ANALYSIS OF GENE TARGETING TECHNIQUES FOR HUNTINGTON'S DISEASE AND GENE EXPRESSION IN HUMAN CELLS

Abstract

Huntington's disease (HD) is an inherited neurodegenerative disorder that is caused by a CAG trinucleotide repeat expansion in the huntingtin (*HTT*) gene. Our team performed a literature analysis to investigate the current state of research for treating HD and identified a new technology called prime editing that could be applied to HD in combination with single nucleotide polymorphisms (SNPs). We found that at least 729 SNPs within the *HTT* gene are compatible with our proposed approach. Experimentally, we performed preliminary studies using Western Blots and RT-qPCR to examine the differences in expression of HTT in a variety of cell lines. Our literature-based work suggests that prime editing is a promising tool for addressing the basis of a variety of genetic disorders. Our experimental-based work confirms that human fibroblast cells express HTT and therefore may be used in proof of concept studies of gene targeting techniques to address HD.

Analysis of Gene Targeting Techniques for Huntington's Disease and Gene Expression in Human Cells

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Chapter 1: Introduction

Overview of Research Problem

Huntington's disease (HD) is an inherited autosomal dominant neurodegenerative disorder caused by an elongated, trinucleotide CAG repetition on Exon 1 in the Huntingtin gene, *HTT*.[1] There are between 6 to 35 CAG repeats in a healthy *HTT* gene, whereas individuals with HD have 36 or more copies.[2] The length of the repeats is inversely related to the age of onset of HD symptoms, which can appear in early childhood in patients with 60 or more repeats in their DNA, but progressively worsens with age.[2] The resulting elongated protein (mHTT) is processed into smaller pieces that aggregate and toxically accumulate in neurons disrupting the function of the cells over time.[3] This fatal genetic disease affects five to seven in 100,000 people of European descent, whereas it is known to be less common among certain populations such as African, Chinese, and Japanese ancestry.[4]

HD exhibits distinct symptoms of neurological problems in affected patients, ranging from physical deterioration to psychosis. Chorea, incoordination, and cognitive decline are the most common indicators of HD.[5] HD patients additionally experience significant difficulty in walking, balancing, talking, and swallowing. These abnormal behaviors may manifest at any time during an individual's life, but HD typically has a later age of onset. Unfortunately, those with adult-onset HD usually live for only 15 to 20 years after diagnosis due to retardation of muscular and cognitive functions.[6,7]

The current treatments focus on improving the quality of the patient's life by alleviating symptoms and slowing the progression of the disease with medication. Medications such as dopamine-depleting drugs are used to address the most prominent phenotype of the disease, chorea, by reducing random, involuntary movements. Other medications target pathways

involving neurotransmitters such as glutamate and GABA to address motor dysfunction. Though such medications are used, a complete cure is unavailable, and the progression of the disease eventually leads to death.

Purpose and Rationale of the Study

Current therapies for Huntington's disease focus on symptom relief, as there is no cure. However, numerous studies are ongoing to develop more therapeutic methods and understand the molecular foundation of Huntington's on a deeper level. Ongoing research hopes to determine specific molecular and genetic factors that impact onset in order to determine what interventions can prevent disease progression.[8] However, mHTT is involved in numerous pathways that may make a chemical or non-genetic method for therapy insufficient, thus motivating the research behind genetic-based methods.

Genetic-based methods can target HTT expression on the RNA or DNA level. A promising RNA-based approach is the use of antisense oligonucleotides, or ASOs, which bind to complementary RNA molecules and lead to endonuclease degradation. ASO can be used to target the CAG repeat, SNPs, or introns to specifically reduce mHTT expression.[9] ASO-based methods that target intronic and exonic SNPs have shown success through reduced mHTT expression in fibroblasts.[10] Improved HD symptoms were also observed in mouse models using ASOs.[11] However, there is a concern with off-targeting effects and variation among different patients.[9] There is a possibility for personalized ASOs, though these types of treatments can be very expensive. Additionally, ASOs do not cross the blood-brain barrier, serving as another obstacle for clinical applications.[12]

Another mode of genetic therapy is RNA interference or RNAi. RNAi is a method in which spliced mRNA fragments are targeted with the intent of altering protein expression. The

RNA transcripts are delivered with the help of a lentiviral or adenoviral vector to protect the mRNA from degradation and ensure that it locates its target.[13] MicroRNA, short hairpin RNA (shRNA), or small interfering RNA (siRNA) have all been proven to be effective in reducing protein expression in numerous studies.[14] For HD, the RNAi method is typically used to reduce the expression of both WT and mutant Htt. However, some innovative techniques such as the use of single-stranded RNA (ss-RNA) to inhibit translation by degrading mRNA have seen allele distinction when using an ss-RNA that targets the expansion on the mutant transcript.[15]

One drawback to both ASOs and RNAi is the need for constant administration to the patient. Thus, these therapies would serve more as a treatment for HD than a cure. A more permanent genetic therapy targeting the HD mutation could lower costs and patient burden. This makes genetic engineering with the CRISPR-Cas9 system attractive. CRISPR-Cas9 has the potential to permanently delete the expanded CAG repeats, halting the production of mHTT for an entire lifetime. This method uses a guide RNA (gRNA) consisting of 16-20 nucleotides that recognize a specific DNA sequence to be cut using a fused Cas9 protein. The protospacer-adjacent motif (PAM) that directly follows the DNA sequence of interest is essential in promoting the Cas9 protein to cut.[16] This method of editing may be done without distinguishing between alleles by deleting the polyQ domain of *mHTT* in both copies. However, it is unclear what effects may arise from lowering wild-type HTT expression as the protein function is not fully understood. It has been observed that completely silencing Htt expression early in life can be lethal in mice, and inactivation in adult mice elicits neurodegeneration.[17] Thus, an allele-specific CRISPR-Cas9 approach may be better tolerated, in which single nucleotide polymorphism (SNP) haplotypes are used to distinguish between copies of the alleles. Current Cas9 based tools cannot completely discriminate between CAG repeats of greater than

about 20 nucleotides lengths but can distinguish between SNPs, allowing for a selective reduction in mutant Htt expression and potentially reducing the number of aggregates.

A relatively new innovation on the traditional CRISPR-Cas9 system is prime editing. Prime editing does not require double-stranded DNA breaks and a donor DNA template for homology-directed repair, yet still successfully achieves targeted insertions and deletions in DNA.[18] The manner of gene editing is more precise and controlled than the original CRISPR system through the use of a prime editing guide RNA (pegRNA) and a prime editor that fuse together in a complex. The Cas9 nickase allows for a single-stranded, rather than double-stranded, break. This decreases the chance for errors in DNA repair associated with double-stranded breaks and therefore may be an appropriate approach for HD.

This study seeks to investigate the applications of the various gene targeting techniques for the treatment of HD. The information discovered and results acquired are split broadly into literature-based and experimental-based sections.

Method Framework

I. Literature-based

The team sought to understand the current state of Huntington's Disease research and available gene-editing tools. We used online library services at the University of Maryland to access journal articles describing the pathophysiology of HD and current therapeutics available. We categorized current clinical trials related to HD into different treatment strategies including stem cell therapy, gene silencing, and small-molecule drugs. All of these treatments were aimed at slowing neurodegeneration and alleviating motor impairment symptoms associated with HD. However, there were no active clinical trials aimed at targeting the HD gene, which is ultimately responsible for HD. We identified certain applications of gene editing as a gap in the research

with potential clinical applications and decided to focus on gene editing strategies to treat HD. To this end, we explore both allele-specific and non-allele-specific approaches to reduce the expression of mHTT.

Through our literature search, we recognized that most CRISPR-Cas9 approaches were not allele-specific. In an HD patient's cell, these methods would result in editing of the healthy HTT allele, which is required for healthy neuronal development and function. Gene editing tools that selectively edited the diseased allele were most desirable for HD applications. We began to search for research groups that have achieved allele-targeted editing. One group, Shin et al., developed an allele-specific gRNA based on single nucleotide polymorphisms (SNPs) which were commonly observed in a haplogroup representing 80% of HD patients of European descent. We included this model system in our literature review.

Another group, Anzalone et al., developed prime editing, an accurate CRISPR-Cas9 editing system that avoids off-targeting and errors in DNA repair associated with double-stranded breaks that resulted in previous CRISPR systems.[19] We decided that this method of gene editing would be most desirable for gene editing since double-strand breaks can induce cell death via apoptosis.

We determined that allele-specific prime editing would be an accurate and safe gene-editing tool with a significant impact. The team developed a program that generated sgRNA and reverse transcriptase template RNA sequences of prime editing systems using input SNPs, identified from NCBI. We identified SNPs in the first exon of the HTT gene as candidate sites for primer binding such that a stop codon could be inserted, prematurely terminating translation and halting mHTT expression. This program could be used as a standard tool for researchers to generate gene editors for HD sub-populations defined by their haplogroup.

II. Experiment-based

In the first phase of experiments, we sought to compare the expression of mutant and healthy huntingtin (HTT) in primary cell lines using Western blotting. HD patients exhibit neurological impairments so initial cell-based studies focused on HTT expression in neurons. However, it is worth mentioning that HTT is expressed in nearly all tissue types, which may provide an explanation for HD symptoms outside of the central nervous system. We decided to include fibroblast cell lines in our studies to investigate their HTT expression levels to probe this question. Beyond this, we sought to examine HTT expression in Hutchinson-Gilford progeria syndrome, where the mutated protein has numerous signaling effects. This rare genetic disease results in the expression of a mutated lamin A protein, known as progerin. This protein downregulates proteins with repetitive sequences, such as the polyQ tract observed in HTT (Arancio, 2019).[20] To verify this, we included fibroblasts from a progeria patient in our initial studies. Additionally, it is known that HTT is required in neuronal developmental processes.[21] As such, a developmental sequence of three neurons was included in initial experiments following a 14-day neuron differentiation protocol: D0, D7, D14 neurons.

General Research Questions

Previous studies have shown the efficacy of using the traditional CRISPR-Cas9 system to silence the mutant *HTT* gene through heterozygous PAM sites in patient-derived fibroblast cells.[22] The gene-editing technology was also able to selectively target the disease allele and reduce mHTT expression in neural cells of the mouse model.[23] The success of the CRISPR-Cas9 system relies on targeting SNPs located within both the promoter region and shortly after exon, which significantly limits the targetable SNPs. To combat this limitation, prime editing technology has been developed. It utilizes Cas9 nickase, which is a heavily mutated version of

the Cas9 protein, to target SNPs outside of the promoter and first intron region and to increase the rate of insertion of a desired sequence. In this system, the Cas9 nickase nicks the desired site, exposing a 3' end which the reverse transcriptase can use alongside designed RNA sequences to reverse transcribe a short new sequence directly into the DNA. On the other side of the cut, a 5' end is exposed, which is targeted by exonucleases for degradation, allowing the newly synthesized sequence on what was the exposed 3' end to replace it. This technology has significantly reduced off-targeting chances through the use of Cas9 nickase. As a result, insertion of a short sequence containing stop codons in the DNA would effectively terminate translation of the mHTT protein.

Currently, there are no studies that have achieved allele-specific targeting of the mutant HTT gene with a single CRISPR-Cas9 target site. In order to explore this novel approach, the PAM generating SNPs within the gene has to be determined first to distinguish between the healthy and mutant alleles. Then by using the prime editing technology, a short stop codon containing sequence would be inserted into the mutant allele to achieve silencing of the mutant protein.

Cell models are critical tools for studying Huntington's Disease in vitro, as they accurately reflect the conditions of HD-affected neurons, providing insight into how the disease affects the cells of a patient. Therefore, it is important to determine which types of cell lines can be used for applying gene editing-based techniques. To assess the relative expression of mutant and healthy huntingtin (HTT), results of the Western blots were quantified across four different types of cell lines of which two were fibroblasts and two were neuronal cells. Quantitative analysis can directly compare the expression within one type of cell line. This is an important

factor to consider since the level of HTT is enriched in neuronal cells, particularly the progenitor cells, compared to that in fibroblasts.

Another method to specifically target the mutant allele is the use of SNPs within the disease allele to allow for allele discrimination based on the creation or destruction of a PAM sequence by a particular SNP. Due to the inherited nature of the disease, HD patients can be categorized into specific haplogroups based on common ancestry. Previous analysis of the HTT gene across the European Huntington's population determined that a single prominent inherited SNP haplotype accounts for 50% of HD chromosomes.[24] The results also determined that seven of the most abundant haplotypes could account for approximately 83% of the chromosomes across the studied population. These associations among HD patients would likely allow for a genetic correction approach in one patient's cell line to apply to a large number of patients. To determine the applicability of the use of SNPs, the frequency of SNP creating or destroying a PAM sequence that can be used in the proposed gene editing approach must be found.

Chapter 2: Literature Review

The following literature review is largely taken from a paper that was written by this team and published by Aging Research Reviews on June 5, 2021. The section on Huntington's Disease Models is not in the original paper and some sections from the original review paper are not included in this chapter.

Fields, E.; Vaughan, E.; Tripu, D.; Lim, I.; Shrout, K.; Conway, J.; Salib, N.; Lee, Y.; Dhamsania, A.; Jacobsen, M.; Woo, A.; Xue, H.; Cao, K. Gene Targeting Techniques for Huntington's Disease. *Ageing Research Reviews* 2021, *70*, 101385. https://doi.org/10.1016/j.arr.2021.101385.

Introduction: Huntington's Disease

Huntington's disease (HD) is an inherited neurodegenerative disorder caused by an elongated, trinucleotide CAG repetition in the *HTT* gene.[2] Repeat lengths greater than 36 glutamines are predictive of HD, while <36 repeats carry essentially no HD risk.[25] The length of the repeats on the mutant allele is inversely related to the age of onset of HD symptoms.[25] HD exhibits neurological symptoms in affected patients, which can range from physical deterioration to psychosis.[26] The most common indicators are chorea, incoordination, and cognitive decline, which can include irritability and depression.[5] HD patients typically experience physical symptoms as well, including significant difficulty in walking, balancing, talking, swallowing, and weight loss.[27] A decrease in BMI and weight loss has been found with a direct proportion to the length of the CAG repeats, stemming from a rapid metabolism.[27] These abnormal behaviors can manifest at any time in an individual's lifetime. Those with adult-onset usually live for only 15 to 20 years after diagnosis due to retardation of muscular and cognitive functions.[6,7]

Current Therapeutic Approaches

Although a cure for Huntington's Disease has yet to be developed, there are treatment options currently available to alleviate symptoms and slow the progression of the disease. Loss of medium spiny neurons (MSNs) in the striatum is the major contributor to HD pathogenesis and physical symptoms as previously mentioned. Recent cell conversion therapy approaches have successfully converted nearby astrocytes into functional MSNs to restore the damaged striatum.[28]

Additionally, since HD patients endure degeneration of dopamine, glutamate, and GABA neurotransmitters, many therapeutic drugs target these neurotransmitters as a means of addressing motor dysfunction. One of the major symptoms of HD is chorea, a motor disorder resulting in involuntary movements and fidgeting. Several drugs aim to combat chorea by

treating the depletion of dopamine levels in the brain. This has been achieved through drugs that inhibit dopamine transport and block D2 receptors.[29–31] While these types of treatments may provide temporary relief, many of them are significantly limited by harmful side effects or patient adherence to the regimen. Ultimately, these treatments may be a temporary fix for those suffering from Huntington's Disease, but additional research is needed for a permanent cure.

Recently, several HD review articles were published discussing a variety of topics, including DNA repair mechanisms involved in HD pathology and therapeutic opportunities, the role of neurodevelopment in HD pathology, accelerated aging as a contributing factor to pathogenesis in HD, and the importance of the Tau protein in HD neurodegeneration.[32–39] This review will focus on current DNA and RNA-based approaches to downregulate the mutant allele and discuss the potential application of the recently developed prime editing technology in disease treatment.

The Function of HTT and Mutant HTT (mHTT) Proteins

The *HTT* gene is highly conserved. The gene's embryonic knockout in mice has shown to be lethal.[40] While its exact function remains unclear, wild-type HTT has been attributed to a variety of functions such as trafficking vesicles, coordinating cell division, regulating cell movement and transcription, mediating autophagy, and cell survival **(Figure 1)**. HTT expression is present in spindle poles and microtubules.[41] The protein interacts with motor machinery directly through dynein or indirectly through huntingtin-associated protein.[42] HTT is also an important requirement for ciliogenesis as it is associated with pericentriolar material 1 protein at the centrosome, mediating dynein-dependent transport.[43] In *HTT* knockout mice, primary cilium was unable to form.[44] HTT also interacts with the proteins involved in endocytosis (HIP1 and HIP12) and activates the small GTPase Rab11.[45] HTT plays a role in the recruitment of

endosomes in a switch from MTs to actin filaments in the trans-Golgi network.[42] HTT is believed to regulate autophagy, as autophagosomes are overexpressed in HD.[42] However, despite the increased presence of autophagosomes, the HD cells are inefficient at degrading cellular debris due to a defect in the loading of the vesicles.[46] Finally, HTT has a wide range of regulatory effects on transcription. It is known to interact with p53, thus affecting DNA damage repair, cell cycle, and apoptosis.[47] It is also proposed that HTT may act as a scaffold for complexes involved in transcription and serve as a cofactor itself.[42]

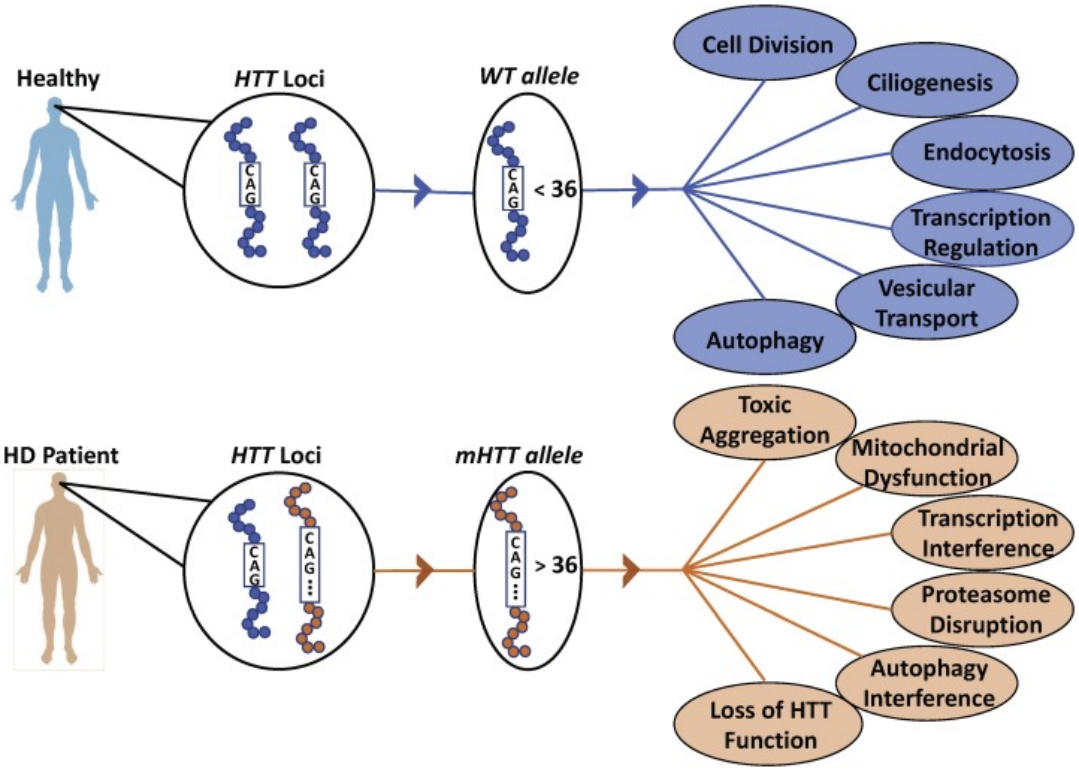

Figure 1. HTT has been experimentally linked to a variety of different cellular functions including cell division, vesicular transport, endocytosis, ciliogenesis, autophagy, and transcription regulation. The mutant allele with a CAG expansion of greater than 36 repeats has been linked to a variety of gain of function effects including toxic aggregation, mitochondria

dysfunction, transcription interference, proteasome disruption, autophagy interference, and potential loss of healthy HTT function.[3,48–50]

Studies suggest that the mHTT destroys some regular functions of HTT (loss of function) while also creating additional toxic gain-of-function mutations, which leads to detrimental effects such as oxidative damage, synaptic and mitochondrial dysfunction, the diminished function of the nuclear pore complex, and other pathological mechanisms **(Figure 1)**.[48–50] On a cellular level, the mutant protein is processed into smaller pieces that aggregate and accumulate in neurons.[3] mHTT aggregates cause dysfunction in the ubiquitylation system. Therefore, in HD neurons, both the ubiquitin-proteasome pathway and autophagy routes that assist with protein aggregate degradation are affected.[51] Studies further demonstrated that reduction of mHTT in animal models resulted in a reversal of neuropathology and motor dysfunction as well as improved behavioral deficits associated with HD, supporting the importance of lowering mHTT as a promising treatment direction for HD.[45,52,53]

Interestingly, exon 1 of HTT, containing the expanded CAG repeats, is capable of causing HD phenotypes even in the absence of the rest of the HTT protein.[54] Previously, the source of this destructive exon was thought to be due to the cleavage of the mHTT protein; however, recent studies have found a potential source in a variant of *mHTT* mRNA resulting from incomplete splicing, as it encodes a short exon 1 HTT protein that is formed more easily when *HTT* is CAG-expanded.[55] *HTT* mRNA also undergoes repeat-associated non-ATG (RAN) translation, generating unconventional protein species from a variety of sense and antisense strands.[56] Further research has found that some of these protein species and even the expanded *HTT* RNA itself may be toxic.[57,58]

Differential Post Translational Modifications of mHTT:

The post-translational modifications of HTT are of increasing importance in Huntington's research, as they modulate its activity and proteolysis.[59] There are several post-translational modifications that HTT is subject to, including phosphorylation, acetylation, palmitoylation, ubiquitylation, and SUMOylation.[59] Many of these processes have been found to differ between the wild type and the mHTT, with some increasing toxicity and others doing the opposite.[60–64] A recent study reports the discovery of an SNP that prevents the post-translational process of myristoylation in HTT, prompting cellular toxicity.[46] In addition, the same mutation caused by the rs118005095 SNP was found to increase the proteolysis of wild-type HTT at D513, contributing to further toxicity coupled with proteolysis at D586.[46]

Huntington's Disease Models

Cell models are a critical tool for studying Huntington's Disease *in vitro*, as they accurately reflect the conditions of HD-affected neurons, providing insight into how the disease affects the cells of a patient. Typically, fibroblasts and induced pluripotent stem cells (iPSCs) have been used to model and study Huntington's Disease. A recently developed cell model, termed TruHD cells, was created by applying human telomerase reverse transcriptase in order to immortalize fibroblast cells taken from Huntington's Disease patients.[65] This immortalization prevents the senescence of the cells that typically occurs after repeated passages and causes changes in cell morphology and gene expression. Additionally, the TruHD cells resembled the phenotypes of HD cells, including altered morphology, smaller size, and increased cell growth rate. Since the TruHD cells are stable after many passages and accurately express the phenotypes seen in HD cells, they provide a solid platform for studying Huntington's Disease.

Induced pluripotent stem cells originate from the discovered ability to reprogram human adult somatic cells to a pluripotent status. Takahashi and Yamanaka reported the four transcription factors that allow for this reprogramming and successfully generated iPSCs from human fibroblasts.[66] Since then, many other groups have found success in establishing iPSC lines from diseased patients, leading to established methods for this process.[67,68] This ability to establish immortal patient-specific stem cells alleviates previous concerns of immune rejection and allows for genetic editing preceding transplantation. Studies have developed a technique to directly reprogram fibroblasts into self-renewing neural stem cells (NSCs) without an intermediate pluripotent stage, thereby avoiding the risk of teratoma formation that is associated with iPSC-derived NSCs.[69,70]

Animal models have also become instrumental in the study of Huntington's disease in humans, as they reproduce similar disease characteristics that can undergo further study and testing.[71] However, because Huntington's disease is an age-related disease, it can be difficult to accurately model with animals since their lifespan is shorter than what the disease requires to progress as it does in humans. The CRISPR-Cas9 system has allowed for the generation of rapid and more cost-efficient mutant mouse models than previous gene-editing techniques.[71] The most common transgenic mouse model of HD is the R6/2 cell line model produced by overexpressing exon 1 of the human gene encoding Huntingtin (IT15) with CAG-repeats that range from 141 to 157 base pairs long, causing early and severe HD symptoms. R6/2 mice have an onset of severe motor deficits at 5-6 weeks and are often unable to survive more than 13 weeks, making this model plausible for the study of juvenile-onset HD.[72] Other mouse models include; (1) the N171-82Q transgenic HD mice, which express a different N-terminal mutant Htt fragment than R6/2 mice, (2) BAC and YAC transgenic mice, which express full-length mutant Htt with

expanded polyQ repeats, and (3) knock-in mouse models with expanded CAG repeats or human mutant Htt exon 1.[72]

Though less common than mouse models, pig models have proven to be an increasingly attractive model in the study of HD due to their genetic, anatomic, and neurologic similarities with humans.[73] Holm et al., created and tested three genetically modified pig models to further assess the effects of HD in animals, however, each model had its own limitations.[73] In the first model, mutant 75Q *HTT* was induced in the fertilized egg of a miniature pig via pronuclear microinjection, but it was unsuccessful in demonstrating phenotypes of HD. The second model used somatic cell nuclear transfer to introduce a transgene that expressed the N-terminal mutant Htt in N208-105Q pig fibroblast cells. However, the incomplete cell reprogramming resulted in early postnatal death for most of the pigs, preventing the models from being regenerated. The second model lived longer, expressing the first 548 amino acids of HTT using transducted zygotes and lentiviral vectors. Similar to HD symptoms expressed in humans, these transgenic pig models experienced DNA fragmentation and apoptosis in the brain, which suggested that they could serve as a successful animal model to study the neurodegeneration pathways of HD.[73] This strategy for allele-specific genome editing was confirmed in mice models for its effectiveness in reducing expression.

Non-allele specific gene editing-based approaches

I. RNA-based

Simultaneous reduction of both *HTT* and *mHTT* has been shown to be well-tolerated in both mouse and nonhuman primate models, suggesting that non-allele specific approaches may be effective.[14,52,74–76]

One such study used lentiviral mediated silencing of *mHTT* in rodents. Four short hairpin RNA (shRNA) that target exons 1-4 and 8-9 of human *HTT* mRNA were designed and transfected into lentiviral vectors.[14] After nine months, the reduction in wild-type *HTT* expression was observed to be well tolerated in the mice with 65-75% reduced expression.[14]

Another similar approach saw the use of inhibitory RNAs expressed in the striata of HD-n171-82Q mice.[52] Boudreau et al. used artificial viral-mediated miRNA, which has been shown to have lower levels of toxicity than that of shRNA vectors, to target exon 2 of the *HTT* gene. The RNAi-treated mice showed improved behavior and prolonged survival, despite the low levels of wild-type HTT protein expression. This knockout was well tolerated for up to a 75% decrease in WT HTT mRNA for four months in mice despite changes to the transcriptome.

More recently, an adeno-associated viral vector (AAV) was designed that interferes with the HTT transcript in the YAC128 mice model by degrading it with small interfering RNA (siRNA).[77] This approach reduced levels of both wild-type and mutant HTT by 40% and achieved transduction in more than 80% of cells in the striatum.[77] These reductions were accompanied by observed behavioral improvements and reduction of cellular aggregates with no noticeable toxicity. The researchers further postulated that it may be possible to design siRNAs that specifically target SNPs on the diseased allele, yet concluded no one SNP has yet been identified that would allow this to happen at the time of the study.[77]

II. DNA-based antisense oligonucleotide approaches

Antisense oligonucleotides (ASOs) use single-stranded DNA and typically range from 18-30 nucleotides.[78] ASOs often recruit RNase H endonuclease to degrade the RNA-DNA duplex, although they can also prevent the formation of the 5' cap, alter the splicing process, and sterically hinder the ribosome, all of which downregulates translation of the mRNA.[79] An

ASO-based approach differs from RNAi methods due to the site on which the molecular machinery acts; ASOs target exons and introns, whereas RNAi compounds act on spliced mRNA.[80,81] Thus, ASOs work to reduce the production of toxic mRNA but may be unable to prevent the formation of truncated mRNA. Since ASOs do not saturate any endogenous pathways and have clear reversible effects, it is advantageous.[82] Repeated administration of ASOs may be needed to retain the therapeutic benefits.

An ASO designed to target a sequence consisting of seven CAG repeats has been used in fibroblasts derived from HD patients.[83] In this study, the ASOs resulted in a reduction of mutant *HTT* mRNA transcripts by 83% while also reducing the wild-type allele by 43%. In addition, the ASOs reduced transcripts of other CAG repeat genes, including *ATXN3*, *ATXN1*, and *ATN1*.51. Off-targeting to this extent is not ideal, both with respect to wild-type *HTT* and the other CAG repeat genes. In mice, however, administration of this ASO significantly reduced mHTT and helped restore neurological function.[83]

IONIS-HTT_{Rx} is an example of a human targeting HTT ASO. In BACHD mice with a full-length human mutant *HTT* gene, IONIS-HTT_{Rx} was able to reduce mRNA expression up to 80% and protein expression by around two-thirds.[84] These reductions lasted for around 4 months and disease phenotypes were mitigated in three different mouse models. In two models (BACHD and YAC128), motor deficiencies were almost completely reversed. In a third, R6/2, brain atrophy was diminished and survival rose.[75] Notably, even after the expression of HTT and the presence of the IONIS-HTT_{Rx} lessened, the phenotype reversals remained.[85] Another study demonstrated that doses of IONIS-HTT_{Rx} resulted in a nearly 50% reduction in cortical HTT and 15-20% reduction in striatum HTT.[86] These reductions together signaled an overall improvement in the BACHD mouse model.[86] In a recent human clinical trial (NCT02519036), patients were

administered IONIS-HTT_{Rx} in four monthly doses. While the primary focus of the study was safety, the investigators did report that there were pronounced reductions in mutant huntingtin concentration in the cerebrospinal fluid.[87]

III. CRISPR-Cas9 gene editing

In addition to RNAi and ASO treatments, non-allele-specific CRISPR-Cas9 has been considered a therapeutic strategy to eliminate the polyglutamine aggregates formed in HD. The traditional CRISPR/Cas9 system is derived from the bacterial immune system which plays a role in recognizing and destroying foreign DNA. In gene-editing techniques, the Cas9 protein recognizes the DNA sequence to be cut through specific and guide RNA constructs (gRNA) of 16-20 nucleotides. If a protospacer-adjacent motif (PAM) does not follow directly after the DNA sequence that the gRNA recognizes, however, the Cas9 protein will not cut. Different Cas proteins have different PAM sequences, but they are typically short sequences of 2-5 nucleotides, such as NGG for the Cas9 protein where N is any nucleotide.[16]

A study conducted by Yang et al. deleted the polyQ domain of *mHTT* using CRISPR in both the WT and mutant copy of HEK293 cells as well as mice.[88] Four guide RNAs were designed to target the DNA regions that flank the CAG repeat in exon 1 of human *HTT*. Reduction of mHTT was seen to alleviate reactive astrocytes while other proteins studied in neurodegenerative disease were unaffected by the treatment. Since CRISPR is able to permanently eliminate expression, this treatment offers long-term benefits as compared to previously used RNAi methods, which require constant administration. However, the efficiency of the mHTT reduction varied among individual mice tested.[85] Further, the loss of the wild-type HTT is undesirable and has been shown to have negative effects in mice.[85]

However, in these non-allele-specific approaches discussed above, the effects of lowering wild-type HTT production must be considered, as the function of HTT is not fully understood yet, particularly in adults. Complete inactivation of *HTT* in adult mice elicits neurodegeneration, and the long-term survival of neurons in the hippocampus was significantly diminished by the loss of HTT.[17,89] Conditional gene inactivation that results in an 84% reduction in HTT protein in adult mice triggers degeneration of neurons and contributes to abnormalities in neurogenesis.[90] HTT is necessary for early development, as a knockout of the HTT gene in mice caused the mice to die of acute pancreatitis at about two months of age.[91] Notably, in this same study, a knockout of HTT was done in adult mice with no change in neuronal survival or behavioral or movement phenotype, showing the function of HTT is at least partially age-dependent.

Allele-specific Approaches

I. RNA-Based

Single-stranded siRNA (ss-SiRNA) inhibits translation by recruiting argonaute, AGO2, for mRNA degradation via the RNAi pathway.[92] Yu et al. successfully downregulated mutant HTT mRNA while maintaining relatively high wild-type mRNA levels using an ss-siRNA RNAi system. In order to achieve discrimination between healthy and mutant transcripts, the group targeted the CAG expansion itself using an anti-CAG ss-siRNA.[93] The expanded mutant allele allowed for more siRNA binding events per transcript compared to the wild-type mRNA, leading to less reduction in wild-type mRNA. While this technique is not completely allele-specific, it is worth mentioning for its allele discrimination capabilities. Several cell types with different CAG repeat lengths were tested and a specificity of 29 CAG repeats was achieved in the cell line with the most similarity between the two transcripts. In this extreme case, 6 binding site events per

mutant mRNA occurred compared to 3 per healthy transcript.[93] This resulted in lower levels of mHTT while preserving mRNA levels in the HD patient-derived fibroblasts.

II. SNP Haplotypes Combined with Traditional CRISPR-Cas9 Technology

The only universal difference between healthy and disease alleles is the CAG expansion (Figure 1), which cannot be easily manipulated to achieve allele-specific silencing or excision. This is because the tools used cannot necessarily discriminate between the two CAG tracts because it would require specificity up to distinguishing between ~35 CAG repeats and above, which corresponds to about 105bp specificity which is not achievable using current methods. Additionally, there are other genes that also have repetitive CAG tracts that would also be affected by targeting the expansion itself.[1] As a result, less direct ways to target the mutant allele have been employed which make use of the presence of single nucleotide polymorphisms (SNPs) within the gene.

Chao et al. built a website that includes descriptions of various SNPs (including chromosome position and sequence), definitions of certain haplotypes of the HTT gene (16 variations), and the genotype data available: https://chgr.partners.org/htt.haplotype.html.[94] Many patients may fall under the identified haplogroups, allowing for the potential for a treatment approach utilizing the three most common SNPs to treat around 80% of European patients.[94] When utilizing all of the genetic variations from this study, they found that the CAG-expanded chromosome repeat could be isolated in 95% of European patients. In order to apply this concept to the majority of HD patients, rather than those of only European descent, haplotypes present in subject-derived fibroblasts, induced pluripotent cells, and embryonic stem cells should be examined to create a method of allele-specific silencing of the *HTT* gene.

Additionally, out of the 45115 SNPs listed in the NCBI SNP database associated with the *HTT* gene, 89.7% are intronic, which limits the potential for targeting coding sequences to only 10.3% of exonic SNPs.[95] A potential challenge is screening patients for particular SNPs and determining which allele the SNP is present on, which can be accomplished through a sequencing technique called phasing.[96] For patients who do not fall under the most popular haplogroups, such as many Asian populations, individualized approaches would have to be applied and tested for efficiency and safety, which could be a considerable task.

Because the mutation that causes HD involves expanded CAG repeats that contain a greater number of nucleotides than the Cas9's gRNA construct can recognize completely, indirect methods have to be used to specifically target the disease allele outside of the mutation. With the Cas9 system, the most promising method has been the use of SNPs within the disease allele to allow for allele discrimination based on the creation or destruction of a PAM sequence by a particular SNP, leaving a PAM solely on the disease allele at a given location.[97] The CRISPR-Cas9 system has been used to achieve silencing of the mutant *HTT* gene through heterozygous PAM sites in patient-derived fibroblast cells, significantly reducing expression of the mHTT protein.[22] In this approach, two SNPs and two cut sites were required to excise large portions of the gene, including the promoter and exon 1.[22] Similarly, the CRISPR-Cas9 system was able to selectively target the disease allele and reduce mHTT expression in the neural cells of the BACHD mouse model using a similar approach requiring a single SNP and a shared target site in an intronic region.[98] Both of these approaches require at least two target sites in the genome, which increases the risk of off-targeting. Additionally, the success of these approaches relies on SNPs within both the promoter region and just shortly after exon 1, which significantly limits the targetable SNPs, as there are 67 exons in the gene. A more robust approach would

allow for the use of SNPs outside of only the promoter and first intron, and would ideally only require 1 target site to reduce off-targeting.

Chapter 3: Materials and Methods

Materials

I. Cell Lines

Six different cell lines were used in the western blot experiment: ReNcell VM, the neural progenitor cell line, was purchased from Sigma Aldrich. HGFDFN168 is a fibroblast cell line from a healthy 40-year-old male procured from the Progeria Research Foundation. HGFDFN167 is a fibroblast cell line from a 10-year-old progeria patient obtained from the Progeria Research Foundation. D0 is a neural progenitor cell line derived from ReNcell VM on the first day of the 14-day differentiation protocol. D14 is the same cell line at the end of the 14-day differentiation protocol. HD fibroblast cell lines from a 50-year-old male patient heterozygous for HD (HD1) with a disease allele with 44 CAGs and a healthy allele with 17 CAGs and a 23-year-old female patient homozygous for HD (HD2) whose HTT alleles contain either 50 or 40 CAG's were purchased from the Coriell Institute with catalog numbers GM01169 and GM04857 respectively.

D0 cells underwent the ReNcell VM Human Neural Progenitor Cell Line differentiation protocol. Following the Coriell Institute protocol for fibroblast culture and subculture, fibroblasts were cultured in T25 flasks with fibroblast growth media consisting of EMEM and 15% FBS and then incubated in a VWR symphony incubator. A Zeiss observer A1 microscope was used to observe cell attachment. Confluent cells were split and counted following the same protocol using 0.53 mM EDTA in HBSS, 0.04% trypsin in 0.53 mmM EDTA in HBSS, new growth media, and an Eppendorf centrifuge 5424.

II. Antibodies

There were two different HTT antibodies used in the Western blot experiment. The primary targeting wild-type HTT (wtHTT) was the Huntington Polyclonal Antibody (PA1-003) in WB. The PA1-003 primary targeting wtHTT was purchased from ThermoFisher Scientific through Invitrogen and sourced from rabbits. It recognizes SDPAMDLND (544-552) residues of Human HTT and yields an approximately 62 kDa product. The PA1-003 antibody targets a cleaved protein product commonly observed in cell types. The PA1-003 has a concentration of 0.4 mg/mL and was stored in a PBS buffer with 1 mg/mL BSA. PA1-003 antibody has a 1:1000 dilution when used (10 μL: 5 mL).

The primary targeting mutant HTT was 1C2 (MAB1574). The MAB1574 primary targeting wtHTT was purchased from Sigma Aldrich and sourced from a mouse. It targets a polyglutamine stretch of HTT (varies in kDa per person). The MAB1574 was stored unpurified in ascites fluid and required a 1:500 dilution. The secondary antibody used was the Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L), purchased from Jackson ImmunoResearch Laboratories (111-035-003). It is a ~160 kDa divalent polyclonal antibody from a goat host that targets rabbits, stored in 0.01M sodium phosphate and 0.25M NaCl buffer, and stabilized by 15 mg/mL Bovine Serum Albumin (BSA). The secondary antibody was diluted 1:5000 in 1% milk in TBST before application to the membrane.

III. Gels

The Mini-PROTEAN TGX (Tris-Glycine extended) 7.5% Precast Protein Gels were purchased from Bio-Rad Laboratories. They are used for the separation of polypeptides fewer than 30 kD in length. The gel dimensions (width x length x thickness) in centimeters are 8.6 x 6.7 x 0.1. The cassette material is Styrene copolymer, and the comb material is Polycarbonate.

The gel contains a 10-well comb with a volume of 30 µL. Agarose gels were prepared at 1% agarose.

IV. Buffers

For the western blot experiments, multiple buffers were used. The sample buffer was prepared using 50 µL of Bio-Rad **β**-mercaptoethanol and 950 µL of Bio-Rad Laemmli Sample Buffer. The 1x Tris-Glycine SDS running buffer was prepared with 500mL 10x Tris-Glycine, 5g SDS, and 4.5L ddH_2O. The transfer buffer was prepared using 200mL of Bio-Rad Trans-Blot Turbo 5x Transfer Buffer, 600mL of nanopure water, and 200mL of ethanol. The blocking buffer solution used consisted of 2.5 g of dry milk that was added to 50 mL of Tris-buffered Saline with Triton, which was then stirred and dissolved.

For cDNA synthesis for the RT-qPCR experiment, the iScript Select cDNA Synthesis Kit was used according to the provided Bio-Rad protocol. For qPCR, iQ™ SYBR® Green Supermix (Bio-Rad 170-8880) was used according to the provided Bio-Rad protocol.

V. Transfer system

For the western blot experiments, the Biorad Trans-Blot Turbo Transfer system was used to transfer protein from gels to a nitrocellulose membrane. The transfer ran at 25V for 15 min with a current of 1.3A.

VI. Visualization and Analysis

The gel transfer system machine used was an ECL Plus Western Blotting Detection System (GE Healthcare catalog #RPN2132) from Bio-Rad Laboratories where ECL substrate solution was used. For the visualization of the western blot, a BioMaX Cassette using BioMax XAR film was used and the exposure solution was prepared according to the Bio-Rad Clarity

Western ECL Substrate protocol. Seven mL of exposure solution (0.1 mL solution/cm^2 of membrane) was prepared by mixing both substrate kit components in a 1:1 ratio.

VII. Primers

The following primer sequences were used in the RT-qPCR runs.

Forward: 5' CTCTGGTGTCAGATACTGCTGC 3'

Reverse: 5' CTCCTCTTCTCCAGACATCTGG 3'

Primers were purchased from Integrated DNA Technologies (IDT). IDT's primer design process includes several tools used to identify oligonucleotide properties such as melting point, hairpins, dimers, and mismatches, as well as dilution volumes. These tools also identify any on- and off-targeting potential of the sequences. The forward sequence has a melting point of 57.4℃ and the reverse sequence has a melting point of 56.3℃ for an amplicon of 130 bp.

VIII. Nucleic acid quantification

For quantification of total RNA and DNA in solution, a Nanodrop 2000 spectrophotometer from Thermo Scientific was used to measure absorbance. For quantification of DNA with PCR reactions, the Biorad CFX Connect Real-Time PCR Detection System was used.

Methods

I. Aim 1: Selection of PAM Creating SNPs

The first aim was to quantify the compatibility of known *HTT* SNPs with prime editing towards achieving allele specificity. Naturally occurring SNPs within many genes are reported in The National Center for Biotechnology Information (NCBI) Database of Single Nucleotide

Polymorphisms (dbSNP). The search was initiated for SNPs within the *HTT* gene that would result in the presence of a heterozygous PAM sequence for prime editing. The establishment of a heterozygous PAM sequence would allow for allele specificity in prime editing targeting. Subsequently, the search results provided several hundred pages of single nucleotide variations that were of interest, but the results were narrowed further by filtering for only coding sequences. Each SNP is verified to be either located in an exon or very close to an exon before the single nucleotide variation is studied. As the PAM sequence needed is a 'GG' or 'CC', each potential SNP was assessed to confirm whether it replaced an existing different nucleotide with guanine (G) or cytosine (C) or vice versa in the genomic DNA. After observing the flanks, each SNP was identified as a potential candidate, if a 'GG' or 'CC' was created or destroyed by the single nucleotide variation. These SNPs were all compiled into one location for future study.
Using the compiled SNPs, a utility program (CHANGE Editor) was designed to produce a pegRNA for a given PAM creating or destroying SNP. The program requires an input of DNA sequence with its corresponding SNP labeled with an 'R' and base pair mutation and provides the corresponding pegRNA sequences. The program reports sequences 5' to 3' that correspond to the correct DNA strand, especially when the PAM generated is on the reverse strand.

II. Aim 2: Protein Detection and Analysis

The second aim was to determine and compare the levels of wild-type and mutant HTT protein expression in different cell types. To this end, western blot analysis was performed with cell lysates from cultured samples. To the cell lysates, ~50uL of sample buffer per 5 million cells was added and mixed. Samples were then boiled at 95°C for 5 minutes and subsequently allowed to cool for 5 minutes at room temperature. 10uL of boiled samples were then transferred to the wells of a 7.5% Tris-Glycine gel (Mini-PROTEAN TGX Gel, Bio-Rad) submerged in a running

buffer. As controls, additional lanes were included for cell lysate for later analysis with B-actin antibodies and for a molecular weight ladder (Bio-Rad Precision Plus Protein Standards Kaleidoscope #1610375) control. The gel was run with an applied voltage of 60V for 30 minutes and then increased to 120V and ran until the protein ladder reached the end of the gel.

The gel was then removed from the voltage source and placed on top of a nitrocellulose membrane and sandwiched with filter paper. The proteins in the gel were then transferred to the nitrocellulose membrane using a Bio-Rad Trans-Blot Turbo system using a current of 1.3A and voltage of 15V for 15 minutes. After transfer, the nitrocellulose membrane was isolated and washed briefly with TBS. The membrane was then soaked in blocking solution for 10 minutes. Following blocking, the membrane was cut to isolate regions based on which antibody would probe in that region of the gel. The membranes were soaked overnight at 4°C in 5mL of antibody solutions prepared with dilutions according to the manufacturer. The primary antibody solution was composed of the blocking solution, while the secondary antibody solution was 2% milk. After soaking overnight, membranes were washed three times with TBST. After washing, the membranes were soaked for 1 hour with appropriate secondary antibody solutions diluted as specified by the manufacturer in 1% milk in TBST. The membranes were then washed three times with TBST again. For visualization, the ECL Plus Western Blotting Detection System was used with substrate solutions prepared according to the manufacturer. The membranes were soaked in the exposure solution for one minute and then prepared with BioMax XAR films and exposed with the ECL system in BioMax cassettes. The resulting images' exposures were manually tuned to identify bands.

III. Aim 3: HTT and mHTT mRNA Quantification

The third aim of the team was to compare the amount of transcription of the wild type against that of the mutant alleles in various cell types. Reverse transcriptase quantitative polymerase chain reaction or RT-qPCR is one technique by which gene expression can be measured.

The following reagents are thawed at room temperature, vortexed, briefly centrifuged, and placed on ice: iQ™ SYBR® Green Supermix (Bio-Rad 170-8880), primer 1, primer 2, sterile water, DNA template. Two master mixes are prepared for this experiment. Master mix 1 consisted of solely SYBR Green and sterile water, which was then used to prepare master mix 2 with the added primers. The DNA template is then added to individual aliquots of master mix 2, before they are added to different wells in Multiplate® PCR Plates/Low 96-well Clear (Bio-Rad MLL9601). A three-stage temperature program is then performed on the plate according to Table 1.

Table 1. The stages of qPCR below were used with the corresponding temperatures and durations.

Stage	1	2	3			Dissociation Stage (4)		
Temp (°C)	50	95	95	58	72	95	60	95
Time (min)	2:00	10:00	0:15	0:15	0:30	0:15	0:15	0:15

Scientific Search Engines and Citation Management

In order to keep track of the numerous resources used to inform this project, a citation manager was implemented. The platform Zotero was adopted, and a team library was established

upon recommendation by the team librarian. Any relevant literature for the team was subsequently stored in separate folders, i.e. ‘Animal Models’ and ‘Stem Cell Techniques’.

Chapter 4: Results

Literature Based:

I. NGG PAM Altering SNPs in HTT Exons

We cataloged all reported SNPs within the first 40 exons (out of 67 total exons) of the *HTT* gene that create or destroy NGG PAM sequences, which is required for catalytic activity of Cas9 proteins. This includes PAM sequence construction or destruction in both the sense and antisense strands. Out of ~1600 reported SNPs in the NCBI’s SNP database, we identified 729 SNPs that created or destroyed a PAM sequence. The following is a google link to the SNPs compiled, which includes each SNP’s rs ID number, the specific mutation, the exon it is located in, and whether it creates or destroys a PAM sequence: https://docs.google.com/spreadsheets/d/1qa1plxnlqkmpSIgPZUZkfDsLhUq3a__7UzeMqS6W6aU/edit?usp=sharing. A brief list is also presented below in Table 2.

Table 2. The first 7 identified SNPs within the first exon that create or destroy a PAM sequence are listed.

SNP ID (rs#)	Reference Allele	Alternative Allele	Exon	PAM (NGG)
rs764460507	G	A,C	1	creates
rs587777899	A	C	1	creates
rs375917	C	A	1	destroys
rs61792465	A	C	1	creates
rs61792466	A	C	1	creates
rs76533208	A	C,G	1	creates
rs747806296	C	A	1	destroys

CHANGE Editor

We developed a utility program that designs pegRNA and reverse transcriptase template RNA for a given PAM creating SNP for use with prime editing. The program takes in the DNA sequence with its corresponding SNP labeled with an R along with the specific base pair mutation and outputs all of the corresponding pegRNA sequences. The program ensures that all output sequences are reported 5' to 3' and that the sequences generated correspond to the correct DNA strand in instances where the PAM generated is on the reverse strand (Figure 2).

(a)

```
PAM sequence: NGG
Insert sequence: ATCCATCCATC


rs2530594 A>G
GGCCGATCCGCCTGGGCGGCGGTGAGAGGCAGGGCCGGGAGCCGGGCCGCTGGGTTTGGGCCTGGCCCGCTCGCCGCAATATTGATGGCCCGTCAGTGCRG
CCCTGATTCCTGTGCTTTCAGTTAAAAGGTTTCTGTTGTTGTAGCTTATGCAGTTGCTCTGTTGCTATGGAAACGTGACATCAAAATGACGTTTCCCGT


rs192642753 G>C
TTCACAAAAATTGTAAGGGCTTGGCCAGGTGTGGTGGCTCACACCTGTAATCCCATCACTTTGGGAGGCTGAGGCAGGTAGATCACGAGGTCAGGAGATCR
AGACCATCCGGGCGTGGTGGCGGGCGCCTGTAGTCCCAGCTACTCAGGAGGCTGAGGCAGTAGAATGGCATGAACCCAGGAGGCAGAGCTTGCAGTGAG
```

(b)

```
Pam sequence was stored as: NGG
Insert sequence was stored as: ATCCATCCATC
SNP id: rs2530594
SNP mutation: A>G
Sequence:
GGCCGATCCGCCTGGGCGGCGGTGAGAGGCAGGGCCGGGAGCCGGGCCGCTGGGTTTGGGCCTGGCCCGCTCGCCGCAATATTGATGGCCCGTCAGTGCRG
CCCTGATTCCTGTGCTTTCAGTTAAAAGGTTTCTGTTGTTGTAGCTTATGCAGTTGCTCTGTTGCTATGGAAACGTGACATCAAAATGACGTTTCCCGT
SNP primer binding site sequence is: UGACGGGCCAUCA
sgRNA sequence is: AUAUUGAUGGCCCGUCAGUG
RT Template sequence is: GAUGGAUGGAU

SNP id: rs192642753
SNP mutation: G>C
Sequence:
TTCACAAAAATTGTAAGGGCTTGGCCAGGTGTGGTGGCTCACACCTGTAATCCCATCACTTTGGGAGGCTGAGGCAGGTAGATCACGAGGTCAGGAGATCR
AGACCATCCGGGCGTGGTGGCGGGCGCCTGTAGTCCCAGCTACTCAGGAGGCTGAGGCAGTAGAATGGCATGAACCCAGGAGGCAGAGCTTGCAGTGAG
SNP primer binding site sequence is: CAUCCGGGCGUGG
sgRNA sequence is: GCCACCACGCCCGGAUGGUC
RT Template sequence is: CUACCUACCUA
```

Figure 2. (a) Shows an example of input data with 2 SNPs. The input requires the name of the SNP, its point mutation, the DNA sequence around the SNP with at least 20 bases upstream of the SNP labeled with R. **(b)** Shows the example output for the input given**.** It reports all pegRNA sequences (primer binding site, sgRNA sequence, RT template sequence) 5' to 3'. Note that even though the same DNA sequence is to be inserted into the gene, the RT template is different for both of the SNPs because the second SNP in the input file creates a PAM sequence on the reverse strand, so the RT template for that SNP is the complement of the first's RT template.

II. Gene Targeting Therapeutic Approaches

Examination of literature and clinical trials identified a variety of approaches directly

targeting either genomic *HTT* DNA or mRNA transcripts, which are shown below in Table 3 (taken from our review article). They were categorized into non-allele-specific and allele-specific to distinguish between approaches that would also affect the expression of the healthy HTT gene.

Table 3. This table categorizes and provides a brief description of currently identified gene targeting approaches for Huntington's Disease.

Approach	Modality	Methods	Advantages	Limitations	References
Non-Allele Specific	**Small Molecules**	Small molecule splicing modulators alter splicing of pre-mRNA in favor of the functional protein. Oral delivery (potentially).	More accessible delivery; reversal is possible	Affects both WT and mutant alleles; difficult to selectively target *HTT*	(Doherty, 2017)
	RNAi	siRNAs pair with RISC to target and degrade mutant HTT mRNA. Intracranial delivery.	Effective reduction of HTT mRNA after a single treatment	Affects both WT and mutant alleles; invasive delivery; reversal not possible	(Boudreau et al., 2009) (Harper et al., 2005) (Franich et al., 2008) (McBride et al., 2011)
	ASO	ASOs pair with target mRNA and recruit RNase H to degrade mutant mRNA or RNA-DNA duplex. Intrathecal delivery.	Effective reduction of HTT mRNA; requires only a single drug	Affects both WT and mutant alleles; significant off-targeting effects; short effective time	(Scoles et al., 2019) (Di Fusco et al., 2019) (Datson et al., 2017)
	CRISPR/Cas9	gRNAs guide Cas9 to cut out and remove a desired sequence of DNA. Delivery via viral or non-viral vectors.	Permanent deletion of CAG repeat; only one treatment required	Varied efficiency; affects both WT and mutant alleles, possible off target effects	(Yang et al., 2017)
Allele Specific	**RNAi**	siRNA targeting CAG expansion. Allele discrimination based on increased rate of binding of long CAG expansion	Effective reduction of mHTT mRNA with limited effect on WT RNA	Off targeting on other genes with CAG expansions	(Yu et al., 2012)
	SNP-based CRISPR/Cas9	gRNAs guide Cas9 to cut out and remove a desired sequence of DNA. Requires at least two target sites and at least one targetable SNP. Delivery via viral or non-viral vectors.	Selective targeting of mutant allele; effective, permanent silencing of *HTT* gene	Increased risk of off-targeting effects; SNP specificity limits RNA-binding sequences	(Shin et al., 2016) (Monteys et al., 2017)
	ASO	ASOs pair with target mRNA and recruit RNase H to degrade mutant mRNA or RNA-DNA duplex. Intrathecal delivery.	Selective targeting of mutant allele,	Off-targeting effects: SNP specificity limits RNA-binding sequences	(Steven Hersch et al., 2017) (Butler et. al, 2015)

III. Review Article

During the pandemic, the team analyzed literature and synthesized it into a relevant review article that we were able to publish in Ageing Research Reviews in 2020. As of March 2022, the paper has been cited 6 times. An image of the title page is shown below in Figure 3.

Contents lists available at ScienceDirect

Ageing Research Reviews

journal homepage: www.elsevier.com/locate/arr

Review

Gene targeting techniques for Huntington's disease

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ABSTRACT

Huntington's disease (HD) is an autosomal neurodegenerative disorder caused by extended trinucleotide CAG repetition in the *HTT* gene. Wild-type huntingtin protein (HTT) is essential, involved in a variety of crucial cellular functions such as vesicle transportation, cell division, transcription regulation, autophagy, and tissue maintenance. The mutant HTT (mHTT) proteins in the body interfere with HTT's normal cellular functions and cause additional detrimental effects. In this review, we discuss multiple approaches targeting DNA and RNA to reduce mHTT expression. These approaches are categorized into non-allele-specific silencing and allele-specific-silencing using Single Nucleotide Polymorphisms (SNPs) and haplogroup analysis. Additionally, this review discusses a potential application of recent CRISPR prime editing technology in targeting HD.

Figure 3. The team published a review article in *Ageing Research Reviews* in 2020 and an image of the publication with the title and abstract is displayed.

Experiment-based

I. Western Blot Analysis

Western blot analysis was performed on cell pellets from non-HD cells using antibodies targeting both the wild type and mutant proteins. The D0 neurons produced a high intensity band between 50-75 kD when stained using the wtHTT antibody (Figure 4). This band was not observed for the same cell sample when stained using the mutant antibody. The healthy

fibroblasts produced a faint band in a similar location as the D0 band. Again, this band was not observed when the mutant antibody was used (Figure 4). The progeria and D14 neural cells did not produce a noticeable band on either the wildtype or mutant gel (Figure 4). The size of the product from the reaction was calculated to be ~53 kDa.

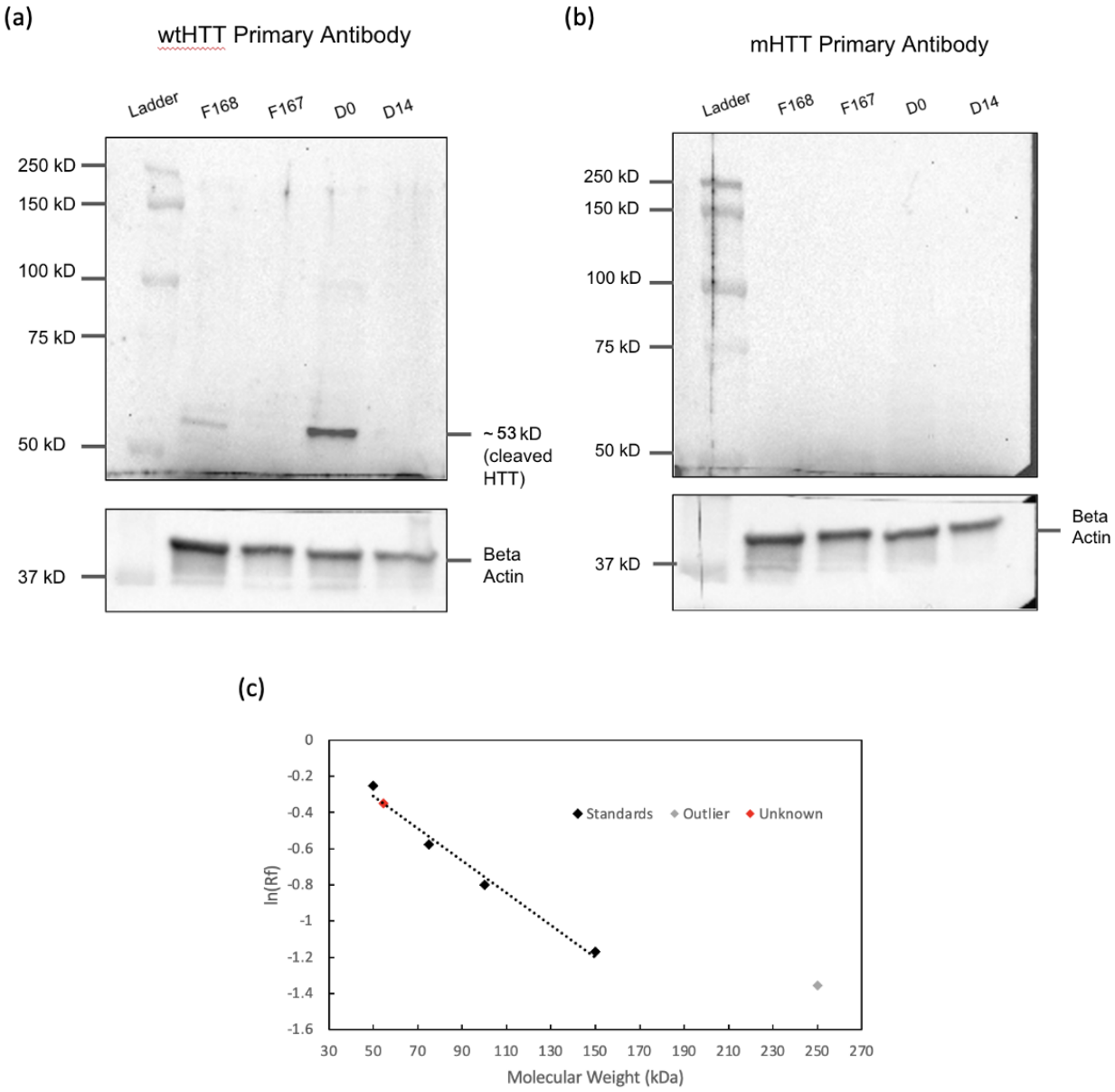
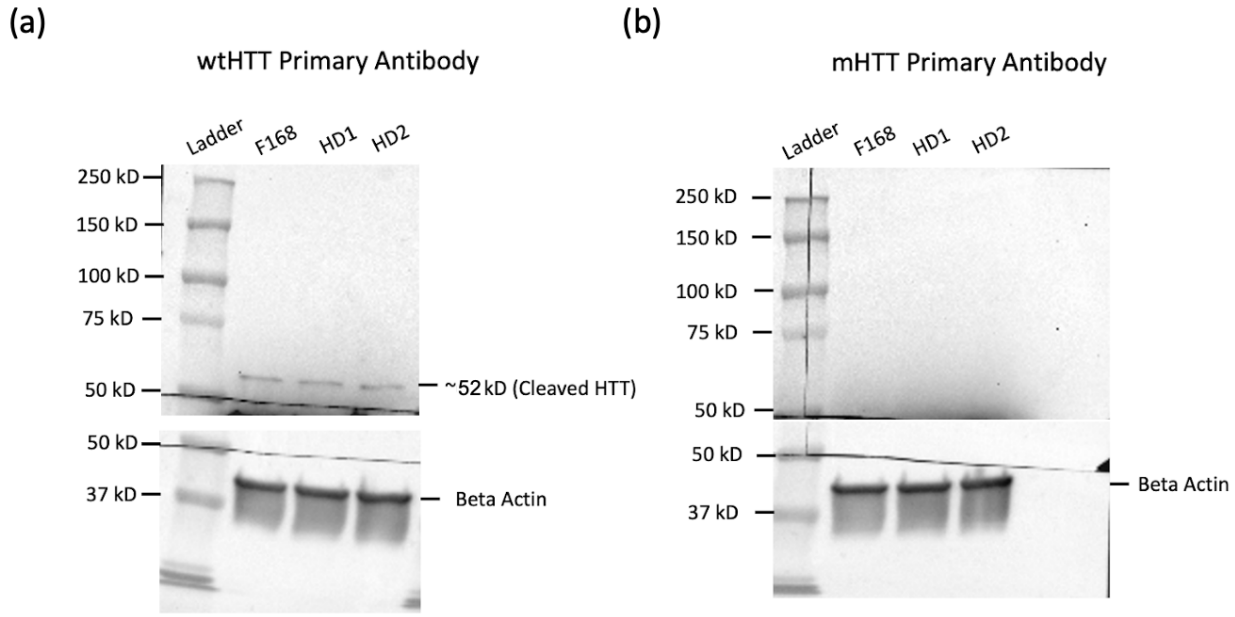


Figure 4. Western Blot images using healthy male fibroblast cells (F168), fibroblast cells derived from a progeria patient (F167), neural progenitor cells at day 0 of differentiation protocol (D0), and neural progenitor cells at day 14 of differentiation protocol (D14) with **(a)** membrane stained using PA1-003 targeting HTT cleaved product and **(b)** membrane stained using MAB1574 targeting polyglutamine stretch. The ladder bands were used to calculate the size of the observed products with the wtHTT antibody to be approximately 53 kD **(c)**.

Another Western Blot was performed with the HD fibroblast cell lines and a healthy fibroblast line and are shown below in Figure 2. The antibody targeting normal wild-type HTT again produced observable bands at around 52kD while the antibody targeting the polyglutamine stretch (mHTT) did not (Figure 2).



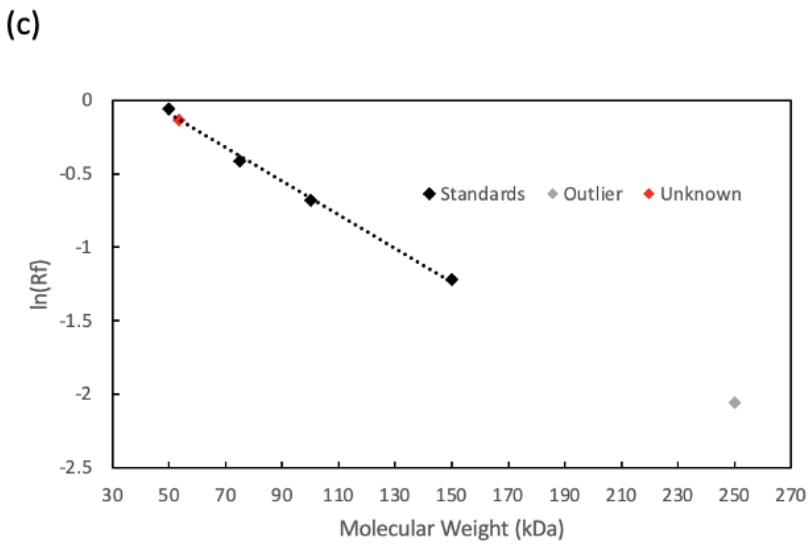


Figure 2. Western Blot images using healthy male fibroblast cells (F168), fibroblasts derived from a 50-year-old male patient heterozygous for HD (HD1), and fibroblasts derived from a 23-year-old female patient homozygous for HD (HD2) with **(a)** membrane stained using PA1-003 targeting wtHTT cleaved 62 kD product and **(b)** Membrane stained using MAB1574 targeting polyglutamine stretch. The ladder bands were used to calculate the size of the observed products with the wtHTT antibody to be approximately 52 kD **(c)**.

Using the HD fibroblast cell lines and a healthy fibroblast cell line, cellular mRNA was extracted for RT-qPCR. The corresponding RNA isolated from the healthy fibroblast cells had a concentration of 19.4 ng/uL whereas the RNA isolated from the HD fibroblast cells had concentrations of 48.7 ng/uL for the 50-year-old male and 53 ng/uL for the 23-year-old female. 200 ng of the healthy fibroblast RNA and 600 ng of the HD fibroblast RNA were used for cDNA synthesis. The resulting cDNA was diluted to 10ng/uL for qPCR and the resulting values for Cq are shown below in Table 4. The values for HTT Cq are fairly similar for all cell types.

Table 4. Table of Cq values from RT-qPCr experiment for healthy male fibroblast cells (F168), fibroblasts derived from a 50 year old male patient heterozygous for HD (HD1), and fibroblasts derived from a 23 year old female patient homozygous for HD (HD2).

Cells	HTT Cq	B-Actin Cq
F168	28.78±0.85	18.95±0.15
HD1	26.80±0.38	17.61±0.29
HD2	26.13±0.20	16.85±0.98

The values of Cq from Table 4 were analyzed further to determine the fold change in HTT expression between the HD cell lines and the healthy fibroblast cells. The fold change based on Cq data is dependent upon the efficiency of the polymerase during amplification of the specific sequence, which is unknown in this case. Nevertheless, calculations assuming 100% polymerase efficiency (E=1) determined an approximately 1.5 fold increase in HTT expression in the HD cells compared to the healthy fibroblast line while assuming 50% polymerase efficiency (E=0.5) determined an approximately 1.3 fold increase in HTT expression in the HD cells compared to the healthy fibroblast line (Table 5).

Table 5. Fold change in the amount of DNA present in healthy male fibroblast cells (F168), fibroblasts derived from a 50 year old male patient heterozygous for HD (HD1), and fibroblasts derived from a 23 year old female patient homozygous for HD (HD2) are calculated based on Cq values listed in Table 4. The change in expression of HTT is normalized against the change in expression of the housekeeping gene B-actin to yield the ΔΔCq value for both HD1 and HD2 cell lines. The fold change in expression of HTT between the HD cell lines and F168 is calculated to $(1+E)^{-\Delta\Delta Cq}$ for E values of 1 and 0.5 where E is the efficiency of the polymerase that in reality

may be anywhere from 0 to 1.

ΔCq (HTT-B-actin)			ΔΔCq (HD - F168)		HTT Fold Change (E=1)		HTT Fold Change (E=0.5)	
HD1	HD2	F168	HD1	HD2	HD1/F168	HD2/F168	HD1/F168	HD2/F168
9.195	9.285	9.832	-0.6370	-0.5466	1.555	1.460	1.295	1.250

The resulting DNA products from the qPCR reaction were run on a 1% agarose gel and are shown below in Figure 3. There are multiple bands observed in the healthy fibroblast cells and the 50-year-old male HD patient cells, but only a single faint band in the female 23-year-old cells.

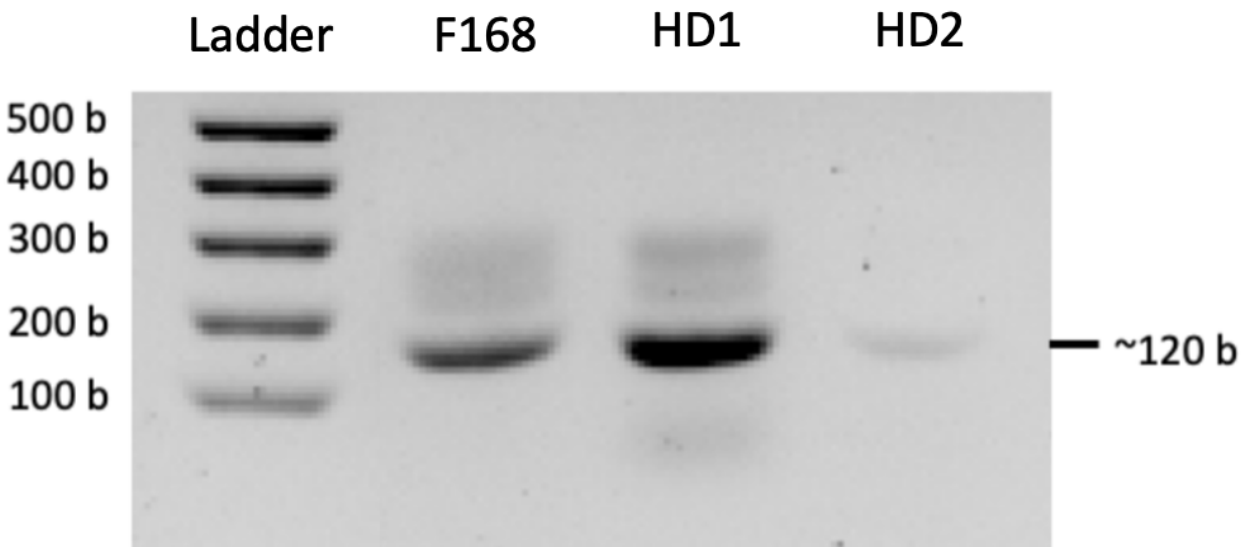


Figure 3. UV-exposed image of 1% Agarose gel with cDNA samples from RT-qPCR procedure with healthy male fibroblast cells (F168), fibroblasts derived from a 50 year old male patient heterozygous for HD (HD1), and fibroblasts derived from a 23 year old female patient homozygous for HD (HD2).

Chapter 5: Discussion

Literature Based:

The following discussion on the limitations of gene targeting techniques is an excerpt from our review article

I. Possible Limitations of Gene Targeting Techniques

There is discussion as to the role the mHTT gene has in altering development and whether the disease could be fully addressed through gene lowering techniques in patients. Barnat et al. investigated the tissue from human fetuses expressing mHTT to determine if there is a difference in development between a healthy fetus and those with mHTT.[99] Using antibody stainings, the researchers found that mHTT was concentrated in the apical endfeet of the tissue rather than spread through the basal region as seen in the healthy tissue. Additionally, it was determined that mHTT impairs endosomal secretion and Golgi trafficking in progenitors, as well as hinders the positioning of tight junctions.[99] Because of this effect on the junction complexes, it was hypothesized that the mutation affects movement through the cell cycle, which was confirmed by tracking expression markers that showed that mHTT lead to cells spending an elongated amount of time in the G_1 and G_2 phases and less time in the S phase transition.[99] These findings indicate that mHTT disrupts neurodevelopment and intervenes with the cell cycle, leading to more progenitor cells entering differentiation. This calls into question the efficacy of lower mHTT levels in adults as the brain is affected by the disease developmentally and counteracts the convention that mHTT is a gain-of-function mutation, pointing that the path to degeneration may be more complicated and less straightforward than it seems.

In addition, these techniques for reducing mHTT in neural cells may not be able to fully address symptoms of HD elsewhere in the body. Recent studies have found that gut dysbiosis is

one of the symptoms of HD that may disrupt motor and psychological functions.[100–102] In two studies, the microbiome composition of male HD mice was found to be significantly different compared to wild-type controls, while in females, there was little to no notable difference.[100,102] In HD mice some bacteria were found at unusually high levels, impacting gut permeability and transit time as well as motor control and cognitive functions, including memory performance, processing speed, and attention.[101,102] Lots of work suggests such disruptions in microbiota may lead to metabolic and psychiatric diseases that influence functions of both the brain and the gut.[103–109]

It is also unclear whether the reduction in toxic protein aggregation in a disease like HD would be able to correct manifestations of the disease. In a recent study, immunotherapy techniques were applied to target insoluble tau protein that is related to neurodegenerative diseases.[110] An anti-tau antibody, Gosuranemab, was used to bind to extracellular tau protein found in the cerebrospinal fluid (CSF) to prevent tau aggregation and promote tau depletion.[111,112] Its effects were evident in animal models of primary and secondary tauopathies, such as Progressive Supranuclear Palsy (PSP) and Alzheimer's disease (AD), respectively.[110] Clinical benefits associated with Gosuranemab appear to be quite limited in AD and Parkinson's disease patients.[110]

More recently, prime editing technology was applied to target progerin, another toxic, dominant-negative mutant protein that accumulates in Huntington Gilford Progeria, which is a rare form of accelerated aging diseases. Encouragingly, around 20-60% correction of the disease-causing mutation across various tissues improved vitality and greatly extended the median lifespan of a progeria mouse model.[113] This study shows the potential of prime editing as a possible treatment for progeria and other genetic diseases.

The following discussion on the potential of prime editing is an excerpt from our review article

II. The Potential of Prime Editing

Prime editing, developed by Anzalone et al., is a new version of CRISPR-Cas9 that allows the applications of gene editing to extend beyond inducing gene correction via targeted double-stranded breaks (DSBs) and homology-directed repair (HDR).[18] With prime editing, researchers are able to "search and replace" specific DNA nucleotides to result in much more controlled, precise editing than the original CRISPR-Cas9 system is able to achieve and with less damage to the DNA.[18,19] Prime editing involves both a prime editor (PE) of different variations and a prime editing guide RNA (pegRNA) which together form the PE:pegRNA complex. The prime editor is further composed of a Cas9 nickase fused to reverse transcriptase. The Cas9 nickase is similar to the Cas9 from the original CRISPR-Cas9 machinery, except the altered Cas9 nicks one strand of DNA rather than causing a double-stranded break. As a result, DNA repair can be more precisely controlled, as studies show that repair of DSBs leads to large deletions and complex rearrangements, such as unrecognized recombination events, that could have pathogenic consequences. The pegRNA, with a size of >100 nucleotides, is a variation of the ~20 nucleotide single guide RNA (sgRNA) used in CRISPR-Cas9. The process of prime editing involves the Cas9 nickase nicking the desired site, exposing a 3' end which the reverse transcriptase can be used alongside designed RNA sequences to reverse transcribe a short new sequence directly into the DNA. As a result, both strands have been edited without inducing a double-stranded break.

One major area of investigation when comparing prime editing to CRISPR-Cas9 is prime editing's ability to reduce off-targeting effects and maintain accuracy.[19,113] While prime editing still requires a sgRNA and PAM sequence to maximize on-target efficiency, its sgRNA, or

pegRNA, is modified to require additional hybridization steps that reduce off-targeting. Specifically, prime editing requires complementarity between the target DNA and the pegRNA spacer for the initial binding of Cas9, complementarity between the target DNA and pegRNA PBS to initiate reverse transcription of the pegRNA template, and complementarity between the target DNA and the reverse transcriptase product for flap resolution. After treating cells with either prime editors and pegRNA or Cas9 and pegRNAs, the additional prime editing hybridization steps allow for significantly reduced off-targeting effects. These are notable and promising results that suggest a potential solution to reducing off-targeting in gene editing without compromising efficiency.

III. Future Applications of Prime Editing to HD

The most direct applications of prime editing we have conceived require the presence of endogenous SNPs within a patient's *HTT* gene that results in a heterozygous PAM sequence located on the mutant allele. With a PAM sequence solely on the mutant allele, allele discrimination with the prime editing system can be achieved. Even with the about 700 PAM-altering SNPs we identified within the first 40 exons of *HTT*, most endogenous SNPs are located in non-coding regions. This is partially a result of the noncoding regions of most gene encoding DNA sequences being larger than the coding region and also a result of the fact that SNPs within a noncoding region may be less evolutionarily deleterious than in coding regions. With this in mind, having an approach that is compatible with SNPs located in a promoter, intron, or other untranslated regions would be highly desirable.

If a heterozygous PAM sequence is located in a promoter region, targeted short insertions or deletions of specific sequences could potentially reduce transcription significantly, which would correspondingly reduce mHTT levels. If a heterozygous PAM sequence is located within

an intronic region, using prime editing to strategically introduce erroneous splice sites or delete natural splice sites could lead to frameshifts that would introduce early stop codons. Any approach that introduces early stop codons is likely to trigger cellular nonsense-mediated decay, which would degrade the mRNA transcript and effectively block translation into mHTT protein. If a heterozygous PAM sequence is located within a 5' or 3' UTR, insertion or deletions of short sequences within those regions could potentially reduce mRNA stability to subsequently reduce the production of mHTT protein. If a heterozygous PAM sequence is located within a coding region, prime editing can easily be used to directly insert a sequence of stop codons into the coding region. This would likely result in nonsense-mediated decay of the mRNA transcript, which is particularly desirable because expression of the protein translated from the first exon of mHTT alone may be sufficient to cause disease phenotypes.

Given that many patients will have different sets of SNPs within the *HTT* gene, clinical applications of any of these techniques would require knowledge of a patient's specific genotype, which would be a logistical barrier. The previous research on known HD haplogroups makes SNP-based approaches more applicable to broader groups of individuals, but SNP-based approaches may never be applicable to all HD patients. In such cases, therapeutic approaches other than prime editing may be better suited, such as non-allele-specific downregulation of HTT and mHTT together. Even with SNP-based limitations, investigations into the different approaches to silence mHTT expression using heterozygous SNPs as described would be worthwhile. This is supported by the fact that the therapeutic inactivation of a mutant allele extends past HD alone to any dominant disorder that is not a result of haploinsufficiency.

Experiment-based:

I. mHTT Expression in Different Cell Types

Western blot analysis indicated the presence of HTT expression in healthy fibroblasts and fibroblasts derived from HD patients, which was expected (Figures 4 & 5). The bands resulting from Western blot analysis indicated that the amount of protein in healthy fibroblasts compared to disease fibroblasts were similar (Figure 5). Precise quantitative measurements of the relative abundance of the HTT protein are not achievable through Western blot analysis alone, but quantification of mRNA levels in the different fibroblasts confirmed somewhat similar levels of mRNA expression in both cells (Table 4). Assuming a polymerase efficiency of 50%, an about 1.3-fold increase in HTT mRNA expression was calculated between HD fibroblasts and healthy fibroblasts (Table 4). Translation of HTT protein is largely influenced by the amount of mRNA, but the exact amount of protein may be influenced by other factors such as differential regulation of ribosome machinery or translated protein stability. With only one RT qPCR experiment performed, quantitative analysis of differences in expression between different cell types is unreliable, even with triplicate measurements. We have not found any indication to suggest that the expression would be increased or decreased in fibroblast cells with HD or without HD, but it is known that HTT is involved in gene regulation, so further investigation of expression with more samples from different populations would be worthwhile.

Western blot analysis indicated the presence of HTT expression in neural stem cells at day 0 of differentiation, but not at day 14 (Figure 4). This suggests that the expression of HTT is modulated during the process of directing a progenitor cell to a differentiated state. Additional experiments to trace both the protein and mRNA expression of HTT at different days during

differentiation would be useful in understanding specifically when and potentially why HTT expression differs during differentiation.

Western blot analysis also indicated the lack of HTT in fibroblasts from a progeria patient, which was not initially expected because a relationship between progerin or lamin A and HTT is not fully established. Past research shows, however, that progerin expression leads to downregulation of transcripts with repetitive sequences, such as the CAG expansion in the *HTT* gene.[20] This suggests that the reason HTT was not found in the progeria patient fibroblasts may not be due to specific interactions between HTT and progerin or lamin A, but rather just because of the repetitive nature of the mRNA transcript. Further investigations into this relationship would be worthwhile to see how this relationship may manifest in progeria patients.

Western blot analysis with the mHTT antibody failed to show bands in all cell lines. The mHTT antibody targets expanded polyglutamine stretches, which are present in both wild-type and mutant HTT alleles. The reduced number of CAGs in wild-type alleles leads to less binding of the antibody, which provides the basis of selectivity of the antibody between wild-type and mutant HTT. The antibody is expected to bind to the full protein, which is very large with a molecular weight of about 350 kD. The lack of visible bands in the cell lines without HD may be explained by the fact that their HTT alleles do not have expanded numbers of CAGs, but this does not explain the lack of bands in the two HD cell lines that contain alleles with expanded CAG repeats. The most likely explanation for the lack of mHTT observed in HD cell lines is that the protein was not successfully transferred onto the membrane from the gel with the transfer protocol used. Alternatively, it is possible that the commercial antibody did not work as described, which is not uncommon. To examine this further, performing the experiment again with either increased voltage or duration during transferring to accommodate for the protein's

large size would be worthwhile. If the protein is still not observable through a Western blot after adjusting the transfer protocol, ordering alternative primary antibodies could be more successful.

Equity Impact

The Gemstone Honors Program strives for equity in research in order to reflect the diverse population of students and faculty, and allow for greater applications of data to multiple demographics of individuals. Team CHANGE advocates for equity by benefiting various populations through accessibility, representation, and awareness of treatments of Huntington's disease.

Huntington's disease affects three to seven in 100,000 people of European descent, whereas it is known to be less common among certain populations such as African, Chinese, and Japanese ancestry.[114] However, due to the genetic nature of the disease, Huntington's may adversely affect any population. Team CHANGE's account of SNPs from diverse demographics allows for extrapolation of the data to extend beyond those of European descent and provide a representation of a greater sample. Although HD may affect specific populations, the ultimate approach of the research focuses on individualized treatment, which innately allows for treatment of all individuals, regardless of race or any other trait. The Team's aim is to increase knowledge and accessibility of personalized treatment plans in healthcare, with a more holistic approach of addressing the root of the disease, rather than the symptoms.

In the realm of healthcare, Team CHANGE works towards increasing awareness of Huntington's disease, allowing for better diagnoses and subsequent treatment. The Team recognizes the socioeconomic gradient and its role in accessing gene-editing technology; and works towards making knowledge of gene therapies available through open access journals, minimizing a barrier to adequate health literacy. Through the review of various genetic therapies,

the Team has committed to making editing technology knowledge more accessible to the general population.

COVID Impact

The COVID-19 pandemic impacted the course of the team's research. In March of 2020, after team CHANGE finished the initial literature review, the team planned to progress into available laboratories for wet-lab research procedures. However, the University of Maryland, College Park closed all of campus, including all laboratories and facilities that the team was planning to meet and conduct experiments in. Due to the type of research the team had planned to complete, a lot of new barriers were brought up in successfully following the procedures. In order to complete new research, the team met up remotely and continued to work towards a common goal, with a new perspective on the objective of the research. Throughout the pandemic, team CHANGE worked diligently to compose a review paper on various gene targeting techniques and their application in combating Huntington's Disease. As team CHANGE shifted the design of the research questions, the review paper was published, ultimately creating the focus of the current plan as a largely literature-based project. When COVID restrictions were minimized, the team was able to complete some wet-lab experiments but did not have ample time to complete the original experiment with prime editing. The COVID-19 pandemic limited the direction of team CHANGE's research, however, it did not impede the success of the team's outcome.

Chapter 6: Conclusion

A cure for Huntington's disease has yet to be achieved, but recent gene therapy attempts show great promise. As CRISPR technology is constantly refined and new technologies such as

prime editing are developed, applications to HD and the possibility of patient-specific treatments become more accurate. With the development of prime editing technology, short sequences can be more efficiently incorporated into DNA. This can be particularly useful for altering post-translational modifications that can potentially aid cellular machinery to degrade the mHTT.

Our literature review identified multiple SNPs that would enable allele-specific targeting with prime editing. This provides a viable route for future research and may provide a means of Huntington's treatment in the future. The techniques used to target mHTT can be applied to other diseases as well, particularly the use of SNPs to achieve allele-specific silencing.

The Western blot and RT-qPCR results suggest that HTT is expressed similarly between healthy and diseased fibroblasts but is not highly expressed in progeria patient fibroblasts and at certain points during differentiation of neural stem cells. Despite the lower expression level of HTT in fibroblasts, there is an adequate expression to be used for proof-of-concept gene editing studies. Future work into the application of prime editing as a tool to correct HD is warranted because of immense potential clinical applications for not only HD but many other human diseases.

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