Progress and Challenges in RNA Interference Therapy for Huntington Disease

Scott Q. Harper, PhD

Huntington disease is an incurable, dominant neurodegenerative disorder caused by polyglutamine repeat expansion in the huntingtin protein. Reducing mutant huntingtin expression may offer a treatment for Huntington disease. RNA interference has emerged as a powerful method to silence dominant disease genes. As such, it is being developed as a prospective Huntington disease therapy. Here I discuss the current progress and important remaining challenges of RNA interference therapy for Huntington disease.

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Huntington disease (HD) is an autosomal dominant neurodegenerative disorder affecting approximately 1 in 10,000 people. Patients characteristically develop motor, cognitive, and behavioral deficits in adulthood, although age at onset is variable and rare juvenile-onset cases occur. Symptoms commonly include chorea, lack of coordination, speech difficulties, diminished memory and problem-solving skills, mood swings, and depression. In later stages, patients often become wheelchair dependent and hypokinetic, lose self-care abilities, and eventually die of HD-related complications about 20 years after disease onset.

Huntington disease is caused by CAG trinucleotide repeat expansion in the huntingtin gene (HTT) (GenBank L12392.1). Unaffected individuals have 35 or fewer repeats on each allele, whereas patients with HD have at least 1 allele with 36 or more CAG repeats. Since CAG encodes for glutamine, pathogenic mutations result in an expanded polyglutamine repeat near the amino terminus of the HTT protein, which confers toxic gain-of-function properties, although some loss-of-function effects may also contribute to HD pathogenesis. Despite ubiquitous mutant HTT expression, striatal and cortical neurons are primarily affected. More recent evidence suggests that mutant HD proteins also cause glial dysfunction, supporting a complex pathogenic mechanism that may include both cell-autonomous and non–cell-autonomous disease contributions.

No cure for HD exists; current treatments are palliative and none modify the disease course. Since HD is an autosomal dominant disorder, patients are almost invariably heterozygous, possessing 1 normal and 1 mutant HD allele. Individuals with HD may benefit from therapies capable of suppressing the expression of mutant HTT. RNA interference (RNAi) has emerged as a powerful tool to reduce expression of any gene in a sequence-specific manner. As such, RNAi is a leading candidate strategy for treating HD by targeting mutant HTT messenger RNA (mRNA) for degradation.

THERAPEUTIC RNAi

RNA interference is an evolutionarily conserved gene-silencing mechanism. Small, noncoding, inhibitory RNAs (called microRNAs [miRNAs]) regulate gene expression via base pairing with target miRNAs. Nucleotide sequence complementarity between the guide strand of an miRNA duplex and a target mRNA helps recruit cellular gene-silencing machinery (RNA-induced silencing complex [RISC])
to specific mRNA transcripts. \(^6\) In general, incomplete pairing causes translational inhibition while leaving target mRNA levels unchanged. In contrast, perfect miRNA-mRNA complementarity (approximately 19-22 nucleotides) causes gene silencing through a distinct mechanism involving target mRNA degradation. Thus, sequence specificity is fundamental to RNAi, and the degree of complementarity between inhibitory RNA and target mRNA determines whether the miRNA will be silenced at all and, if so, which mechanism will be induced (translational inhibition vs mRNA degradation).

In the cell, genome-encoded miRNAs arise from longer transcripts and require 3 major processing steps to produce mature, RISC-associated miRNAs. \(^6\) Recent work has focused on understanding miRNA expression and processing. \(^6\) An important consequence of this growing knowledge has been the development of RNAi as molecular therapy to suppress viral infection or dominant disease genes. Designer RNAi molecules can be engineered to mimic natural miRNAs and used to suppress any gene. In general, there are 2 major types of artificial inhibitory RNAs: (1) in vitro synthesized, double-stranded small interfering RNAs (siRNAs) and (2) promoter-expressed short hairpin RNAs (shRNAs) or miRNAs that are produced intracellularly from plasmid or viral vectors (Figure). \(^7,8\) The siRNAs are structurally identical to mature, postprocessed miRNA duplexes and are typically immediately available to complex with RISC proteins after delivery to cells.

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**Figure.** MicroRNA (miRNA) biogenesis pathway. MicroRNAs are tiny, noncoding RNAs that arise from eukaryotic genomes ranging in complexity from single-celled algae to mammals. A long single-stranded primary miRNA transcript (pri-miRNA) forms intramolecular hairpin structures. Pictured here is human mir-30. The mir-30 antisense guide strand is shown in red text. In the nucleus, the pri-miRNA is trimmed to a smaller pre-miRNA by DROSHA and cofactor DGCR8. The pre-miRNA is then shuttled by exportin-5 (EXP5) to the cytoplasm and further trimmed to a duplex miRNA form by DICER. One strand of the miRNA duplex directs gene-silencing proteins of the RNA-induced silencing complex (RISC) to target messenger RNAs via base pairing. Engineered miRNA shuttles, short hairpin RNAs (shRNAs), and small interfering RNAs (siRNAs) enter the pathway at different steps.
although some require 1 processing event prior to RISC association. In contrast, shRNAs and miRNAs are first transcribed intracellularly from DNA templates delivered to target cells via viral vectors or plasmid transfection and must be processed by endogenous miRNA biogenesis pathways. Ultimately, both methods cause gene silencing but differ in persistence. The siRNAs are transient and long-term target gene suppression may require repeated administration, whereas shRNAs and miRNAs are more enduring and, if delivered via an appropriate viral vector, may permanently silence a target gene after 1 administration.

**PRECLINICAL RNAI THERAPY TRIALS IN HD RODENT MODELS**

Since 2005, several preclinical studies have shown that RNAi-mediated HD silencing improved HD-associated phenotypes in rodent models (Table). In the first published study, adeno-associated virus 1 (AAV1)–delivered shRNAs (shHD2.1) were used to knock down truncated mutant human HTT in HD mice. Intrastrial expression of AAV.shHD2.1 reduced hallmark HD-associated inclusions, significantly improved rotated motor performance, and partially normalized gait abnormalities in these mice. In a parallel study, AAV5 vectors were used to deliver 2 different human HTT-specific shRNAs to HD mouse striata. One shRNA (si-Hunt1) reduced mutant HTT mRNA expression and inclusions and improved hind-limb clasping, an indicator of neurological impairment. Moreover, striatal ppENK and DARP32 mRNA transcripts, which are reduced in human and mouse HD brain, were partially normalized in treated mice. Together, these studies demonstrated the first proof of principle that RNAi could be used as a potential HD therapy. Subsequent shRNA studies using different models, RNA sequences, and efficacy assessments (Table) showed similar neuropathological and/or motor improvements resulting from RNAi-mediated knockdown of mutant human HTT.

Later in 2005, beneficial effects of in vivo mutant HTT knockdown using chemically synthesized siRNAs were first demonstrated. In this study, lipid-encapsulated siRNAs were delivered in a single intraventricular infusion to 2-day-old HD mice. The HTT-targeted siRNA-HDExon1 was nearly identical to siHunt2 shRNA used in a previous study. Real-time polymerase chain reaction showed that mutant HTT transcripts were significantly reduced up to 7 days after treatment, but silencing was almost completely diminished 1 week later. This transient suppression is consistent with short-lived persistence of unmodified siRNAs in vivo. Despite this relatively small period of mutant HTT suppression, surprisingly long-lasting behavioral improvements were reported, including reduced hind-limb clasping and improved open-field behavior up to 14 weeks of age, significantly better rotarod retention times, diminished striatal inclusions at 8 weeks, and a 14% lifespan increase.

In a later study, chemically modified and cholesterol-conjugated siRNAs (cc-siRNA-htt) were used to improve in vivo siRNA stability and cellular uptake, respectively, without using potentially deleterious liposome encapsulation. The mouse model used in this study was nontransgenic; HD-like phenotypes were produced in adult wild-type mice following delivery of AAV1/8 vectors carrying expanded human HTT fragments. Co-injected cc-siRNA-htt reduced monomer levels and HTT protein aggregation 3 days and 2 weeks after treatment, respectively, and improved hind-limb clasping and beam walking motor deficits after 1 week. No longer-term improvements were measured. Lipid encapsulated siRNAs and cc-siRNAs were not compared. Both siRNA studies suggest that chronic or pulsed infusion of siRNAs may be used to effectively silence HTT long-term.
No studies listed in the Table used identical methods; different HD animal models, inhibitory RNAs, delivery strategies, and metrics to assess phenotypic improvements were used. However, fundamental similarities in each experiment support that RNAi may be an effective HD therapy should existing challenges be addressed.

**CURRENT CHALLENGES TO RNAi THERAPY FOR HD**

Therapy using RNAi for HD is promising and may someday prove beneficial to human patients. Still, several remaining questions or issues related to safety and in vivo delivery need to be addressed to further advance this technology.

The first is related to off-target effects. The power of RNAi lies in its sequence specificity, and in theory only the gene of interest should be affected. This is not always true in practice. Several studies demonstrated that RNAi can in some instances elicit nonspecific, off-target effects. These can arise from 3 major sources: immunostimulation, unintended sequence-based silencing of nontarget genes, and inhibitory RNA overexpression that disrupts natural miRNA biogenesis. Importantly, off-target effects were reported in 2 recent HD RNAi studies. In one, AAV.sHunt2 expression caused aberrant striatal gene expression profiles in wild-type mice owing to sequence-based unintentional silencing of an undefined transcription factor. Interestingly, no overt off-target effects were reported in 2 other animal models using identical or nearly identical inhibitory RNA sequences. These results suggest that RNAi treatment strategies should be validated for safety in multiple models.

The second is related to long-term HD suppression required? Since mutant HTT accumulation progressively causes HD and the mutant gene is constitutively expressed in patients with HD, long-term suppression may be required to treat the disorder. Viral vector–delivered shRNAs or miRNAs are now the best strategy to achieve long-term HD suppression. Indeed, numerous preclinical studies used viral vectors to indefinitely express therapeutic shRNAs or miRNAs in postmitotic neurons. The major drawback to this strategy currently is control. Once the vector is delivered, shRNAs and miRNAs cannot be turned off. Considering that off-target effects can occur and that long-term effects of artificial inhibitory RNA expression are unknown, an ideal therapeutic RNAi strategy should be adjustable so that the dosage could be decreased or stopped if untoward events should arise. One recent study showed the feasibility of conditional shRNA expression using doxycycline-inducible vectors in rat brain. Nonviral siRNA systems allow dosage control but require invasive, repeat administration if long-term HD suppression is necessary. In contrast, siRNAs are the best available tool if transient HD gene silencing proves beneficial in humans, as was reported in 2 HD mouse studies. 

The third is related to off-target effects. The power of RNAi lies in its sequence specificity, and in theory only the gene of interest should be affected. This is not always true in practice. Several studies demonstrated that RNAi can in some instances elicit nonspecific, off-target effects. These can arise from 3 major sources: immunostimulation, unintended sequence-based silencing of nontarget genes, and inhibitory RNA overexpression that disrupts natural miRNA biogenesis. Importantly, off-target effects were reported in 2 recent HD RNAi studies. In one, AAV.sHunt2 expression caused aberrant striatal gene expression profiles in wild-type mice owing to sequence-based unintentional silencing of an undefined transcription factor. Interestingly, no overt off-target effects were reported in 2 other animal models using identical or nearly identical inhibitory RNA sequences. These results suggest that RNAi treatment strategies should be validated for safety in multiple models. McBride et al showed overexpression-related neurotoxic effects from 2 of 3 different shRNAs despite equivalent target HTT knockdown. Importantly, placing the identical mature sequences into more predictably and efficiently processed miRNA backbones mitigated toxic effects without compromising HTT silencing. These results showed that excessive inhibitory RNAi expression was unnecessary to achieve beneficial gene-silencing levels and suggested that disruption of natural miRNA function was harmful to mammalian brain. These studies underscore the importance of optimizing currently available delivery and inhibitory RNA expression methods to best avoid potential RNAi-related adverse effects. Choosing the best system should include an assessment of the necessity for long-term HD suppression.

Second, are there negative effects of knocking down mutant and wild-type HTT alleles in the same cell? Normal HTT function is not well defined, although knockout studies suggest it plays an important role in embryogenesis and brain development. Heterozygous Htt knockout mice are normal, but Htt-null mice die as early embryos; brain-specific Htt deletion at postnatal day 3 rescued embryonic lethality, but animals showed neurodegeneration and shortened lifespan. Thus, wild-type Htt may be required for normal brain function, but it is unclear whether long-term hypomorphic (<50%) normal HTT expression will negatively affect adult, postdifferentiated neurons. This question reflects a limitation of the first 7 HD RNAi studies. All used HD rodent models that expressed mutant human HTT on a genetic background containing 2 normal mouse (or rat) Htt alleles. The HD-targeted inhibitory RNAs exploited species-specific sequence mismatches to selectively target only the mutant human allele. The cc-siRNA-hTt sequence is an exception; although normal endogenous HTT knockdown was not measured, this siRNA has 20 nucleotides of perfect homology with mouse Htt and could theoretically target the normal allele. Nevertheless, there is no evidence demonstrating the effects of normal Htt knockdown from the first 7 studies listed in the Table, either because it was not measured or its levels were unaffected by human HTT-targeted inhibitory RNAs.

Third, are there negative effects of knocking down mutant and wild-type alleles? In the first study, McBride et al delivered Htt-targeted miRNAs to wild-type mouse brain using AAV1 vectors. Four months after injection, normal mouse Htt mRNA and protein levels were reduced by 70% and 83%, respectively, and animals showed no overt histopathological changes. Therapeutic shRNAs and miRNAs were also delivered to the CAG140 knockin mouse model of HD, but ultimately the investigation by McBride and colleagues was a safety study that did not assess the therapeutic potential of coincident mutant and...
normal Htt knockout. Shortly thereafter, Drouet et al\(^1\) showed the first evidence that co-knockdown of mutant HTT and normal Htt in the same neurons could improve HD-related histopathology. In this study, Drouet and colleagues used nontransgenic mouse or rat HD models in which mutant HTT was expressed in rodent striata using lentiviral vectors. Coexpression of HTT-targeted shRNAs in affected neurons prevented Darpp32 loss and reduced ubiquitin-positive neuronal inclusions up to 9 months after injection. However, the levels of remaining normal Htt associated with these histopathological improvements were unclear. Specifically, Htt mRNA was reduced 86% at 3 weeks following injection but only 50% to 60% at 16 weeks, and no Htt knockdown data were reported for the 9-month time point.\(^1\) No corresponding Western blot data demonstrating how these 50% to 86% mRNA reductions affected normal Htt protein were shown, and behavioral assessments were not performed.\(^1\) In a parallel study using AAV-delivered miRNAs, Boudreau et al\(^1\) reported the first evidence that cosuppression of mutant human Htt and normal mouse Htt in the same neurons could improve HD-associated motor deficits 11 weeks after injection in N171-82Q HD mouse striata. These rotarod improvements were associated with 75% knockdown of normal Htt mRNA, which is consistent with previous findings by this group.\(^17,19\) Together, these 3 studies provided encouraging data supporting the feasibility of non–allele-specific gene silencing strategies to treat HD. However, both Drouet et al\(^1\) and Boudreau et al\(^1\) reported abnormal alterations in molecular pathways that were associated with Htt loss of function. Although it is unclear whether these changes could negatively affect adult neurons over time, these results indicated that further investigation of the safety of RNAi-mediated normal Htt knockdown is necessary. One component of these safety studies should address how long-term Htt gene silencing in rodent brain corresponds to what may be required in humans. Specifically, it is unclear how the 3- to 9-month normal Htt suppression in relatively short-lived rodent models translates to potentially years- or decades-long gene silencing that may be necessary in human patients. It will therefore be important to test the safety of non–allele-specific Htt suppression in larger, longer-living animals such as monkeys or dogs. Should normal HTT knockdown not be tolerated by postmitotic neurons, it is possible to preferentially suppress mutant HTT by targeting disease-linked single-nucleotide polymorphisms.\(^2,23\) Importantly, some HD-linked polymorphisms have been described and specifically targeted in cell culture, suggesting that mutant HD-specific personalized RNAi therapies may someday be possible.\(^2,24-27\)

Finally, what are the cellular targets? Huntington disease is characterized by striatal medium spiny neuron loss, which an effective HD treatment should prevent.\(^1\) Since cell-autonomous factors likely play a role in HD pathogenesis, medium spiny neurons are certainly targets for RNAi therapy. In addition, recent evidence suggests that mutant HTT may also cause dysfunction in glia and cortical neurons, thereby contributing non–cell-autonomous effects to HD, including glutamate excitotoxic effects and reduced neurotrophic support to the striatum.\(^1,3,4\) More-over, in mouse studies, striatal-specific mutant Htt knockdown caused only partial improvements in some HD-associated phenotypes.\(^10,11\) These data suggest that a broader treatment regimen to additional brain regions or cell types may be indicated. Improvements in viral vectors or siRNA delivery systems,\(^2\) which allow efficient uptake into all target cell types, may further benefit HD RNAi therapy development.

CONCLUSIONS

RNAi therapy is a powerful method to silence disease genes. As such, RNAi is a leading candidate strategy to treat HD and other dominant neurodegenerative disorders. Importantly, RNAi technology can also be used to ask important basic questions related to HD pathobiology, which in turn may direct more targeted therapies. This symbiosis may help quickly advance RNAi strategies for treating HD and other dominant neurodegenerative diseases in the near future.

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Correspondence: Scott Q. Harper, PhD, Department of Pediatrics, The Ohio State University Medical Center, and Center for Gene Therapy, The Research Institute at Nationwide Children’s Hospital, 700 Children’s Dr, Room WA2015, Columbus, OH 43205 (harper.104@osu.edu).

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REFERENCES


**Correction**

Error in Table. In the Neurological Review article titled “Progress and Challenges in RNA Interference Therapy for Huntington Disease” by Harper, published in the August 2009 issue of the Archives (2009;66[8]:933-938), an error occurred in the Table on page 935. In the “Species Specificity” column, the footnote symbol “c” in the row referencing the study by Machida et al should have been omitted.