To do so, flow cytometry single cell isolation was initially attempted through the University of Florida Cancer and Genetics Research Center, but this approach was less successful since it resulted in only a few single cells. So, serial dilutions in two 96-well plates were completed. To do so, 90 uL of D10 growth media were mixed with 10 uL of cells. Next, 10 uL of this mixture were then placed into the next wells and 90 uL of growth media were added. This process was continued for a total of four dilutions four times to increase chances of diluting the cells enough to isolate a population of cells containing only the mutation of interest. More emphasis was placed on wells with the third and fourth dilutions since they were more diluted and there was a greater likelihood of those wells containing a single cell. The day after serial dilutions were completed, wells were marked with no visible cells to focus on wells containing cells.

Results

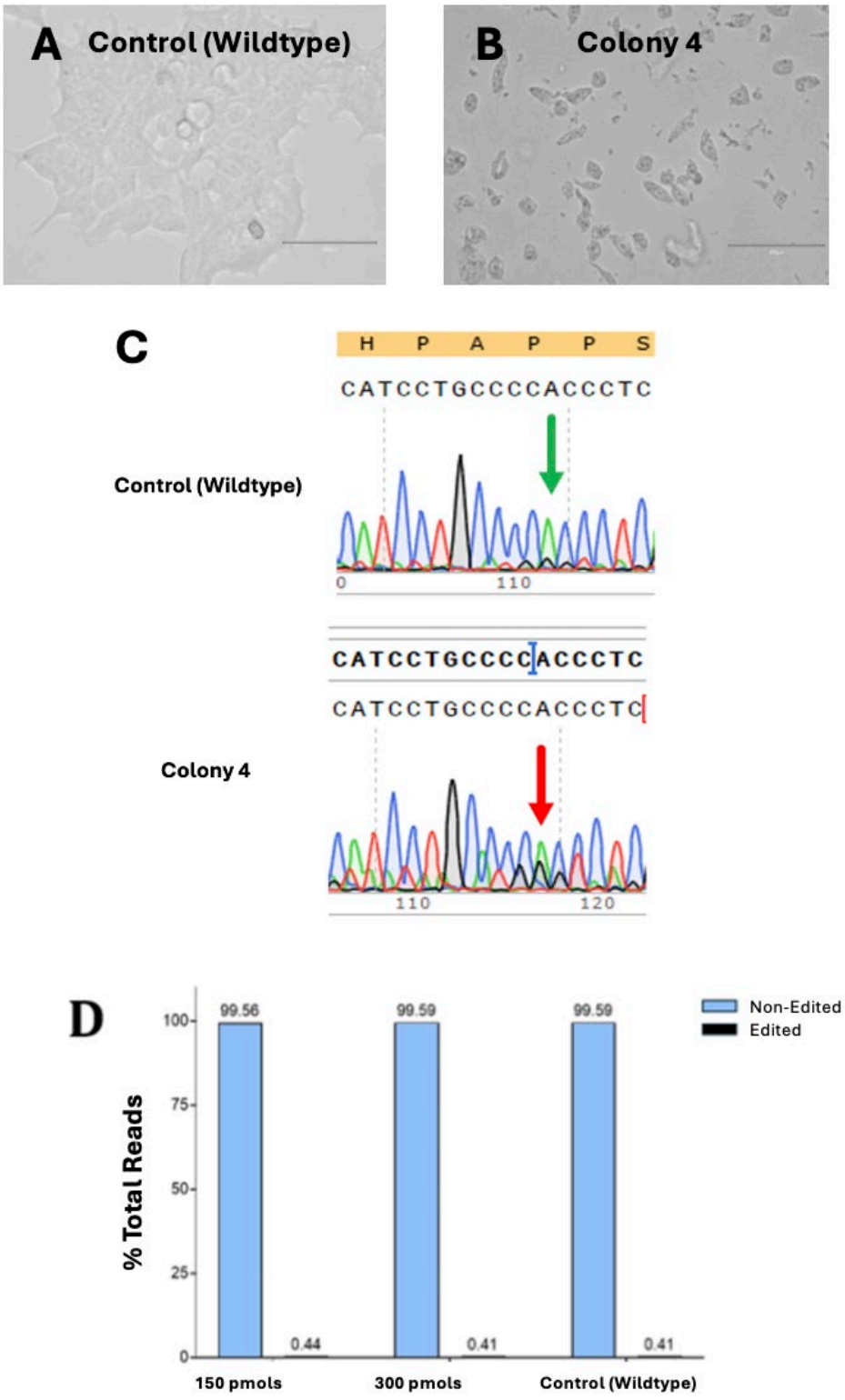

Figure 2. Determining whether deletion was made in HEK293T cells

(A) Bulk Sanger sequencing data after prime editing in HEK293T cells.
(B) Microscopic image of first well of HEK293T cells after prime editing to induce mutation.
(C) Microscopic image of second well of HEK293T cells after prime editing to induce mutation.
(D) Prime Editing efficiency in HEK293T cells as quantified by Next Generation Sequencing. Percent edit efficiency after prime editing using 150 pmols of prime editor protein, 300 pmols of prime editor protein, and untreated control.

To study the ability of prime editors to address the mutation, a cell line containing the mutation of interest must first be established. To do so, prime editing was attempted to create a single base-pair deletion in HEK293T cells. Sanger sequencing revealed a small difference between treated and untreated controls near the edit site, so single-cell isolation was done to determine if the edit of interest was made.

After outgrowth of single colonies, certain colonies displayed phenotypic differences in adhesion. Because usherin is known to be essential for cell-cell adhesion, it was hypothesized that colony 4 contains the desired edit (Fig. 2A and B). However, Sanger sequencing data on a single colony was collected, and the chromatogram illustrates that no edit occurred in this single colony (Fig. 2C). The phenotypic differences may be due to off-target effects that were not characterized in the study. To confirm that the edit was not made in the bulk cell population, next generation sequencing was performed. There was no difference between the untreated and prime edited samples, indicating that the experiment was unsuccessful (Fig. 2D).

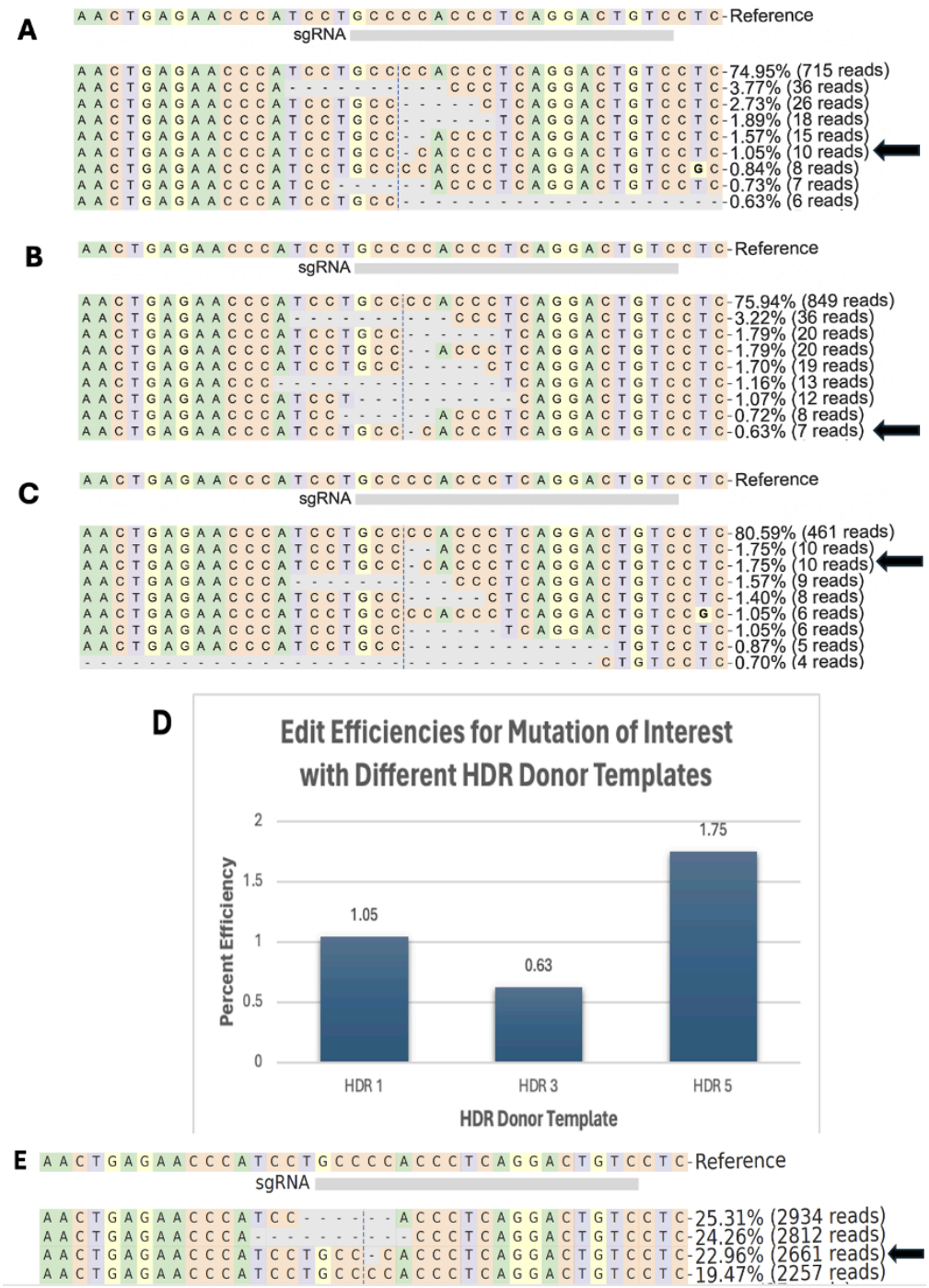

Figure 3. Edit efficiencies after HDR
Next Generation Sequencing (NGS) allele plots of HEK293T cells nucleofected with **(A)** HDR donor template 1, **(B)** HDR donor template 3, and **(C)** HDR donor template 5. Arrows indicate allele containing the target mutation. **(D)** NGS Percent of total reads containing the correct edit for each HDR. **(E)** NGS allele plots of HEK293T cells after single-cell isolation and colony expansion.

Homology-Directed Repair

Since prime editing was unsuccessful, HDR was attempted to induce the desired edit in HEK293T cells. Three different donor templates were selected and tested with one guide RNA. Out of the three donors that were tested, the most effective was HDR donor template 5 with 1.75 percent of alleles containing the edit (Fig. 3A-D). Serial dilutions were completed, producing a population of cells of which 22 percent contained the desired edit (Fig. 3E). Since four unique alleles were identified, it is possible that there were two heterozygous colonies that expanded together, creating four unique alleles. Serial dilutions are ongoing to establish an isogenic population of cells containing the desired single base-pair deletion mutation. Once this is complete, this cell line will be a highly useful in vitro model to enable optimization and testing of prime editing as an approach to addressing different USH2A mutations.

Discussion

Given the lack of basic biological understanding of usherin, along with the high unmet need for a cure in a time-sensitive manner due to the progressive nature of Usher syndrome, this research aims to first establish a cell line with the USH2A mutation of interest. This cell line can be used to investigate usherin protein structure, molecular function, and cellular phenotyping analyses to understand its homeostatic roles in sensory function as well. The cell line can also be used to test prime editing tools to address the mutation of interest and ultimately restore usherin expression and function. This work will also serve as a foundation for identifying possible therapeutic targets capable of restoring usherin protein loss and function in vision and hearing contexts.

Since the first objective of this project is to simply create a cell line with the desired edit for subsequent testing of the optimized prime editor, HDR was attempted to due to its tendency to yield a higher efficiency and despite it being a less safe treatment. Although prime editing was unsuccessful in inducing the mutation of interest in a HEK293T cell line, this technique represents a promising method to address the mutation of interest through restoring the reading frame to remove the premature stop codon. The inefficiency of the prime editor indicates the need to optimize the spacer and 3’ extension sequences for future experiments. Prime editing may have been unsuccessful due to the need for optimization of the guide RNA design, RT template length, or delivery strategy. Since the next objective is to optimize the prime editing system, there will be a greater focus on improving each of these aspects.

Moving forward, the HEK293T cell line will be transfected with prime editors under a variety of conditions to determine those which are the most optimal and yield the highest editing efficiencies. This process may become more complicated if it is determined that the mutation of interest is difficult to target, which may be due to the location of the mutation in the coding sequence. In this case, another option to consider is to either insert one nucleotide or delete two nucleotides through prime editing in another area within that region containing the mutation that is easier to target. The purpose of this approach would be to restore the reading frame and remove the PTC. This approach should serve as a last resort only in the case that the region containing the mutation of interest is difficult to target.

It is essential to consider the limitations of prime editing that should be addressed to avoid undesirable consequences of therapies brought to clinic. Prime editing systems must be tailored to the specific gene and mutation and is dependent on the surrounding sequence, making it difficult to apply the treatment to a patient population with many different mutations. The possibility of off-target effects is also a limitation of prime editing. Off-target editing occurs when the Cas9 protein and sgRNA bind to a different sequence that is not the target sequence, which may be due to sequence similarities. A way to minimize these effects includes improving sgRNA specificity to ensure it does not overlap in sequence similarity to other surrounding sequences.

Upon successful editing of HEK293T cells, prime editors can be tested in patient-derived iPSCs that are differentiated into photoreceptor precursor cells, so there will be two solid models for testing prime editors to address USH2A mutations. iPSCs are derived from a patient's somatic cells and can be reprogrammed into an embryonic-like state that allows them to be differentiated into a specific cell type (Ye, L., Swingen, C., & Zhang, J., 2013). The resulting differentiated cells will have the same genetic material that the patient has. By using patient-derived iPSCs that have the same genetic makeup as the patient and differentiating them into retinal organoids (photoreceptors), researchers can create an excellent model to test proof of concept of a potential therapeutic. Nonetheless, iPSCs can be more difficult and costly to maintain, so it is best to start an iPSC cell line closer to when the prime editing tool has been optimized to address the mutation of interest. HEK293T cells are much less expensive and easier to maintain, so they will be used initially as the prime editing tool is developed.

Collaborations have been established with subject matter experts in Switzerland and the Netherlands. Therapeutics to address the USH2A mutation will be tested in HEK293T cells, patient-derived iPSC cells, mice, and zebrafish to determine their efficacy. To plan ahead, an application for the development of a mouse model carrying the USH2A mutation of interest has been submitted and is under review. The goal of this project is to get a therapeutic to clinical trials.

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