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RNAi: a potential treatment for Huntington's Disease

Roshan Patel

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RNAi: a potential treatment for Huntington's Disease

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Abstract

Huntington's disease (HD) is a fatal progressive brain disorder which causes uncontrolled motor function, emotional changes and a loss of cognition. Caused by genetically dominant trinucleotide CAG repeats in the Huntingtin gene (*HTT*) exceeding 36 repeats, the age of onset of HD is inversely proportional to the number of CAG repeats. Although the *HTT* gene is an obvious target for treatment, it cannot be knocked out during embryogenesis as it is crucial in cellular development, intracellular transport and vesicular trafficking. Therefore, RNA interference (RNAi) has been suggested to knock down mutant Huntingtin protein (mHTT) by intercepting mutant Huntingtin mRNA. In this Aske Project, I will discuss the potential of RNAi as a treatment for HD and compare two types of RNAi, shRNA and siRNA, evaluating their promise in clinical development.

Background

10.6-13.7 per 100,000 people of European ancestry have Huntington's (Bates, 2015), with a possible 50,000 cases in Europe (Huntington Disease, 2020) and 30,000 in America (Glorioso et al., 2015) making it the most common monogenic neurological disorder in the developed world. Cases occasionally occur with more than 36 CAG repeats and always occur above 40 (Bates, 2005) in the $4^{th}/5^{th}$ decade of life, but 5% have juvenile onset when CAG repeats exceed 70 (Gonzalez-Alegre, 2006). Progressive neurological deterioration sets in for 15-20 years, reduced to 10-15 years in juveniles due to the aggressive nature of extended CAG repeats. As HD is genetically dominant there is a 50% chance of inheritance if one parent has the disease, providing improved prediction so treatment can start earlier. However, due to instability of polyglutamine tracts, there is a risk of increased repeat lengths between generations, causing juvenile cases (50-60 repeats) with one parent with 40 repeats (Bates, 2015). With no cures for HD, only tetrabenazine for treating symptoms, RNAi presents a revolutionary treatment to improve the lives of thousands. Moreover, the ability of RNAi to supress symptoms is especially important in HD, as cognitive and motor deficits not only distresses those who have it, but also their family and friends caring for them for decades.

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Pathology

HTT is located at chromosome 4p16.3 and is expressed in all cells but mainly in the brain which is where pathology presents itself (Saudou and Humbert, 2016). Mutant HTT (mHTT) is responsible for initiation of a cascade of molecular changes, leading to the loss of medium spiny neurons due to a toxic gain in function of protein. When spliced, mHTT leaves behind small polyglutamine (polyQ) peptides which aggregate between cells, forming neuronal intracellular inclusions (NIIs). These NIIs can be seen with a light microscope, containing over 100,000,000 molecules of huntingtin-related peptides. However, there is no correlation between NIIs and toxicity, with suggestions that they have protective properties. (Arrasate et al., 2004)

The length of the polyQ chain is indirectly proportional to the age of onset and is directly proportional to the severity of symptoms, accounting for 56% of variation in age of onset (Li et al., 2003). Therefore, juvenile cases can be spotted earlier to prepare more robust treatment. Usually this would be done in teenage years accompanied by genetic counselling. However, from talking to people affected by HD, not everyone chooses to do this even with cases being prevalent in their family, as they would rather not live with such a burden. If someone does test positive, a treatment could be chosen tailored to the age and severity of onset. For example, a higher concentration of siRNAs would be used in juvenile cases to combat more severe symptoms.

There is an average weight loss in advanced HD of 25% (Reiner, Dragatsis and Dietrich, 2011) suggesting a somatic effect of mHTT, however it is important that RNAi treatments are localised to the brain to reduce side-effects. The most prominent pathology is in the striatal section of the basal ganglia with extreme neuronal loss. This worsens the UHDRS motor score, a test of 31 assessments of motor ability, seen through clumsiness, retching and trouble getting dressed. In addition, sections of the brain lose volume, the worse being 60% in the striatum and 55% in the globus pallidus (de la Monte, Vonsattel and Richardson, 1988; Lange et al., 1976; Heinsen et al., 1994).

A grading system has been developed for HD, ranging from 0-4 relating to the degree of clinical instability (Vonsattel et al., 1985). At grade 0, pre-symptomatic, few microscopic abnormalities appear, mainly NIIs, with no gross abnormalities. Grade 1 displays 50% loss of neurons in the head of caudate, a key part of the brain for making memories, however it retains its normal convex shape. There may be subtle motor, cognitive or behavioural changes. Grade 2 is when striatal atrophy is first seen. At Grade 3 there is severe atrophy and flattening of caudate. At Grade 4 up to 95% of neurons in the caudate are lost, becoming concave. This grading system not only aids patients in understanding the progression of their disease, but also clinicians in selecting treatments.

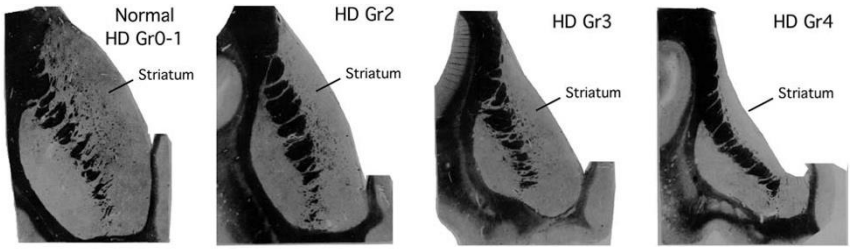

Figure 1 Coronal sections showing grades of HD progression. (Vonsattel et al., 1985)

How would RNAi alleviate symptoms of Huntington's disease?

RNA interference acts in the intermediate stage of the central dogma of biology (DNA to mRNA to protein); mRNA is cut, so protein cannot be made.

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In nature, RNAi controls gene expression, destroying mRNA molecules which would translate into proteins. In eukaryotic cells, most genes are transcribed by RNA polymerase II, then processed by splicing, forming mRNA. These are then exported into the cytoplasm which are translated into proteins by ribosomes. RNA interference acts between the points of transcription and translation (NCBI, 2020). Therefore, it is an extremely powerful tool in research to test functions of genes by knockdown of the resulting mRNA, and treatment as it can affect protein expression without any permanent DNA changes.

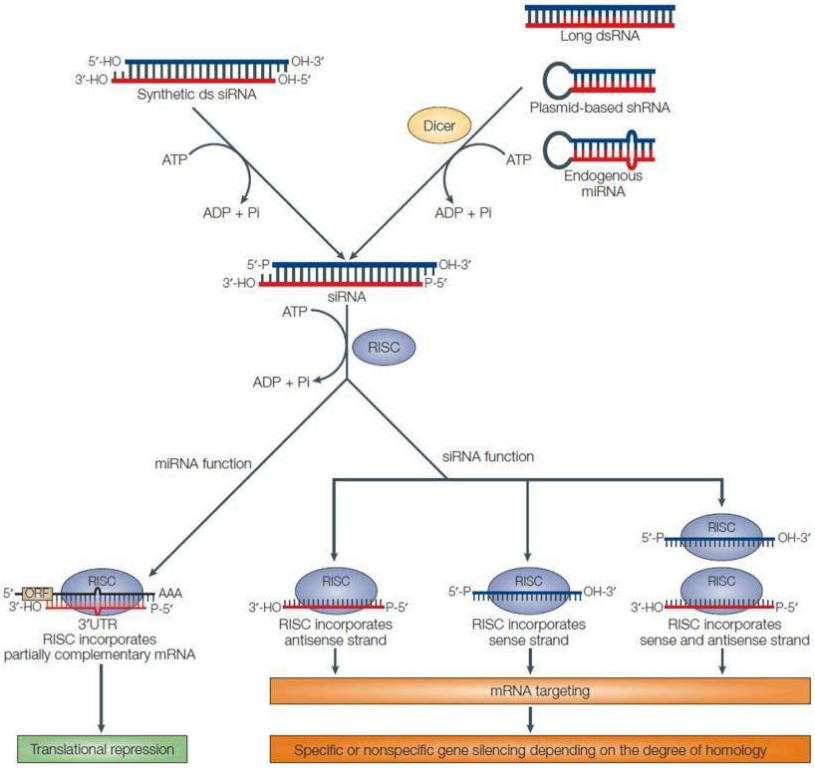

Figure 2 This schematic displays how synthetic siRNA will function under the same mechanism as plasmid delivered shRNA. (Mittal, 2004)

The primary stage of RNAi is cleavage of endogenous double-stranded RNA (dsRNA) into a small-interfering RNA molecule (siRNA) by DICER, an RNase III-like enzyme. The mature siRNA binds to a multiprotein complex called RISC (RNA-induced silencing complexed), with a catalytic argonaute protein, Ago2. The siRNA strands are then separated so the strand with a more stable 5'-end is integrated into the complex. This antisense siRNA acts as a guide strand to guide RISC towards target complementary mRNA. Once the target mRNA and the guide strand bind, Ago2 catalyses cleavage of the mRNA which is degraded, preventing mHTT production. (Rao et al., 2009).

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Therapeutic applications to Huntington's Disease

Strategy	Pros	Cons
Humanized synthetic ZFN-KRAB repressors (synthetic zinc finger repressors)	No risk of a double strand breaks	Off target effects Triggers an immune response The temporary effects depend on protein turnover
CRISPR knockout of mHTT	Permanent Technology readily available	Too large to fit in an adeno-associated virus Requires a PAM site near the polyglutamine tract Elicits innate immune response due to bacterial origins of cas9
siRNA/miRNA and ASOs	Drug like properties are more suited to regulation Easily customized for specific alleles There can be a long-term alleviation of symptoms	Requires long-term dosage Renal and hepatic toxicity caused by off-target effects Inflammatory response
shRNA-based RNAi	Longer lasting but is not permanent Fits inside an AAV as a plasmid shRNA elicits less inflammation	Overdose can be common Off target effects similar to those in siRNA treatment

Figure 3 Table showing pros and cons of using different treatments. shRNA lasts longer than siRNA, however an overdose is common (Author's own, 2020).

siRNA and shRNA have the most untapped potential in HTT treatment, as they can be delivered with ease compared to CRISPR/Cas9 technology or ZFN repressors because these elicit a stronger immune response. Especially in HD, where the huntingtin protein is necessary for embryogenesis, it is not favourable to completely knockout the mutant gene, but knockdown by RNAi will reduce the levels of mHTT to non-toxic levels. shRNA is synthetic RNA, delivered by a plasmid, engineered into the cell's genome. The gene for shRNA transcripts into a molecule which mimics dsRNA, ready to be cleaved by DICER and integrated into a RISC complex just like siRNA.

siRNA injection is similar to endogenous dsRNA and miRNA in terms of mechanism. However, the advantage of siRNA over miRNA is its high specificity with exact complementarity to the mRNA targets. In contrast, miRNA can have over 100 mRNA targets as only a short guide strand is used, which can target many mRNA molecules with similar segments (Pinto et al., 2017). This increases risks during therapy, as it can have more off-target effects (OTEs), occurring when a mRNA molecule which is not an intended target is cleaved, affecting functions of essential tissues and organs. Therefore, siRNA bypasses much of this risk by having perfect complementarity.

RNA interference *in vivo*

One issue for RNAi treatment for HD is how the brain is so inaccessible. Implanted pumps have been used to treat pain with short-term knockdown of pain-related ion channels with siRNA (Hemmings-Mieszczak, 2003), (Thakker et al., 2004), although these can be extremely inconvenient. For shRNA, viruses can be delivered directly into the brain for the inhibition of disease targets. However, a concern of viral delivery is duration, lasting from months to years, warranting a need for an "off" switch to prevent adverse effects. This would be increasingly important if there are OTEs, since both

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shRNA expressing vectors and siRNA formulations affect many parts of the body. In the context of HD, the target would be the striatum, however sometimes vectors can transduce nonneural cells by diffusing further from injection sites (Denovan-Wright and Davidson, 2005).

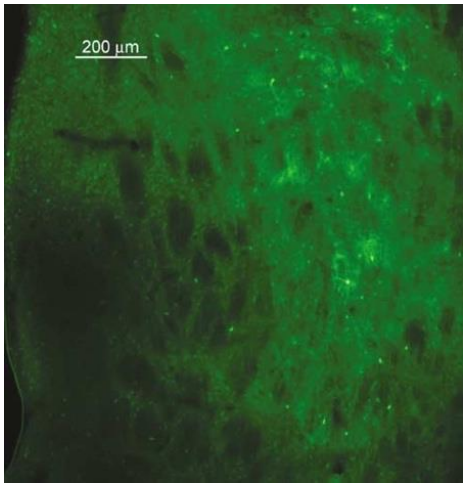

Figure 4 AAV-mediated transduction of mice striatum, with fluorescence of hrGFP identifying transduced cells after 3 weeks. (Denovan-Wright and Davidson, 2005)

Comparing siRNA and shRNA

A key difference between siRNA and shRNA are OTEs and longevity. OTEs have been seen in the induction of interferons by activation of toll-like receptors 7/8. One study showed how a liver-directed adeno-associated virus containing shRNA increased lethality caused by saturation of an endogenous miRNA pathway leading to competing shRNA and miRNA (Rao et al., 2009). Some studies have found that siRNA has more OTEs than shRNA in cancer models. Klinghoffer treated carcinoma cells with siRNA or inducible shRNA targeting the TP53 gene. There was a significant increase in off-target genes expressed differently with siRNA compared shRNA whilst knockdown efficiency was similar (Klinghoffer et al., 2010). Even when expanded for multiple mRNA targets, there were fewer OTEs and better knockdown with shRNA. A typical fix for shRNA OTEs would be to use an shRNA expression which does not overwhelm endogenous miRNA pathways.

Nomenclature	Small Interfering RNA	Short Hairpin RNA
Source	Laboratory synthesis	Nuclear expression
Delivery to the cell	Via synthetic/natural polymers and lipids to the cytoplasm	Via viral and other gene therapy vectors to the nucleus.
Persistence	99% degraded after 48 hours	Expressed for up to 3 years.
Administration	Local or limited systemic injection	Local and systemic injection
Dosage	High (low nM)	Low (5 copies)
Likelihood of specific 'off target' effects	Higher than shRNA	Lower than siRNA
Likelihood of non-specific 'off targets' effects	Higher immune activation, inflammation and toxicity	Lower immune activation, inflammation and toxicity
Application	Acute disease conditions; Where high doses are tolerable	Chronic, life threatening diseases or disorders; Where low doses are desirable

Figure 5 Comparison of siRNA and shRNA (Aguiar, van der Gaag and Cortese, 2017)

One way to increase shRNA specificity is by co-expression of tough decoys (TuDs) which counteracts shRNA sense-strand activity, otherwise known as the passenger strand. By using bioluminescent luciferase, studies found both strands' activity matched each other, highlighting the importance of

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controlling both strands when preventing OTEs. TuDs act as competitive inhibitor RNA, binding to the sense-strand before it has time to act on off-target genes. In practice, TuDs would have perfect complementary to the passenger strand. It has been shown that TuD co-expression inhibits passenger-strands, reducing OTEs by 50% (Mockenhaupt et al., 2015).

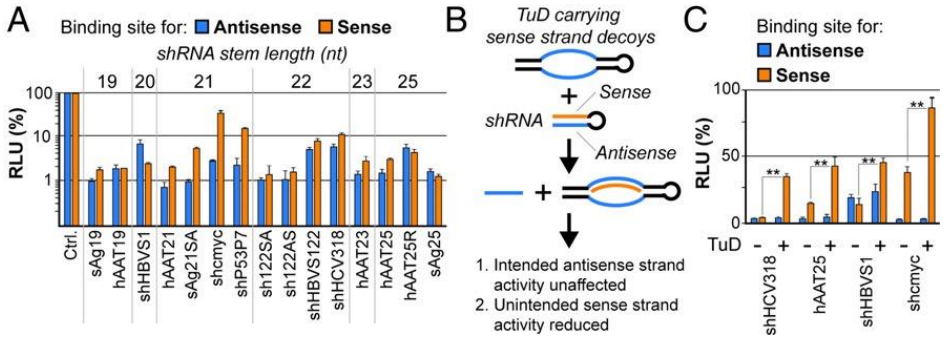

Figure 6 A) Results after co-transfection of luciferase and shRNAs against light emitted. B) Schematic of sense strand counteraction by TuD RNA. (C) TuD functionality when co-expressed with shRNAs displaying OTE reduction specific to sense strand. (Mockenhaupt et al., 2015).

Reducing siRNA bonding strengths reduces OTEs. Guide strand 2'-0-methylation reduces binding efficiency to off-target mRNA, lowering unwanted cleavage (Chen et al., 2007) (Song et al., 2017). Moreover, this does not affect binding to target mRNA, so knockdown efficiency is unchanged. In addition, not only does the siRNA guide strand cause OTEs, but also passenger strands which are not intended to integrate into RISC. Therefore, passenger strand 5'-0-methylation reduces OTEs as it prevents binding. Although these modifications increase specificity, there is still significant generation of OTEs, highlighting limitations of siRNAs.

Both methods of RNAi have their own merits, however shRNAs may be more effective. They act more like endogenous dsRNA as they are continuously expressed. To contrast, siRNA needs to be in greater concentrations and dosed more frequently since it does not cause long-term expression of RNAi and unprotected siRNA can easily be degraded in the cytoplasm, reducing on-target binding.

However, shRNAs do have many considerations, such as its delivery via a viral vector, an adenoviral-associated virus (AAV) which has been known to trigger an immune response. Currently, two AAV-based gene therapies include GlaxoSmithKline's SCID therapy and uniQure's lipoprotein lipase deficiency therapy, proving that, as stated before, shRNA therapy delivered by AAVs would be safe (Miniarikova et al., 2016). However, a main safety issue with shRNA is intracellular overdose causing competition with endogenous miRNAs.

Since HTT interacts with a huge number of proteins it is extremely important to ensure humans can survive knocked-down levels of HTT, especially if treatments target normal HTT and mHTT because it would affect normal cellular activities. As precise roles of HTT are unknown, there must be robust testing into what degrees of protein reduction are manageable. So far, adult knockdown of HTT in the CNS seems to be tolerated, holding promise for future safety, but this is not guaranteed as many more studies must take place. Nevertheless, studies into non-human primates showed 45% reduction in HTT was tolerated (Marxreiter, Stemick and Kohl, 2020). Additionally, the amount of protein reduction necessary to slow disease progression needs to be evaluated to find the sweet spot with maximum disease suppression but minimal side-effects. To summarise: long-term safety of RNAi, especially shRNA, is unknown, warranting deeper and more thorough research in humans.

Overall, shRNA is the most viable RNAi method of mHTT knockdown. Although siRNA may be more effective in acute disease conditions when higher doses are tolerated, there are too many OTEs rendering the treatment potentially deleterious. On the other hand, shRNA elicits fewer OTEs with

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an equal knockdown efficiency. Moreover, shRNA is continuously expressed, reducing the number of procedures needed to apply therapies.

Pre-clinical studies into shRNA therapy for HD models

Harper et al. investigated how RNAi improves motor and neuropathological abnormalities in mouse models. They used shRNAs directed against HD-N171-82Q mRNA expressed in transgenic mice. One shRNA, shHD2.1, reduced Huntington mRNA by 85% and protein levels by a significant 55% compared to controls, with precursor and processed shRNA still produced after 3 weeks. Moreover, using a fluorescent protein GFP, widespread transduction was seen 5 months post-injection and NIIs were absent from AAV.shHD2.1 transduced cells. One key part of the study was the investigation into behavioural phenotypes. shRNA treated mice showed no notable weight changes compared to un-injected HD mice, suggesting RNAi was localised to the site of application. Mice expressing mHTT showed significantly shorter stride length compared to wild type (WT) mice, but gait improved significantly when treated with AAV.shHD2 by 13% in front strides and 15% in rear. However, gait improvements were still significantly different from age-matched WT littermates. Control mice showed greatly impaired movement throughout the study, developing impaired performance by 10 weeks. AAVshHD2.1 treated mice showed only a 3% drop in rotarod performance compared to 22% in untreated mice. Differences in rotarod performance in injected WT mice suggested that there were detrimental OTEs on motor ability. These resolved themselves at 18 weeks indicating there were no long-lasting negative impact. Overall the study found that RNAi dramatically improves HD pathology and behaviour, suggesting its feasibility as a treatment for HD. (Harper et al., 2005)

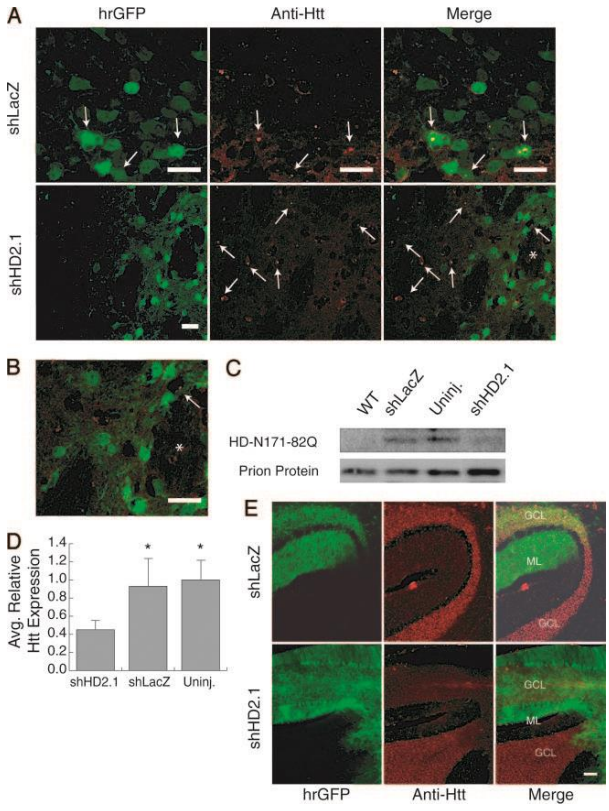

Figure 7 A) Micrographs showing HTT-reactive inclusions B) Higher-magnification micrograph from A showing few NIIs in shHD2.1 cells. C) Western blot demonstrating decreased HD-N171-82Q expression with AAV.shHD2.1 D) AAV.shHD2.1 treated HD mice showing 55% reduction of mHTT mRNA on average. E) AAV-shHD2.1 showed reduced HTT immunoreactivity. (Harper et al., 2005).

This study is a key sign of the potential of RNAi as a treatment for HD. Although there are currently no shRNA treatments for HD in the clinic, there are many trials which are gaining traction in similar areas. For example, the GENERATION HD1 study will use 800 people to test an antisense oligonucleotide (ASO) which acts in a similar way to siRNA.

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Comparison to CRISPR

A contrasting study used CRISPR/Cas9 to disrupt the *mHTT* gene resulting in a 50% reduction in mHTT and improved life expectancy. CRISPR (clustered regularly interspaced short palindromic repeats) Cas9 gene-editing finds a specific area in the genome and cuts the DNA strand so the gene will repair with alterations from the original

CRISPR/Cas9 reduced NIIs by 2-fold, increased lifespan and improved motor deficits in mice. 85% of cells were transduced, with 40% fewer mHTT NIIs and 50% less mHTT. Mice injected with AAV1-SaCa9-HTT showed a 15% increase in survival duration compared to controls. Additionally, end-stage mice with functional saCas9 had 10% more transgenic cells compared to controls, suggesting CRISPR disruption of mHTT protected neurons from mHTT-induced toxicity. (Ekman et al., 2019)

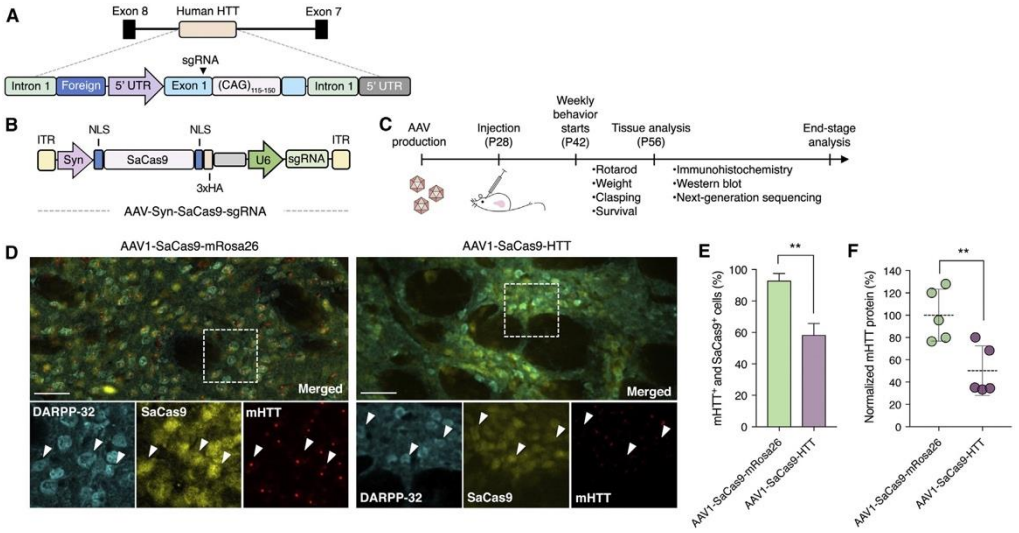

Figure 8 A) HTT transgene which was induced into mice. B) A schematic of the AAV vector used. C) Timeline. D) Immunofluorescent staining of striatum 4 weeks after injection. E) Immunohistochemical results displaying decreased mHTT. F) Normalized mHTT protein after 4 weeks, displaying a clear reduction in mHTT in AAV1-SaCas9-HTT injected mice (Ekman et al., 2019).

CRISPR/Cas9 still has risks of OTEs: if Cas9 was directed to another area of the human genome, there could be genetic alterations which could lead to death. In comparison, shRNA does not cause permanent off-target genetic alterations. shRNAs showed better knockdown compared to CRISPR, indicating that shRNAs may be more efficient than CRISPR. Both studies described substantial benefits of treatments on motor ability, a key facet of treatment for the wellbeing of a patient, however a more unified approach in measurement is needed to make direct comparisons. However, in my opinion RNAi has yielded more promising results with a greater degree of knockdown and an increased efficiency of transduction. Although CRISPR has been hailed as a miracle treatment due to its relative ease-of-use, I believe that it is not the optimal treatment for HD at this point owing to its lower protein reduction efficiency.

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The personal side of HD

When I started this project, I looked at causes, symptoms and treatments but realised impacts on individuals are often overlooked. Because of this I contacted somebody whose son has early-onset HD. Steven had been showing signs of HD from his early teens but was first diagnosed at the age of 25 with 56 CAG repeats even though his father only has 35. Therefore, the disease was a shock, and since symptoms appeared early, it was clear the disease would be aggressive. What struck me from talking to Alex, Steven's father, was the devastating impact on carers and family. Alex often found himself "mentally exhausted" when accompanying his son to appointments, leaving him a "gibbering wreck". Therefore, you can see how it is essential to find a treatment for HD immediately: the lives of everyone surrounding the disease are decimated by HD caused anger outbreaks and isolation. He describes the whole thing as like being on a helter-skelter - and trying to climb up it and falling – and falling. Although HD is a soul crushing disease, families do not give up; rather than giving up their lives to the disease, they give their lives in service of the individual, to make their life as good as possible. Thus, we must raise awareness of HD and encourage research into potential cures. Alex told me that what you have to do is accept what is in front of you - and do all you can to delay the inevitable. Perhaps in the future, a cure will appear to give him the hope which has been lost to him.

Conclusion

In theory, RNA interference appears to be a viable treatment for HD in humans since it has been shown to improve behavioural phenotypes and increase mHTT knockdown considerably. Although methods such as 5'-O-methylation and co-expression of TuDs reduce OTEs, there are substantial risks of lethal side-effects which may hinder progression to clinical trials. When compared to CRISPR, RNAi holds more promise due to reduced ethical concerns surrounding genetic engineering, high risk of CRISPR off-target cleavage and better mHTT knockdown in RNAi models.

Within RNAi, shRNA seems to be the way forward due to its long-term expression. Moreover, shRNA has considerably fewer OTEs and does not need to be re-administered so often, reducing stress on the mind and bodies of patients. Alternatively, siRNAs could be viable in late stage patients in grade 4, where a high dosage of fast acting siRNAs could be their only chance at maintaining their current condition, or even recovering slightly.

In my opinion, shRNA is an untapped goldmine of treatment options for Huntington's Disease. Although more research and trials must be done into reducing OTEs, perhaps with the introduction of a robust "off-switch" to disable shRNA expression, I believe RNAi will become a leading treatment for the currently incurable Huntington's Disease. However, if RNAi treatment does not come to fruition, as Alex pointed out to me, you can have a hundred negative things happen in a week - but that's HD and you've got to let that be taken away with the garbage or be shoved down an imaginary bottomless pit. Therefore, there must be continued research into a treatment for HD, no matter which method is used, to help protect families like Alex's.

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