


Silencing of the Mutant Huntingtin Gene through CRISPR-Cas9 Improves the Mitochondrial Biomarkers in an In Vitro Model of Huntington's Disease

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Abstract

During the 25-year history of the American Society for Neural Therapy and Repair (ASNTR) there have been several breakthroughs in the area of neurotherapeutics, which was the case during the 2014–2015 year when one of us (GLD) had the privilege of serving as its president. During that year, the use of a newly developed gene-editing tool, the CRISPR-Cas9 system, started to skyrocket. Although scientists unraveled the use of “clustered regularly interspaced short palindromic repeats” (CRISPR) and its associated genes from the Cas family as an evolved mechanism of some bacterial and archaeal genomes to protect themselves from being hijacked by invasive viral genes, its use as a therapeutic tool was not fully appreciated until further research revealed how this system operated and how it might be developed technologically to manipulate genes of any species. By 2015, this technology had exploded to the point that close to 2,000 papers that used this technology were published during that year alone.

Keywords

gene therapy, neurodegeneration

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Our lab became interested in CRISPR-Cas9 technology in 2014 as potential means of knocking down the transcription

or translation of the mutated gene that caused Huntington's disease (HD). The mutated gene in HD causes the progressive degeneration of neurons in the brain, leading to cognitive, motor, and psychiatric dysfunction⁷. The mutation occurs in the short arm of chromosome 4, and the transcribed

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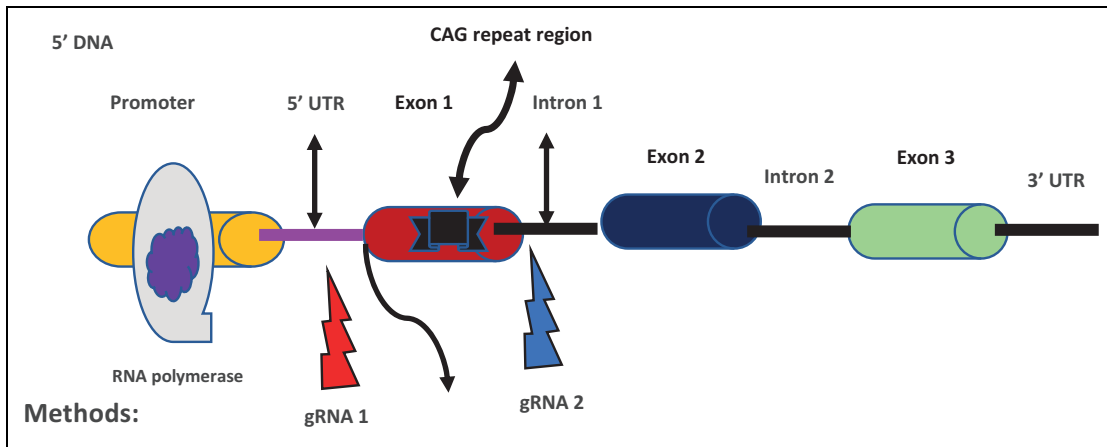


Fig 1. Schematic diagram of the targeted region on huntingtin gene. The target region in the mouse Huntington gene is the open-reading frame (uORF) of the 5'UTR and the exon1-intron 1 boundary.

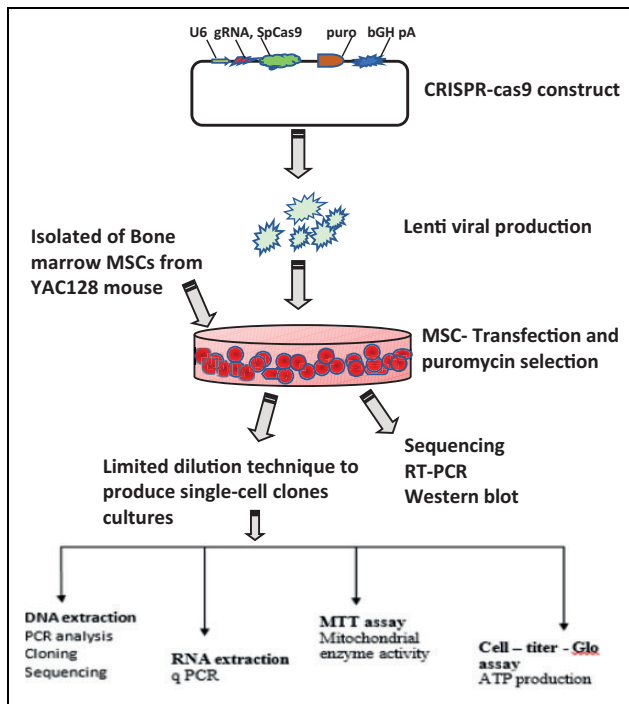


Fig 2. The methodology for CRISPR-Cas9 mediated silencing of the mHTT gene in vitro. Each of the two CRISPR-Cas9 gRNA constructs were administered to the bone-marrow-derived MSCs. The edited sequence of the gene was confirmed using Sanger dideoxy nucleotide sequencing method. The transcription of the mHTT was then analyzed with real-time polymerase chain reaction (RT-PCR). This was further confirmed using single-cell clone cultures that were prepared from CRISPR-treated MSCs using qPCR. We also performed MTT and Glo assays to analyze the mitochondrial function of the CRISPR-treated MSCs.

mutant huntingtin protein (mHTT) contains an abnormally long polyglutamate (CAG) domain, consisting of more than 35 CAG repeats. This increase in the size of the CAG segment leads to the production of an unusually long version

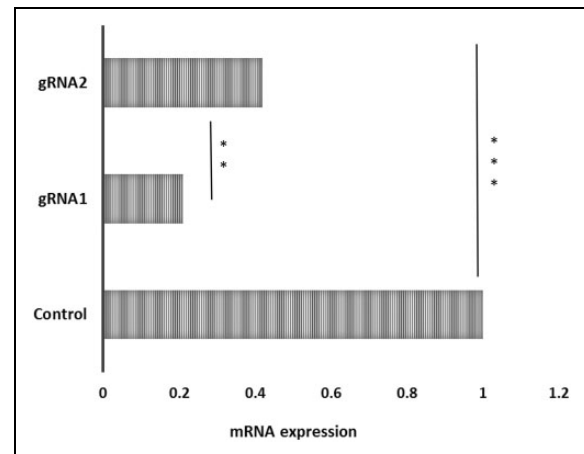


Fig 3. Levels of mRNA from the mHTT gene in treated BM-MSCs. A significant reduction of mHTT (mRNA) expression in gRNA1- and gRNA2-treated cells, relative to controls ($p < 0.01$).

of the huntingtin protein which undergoes inappropriate post-translational modifications and cellular dysfunction, resulting in cell death⁸.

Currently there are no effective treatments for HD, but the use of the CRISPR-Cas9 technology provides one of the most promising new approaches for inhibiting the progression of this devastating disease. The CRISPR system consists of the Cas9 protein, which acts like molecular scissors to induce a double-strand break in the DNA or mRNA, and a 20-base-pair guide RNA sequence that escorts the Cas9 to the genome of interest. When Cas9 is guided to the appropriate target it produces a double-strand break in the DNA or mRNA, the interrupted sequence attempts a self-repair mechanism that often results in non-homologous end joining (NHEJ). During NHEJ DNA repair process, different nucleotides can be inserted and deleted from the DNA strands, causing what are known as indel mutations. These

indel mutations can cause a frame-shift mutation which leads loss of function of the protein.

Work in our lab utilized the endogenous NHEJ mechanism and the resultant indel mutations as a means of knocking down the production of mHTT. We focused on the 5' untranslated region (UTR) of the exon 1 region of the huntingtin gene because of its importance in the production of the HTT. We targeted 5' UTR and exon1-intron boundary in the huntingtin gene with CRISPR-Cas9 plasmids that could interrupt the 5' UTR of the mRNA, thus reducing the translation of the mutant huntingtin gene (Fig. 1).

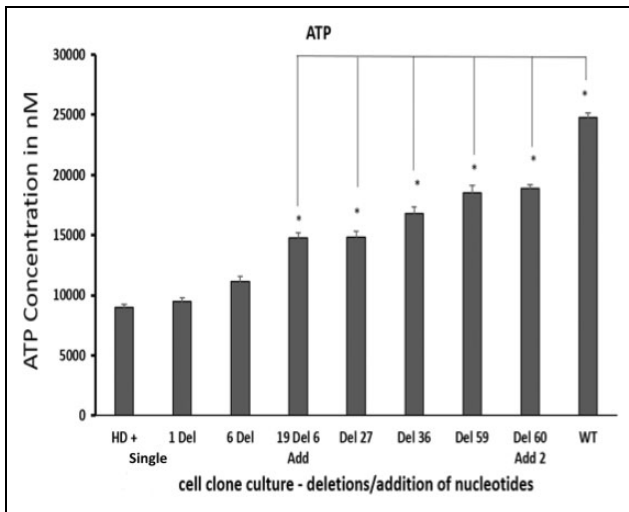


Fig 4. ATP levels in the single-cell clone cultures at various expressed levels of mHTT. The increased number of indel mutations resulted in a significant increase in ATP production (* $p < 0.001$).

Our experimental procedure is outlined in Fig. 2. We constructed two CRISPR-Cas9 plasmids with gRNAs to target the open reading frame (uORF) of 5' UTR and exon1-intron boundary. These gRNAs were designed by using MIT tool, allowing us to specifically target the human huntingtin (HTT) gene without affecting the mouse huntingtin (Htt) gene. We induced indel mutations using CRISPR-Cas9 methods on cultured bone-marrow-derived mesenchymal stem cells (MSCs) that were extracted from YAC128 mice (which contain the full-length human mHTT) by transfecting isolated MSCs from the YAC mice using a lenti-CRISPRv2 plasmid. The methods that we used in the study, such as gene cloning, viral production, isolation of MSCs from YAC 128, and transduction of the Lenti-CRISPR-gRNAs followed our previously established protocols⁹. The single-cell clone culture technique was adapted from standard protocols^{10,11}.

We measured the number of indel mutations and related this to amount of mRNA produced, mitochondrial functioning (ATP production), and cell viability. The mRNA expression and ATP levels were analyzed using a one-way analysis of variance, followed by Tukey's Honestly Significant Difference posthoc tests, when appropriate. We used regression analysis to measure the relationship of indel mutations with ATP production and cell viability.

After treatment with the CRISPR-Cas9, we found that the interruption of uORF through CRISPR-Cas9 causes the increased reduction of translation of mHTT when compared with the disruption of the exon1-intron boundary. It was observed that CRISPR-gRNA1 (UTR targeted) and CRISPR-gRNA2 (exon1-intron region) resulted in 79% and 58% reduction in mHTT production (Fig. 3).

From the sequencing results, we observed the pattern of the nucleotide addition or deletion (indel mutations) at the site of the double-strand DNA break. We selected the

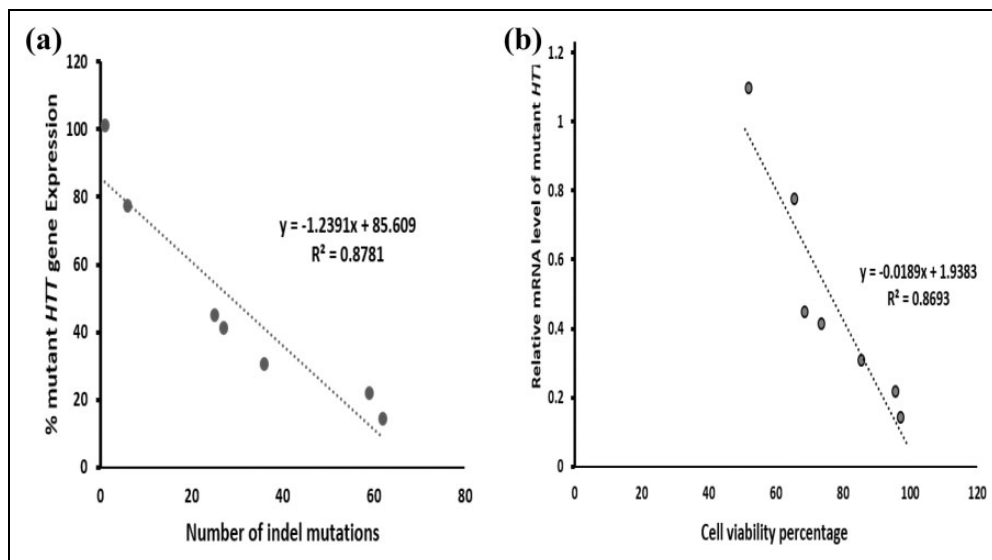


Fig 5. Relationship between mHTT expression with the number of indel mutations and cell viability. Linear regression analyses revealed a strong relationship ($R^2 = 0.8781$) between the indel number mHTT expression (a) as well as a significant relationship ($R^2 = 0.8906$) between mHTT and cell viability (b).

targeted cells located in the untranslated region upstream to the open reading frame and then separated and cultured them into single-cell clones to establish the relationship between the indel mutation number and levels of mHTT protein. In addition, we observed a significant increase [$F(8, 18) = 414.59$, $p = 0.0001$] in ATP production with respect to an increase in number indel mutations at the site of double-strand break (Fig. 4).

Finally, we found a strong relationship between the number of indel mutations and *mHTT* gene expression, $R^2 = 0.8781$ (Fig. 5a) and a significant correlation between mutant huntingtin levels and cell viability, $R^2 = 0.8906$ (Fig. 5b).

Our results provide a proof-of-concept that the use of the CRISPR-Cas9 system can induce indel mutations that reduce neuropathological markers in an *in vitro* model of HD. We are now looking at mechanisms by which mutant huntingtin decreases mitochondrial function and exploring how mitochondrial function might be restored with the decreases in mHTT. Also, further work is needed to show that the strategy we used is safe and efficient, *in vivo*, and can translate to clinical applications in humans. Given that our approach does not specifically target mHTT, the degree to which normal huntingtin protein is affected and its potentially harmful consequences need to be carefully studied. To this end, it is encouraging that a recent study by Yang and colleagues¹² showed that a similar, non-allele-specific approach using CRISPR-Cas9 knockdown of mHTT in HD140Q-knockin mice was effective and safe. As such, this technology may provide the first effective treatment for HD, validating the attention it received in 2015 as a major breakthrough in neural therapy and repair.

Ethical Approval

This study was approved by the Biosafety Committee and the Institutional Animal Care and Use Committee at Central Michigan University.

Statement of Human and Animal Rights

No humans or animals were tested in this study, only cells from YAC-128 mice were used.

Statement of Informed Consent

There are no human subjects in this article so informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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