Huntington's Disease: Silencing a Brutal Killer

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Huntington's disease (HD) is a progressive and invariably fatal autosomal dominant neurodegenerative disorder caused by expansion of the CAG repeat in exon 1 of the *Huntingtin* gene. CAG repeat expansion generates an extended, toxic polyglutamine tract in the mutant Huntingtin protein (The Huntington's Disease Collaborative Research Group, 1993), which disrupts a stunningly large number of cellular pathways. Mutant Huntingtin is especially damaging to the medium spiny neurons of the striatum (Graveland et al., 1985), and degeneration in this region is thought to underlie the motor disturbances or —chorea that characterize the disease. In parallel, loss of striatal and cortical neurons disrupts patient cognition and alters mood and personality (Heinsen et al., 1994). The age of onset of HD varies with CAG repeat length (Brinkman et al., 1997; Duyao et al., 1993) 36 or more repeats is considered pathological, but some patients with 36 to 39 repeats remain asymptomatic well into old age. The vast majority of patients are heterozygous for the HD mutation; most have 39 to 50 repeats and develop symptoms between age 30 and 50. No current treatment can prevent or delay the progression of HD.

In theory, curing HD should be simple: turn off expression of the mutant, disease-causing *Huntingtin* gene. Yet identifying a clinically feasible strategy for silencing an autosomal dominant gene in brain has proved challenging. Perhaps the most promising strategy is RNA interference (de Fougères et al., 2007; Kim and Rossi, 2007), using synthetic small interfering RNAs (siRNAs) to suppress expression of mutant *Huntingtin* mRNA, thereby preventing the accumulation of the toxic, mutant Huntingtin protein (Wang et al., 2005; Machida et al., 2006; DiFiglia et al., 2007). Encouragingly, studies in mice suggest that mutant *Huntingtin* toxicity is reversible (Yamamoto et al., 2000; Regulier et al., 2003; Drouet et al., 2009). However, siRNAs cannot distinguish between normal and CAG-repeat expanded transcripts (Caplen et al., 2002), so most studies of RNAi for Huntington's disease have examined the effect of simultaneous reduction of both the mutant and wild-type *Huntingtin* mRNA (Wang et al., 2005; Machida et al., 2006; DiFiglia et al., 2007). In contrast, two recent papers (Lombardi et al., 2009; Pfister et al., 2009) provide strong support for an alternative strategy: distinguishing between mutant and wild-type *Huntingtin* by targeting heterozygous single-nucleotide polymorphisms (SNPs) as an alternative to direct targeting of the CAG repeat. A third study reports similar results by targeting a common micro-deletion associated with the *Huntingtin* disease allele (Zhang et al., 2009).

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siRNAs can distinguish between mRNAs differing at a single nucleotide (Ding et al., 2003; Du et al., 2005; Schwarz et al., 2006), and such allele-specific siRNAs have been developed for SNPs in the SOD1 and *Huntingtin* genes (Ding et al., 2003; Schwarz et al., 2006). The inherited form of Amyotrophic Lateral Sclerosis (ALS) is caused by specific single nucleotide polymorphism in the SOD1 mRNA, so selecting a SNP to target SOD1 mRNA is straightforward. In contrast, the pathogenic mutation for HD is the expansion of the CAG repeat region, and it was thought that no specific SNPs would be associated with the disease-causing CAG expansion.

Hayden and colleagues provided the first systematic examination of a large number of *Huntingtin* SNPs on both disease and control chromosomes (Warby et al., 2009). They found that the SNP distribution of HD and normal chromosomes differ. The vast majority of HD chromosomes (95%) belong to a single haplogroup defined by 10 SNPs; only 53% of control chromosomes belonged to this same haplogroup. Consequently, ~45% of HD patients have a predictable heterozygosity within the *Huntingtin* locus and could, in theory, be treated with a single siRNA targeting one of the SNPs that defines the haplogroup. However, the Warby et al. study did not distinguish between SNPs occurring in introns, which are not useful for therapy, and those in exons, which are candidate therapeutic targets.

To examine the availability of targetable SNPs, Aronin and coworkers compared the heterozygosity at twenty-two SNP sites in the coding region and 3' untranslated region (UTR) of the *Huntingtin* mRNA in patient and control samples from the US and Germany (Pfister et al., 2009). Forty-eight percent of patients were heterozygous at a single site in the 3' UTR, and the U isoform of this SNP was significantly more common in HD samples than in controls: 48.6% heterozygosity in patients vs. 13.0% in controls, with 87% of controls being homozygous for the C isoform. This suggested that the U isoform is present on the disease transcript and that targeting this SNP isoform with a selective siRNA could provide therapy for almost half of the patient population. The authors directly confirmed the linkage between the U isoform and the CAG repeat expansion for a smaller set of patients using a novel molecular assay—SNP linkage by circularization (SLiC) (Liu et al., 2008). The team then designed and tested siRNAs targeting the U isoform of the SNP associated with HD. Alas, no siRNA containing a single-nucleotide mismatch could distinguish between the two isoforms of this SNP. However, introducing a second mismatch to both isoforms allowed the siRNA to selectively reduce the disease target while sparing the wild-type isoform. The authors concluded that the association of specific SNPs with CAG repeat expansion provides a strategy to target mutant *Huntingtin* in nearly fifty percent of the patient population—at least in theory.

One of the first polymorphisms found to be associated with the disease-causing *Huntingtin* allele is a micro-deletion in exon 58 that removes one to four GAG triplets (Ambrose et al., 1994; Novelletto et al., 1994). Friedlander and colleagues screened siRNAs designed to use the $\Delta 2642$ polymorphism to discriminate between the disease and normal *Huntingtin* mRNAs (Zhang et al., 2009). They report siRNAs that can preferentially silence the mutant *Huntingtin* protein in HD fibroblasts and in neuroblastoma cells. Whereas silencing both mutant and wild-type *Huntingtin* results in activation of caspase-3 and increased sensitivity to cell death, silencing of mutant *Huntingtin* alone did not (Zhang et al., 2009). Therefore, specific targeting of mutant *Huntingtin* using SNPs is not only feasible, but may prove safer than targeting both mutant and wild-type *Huntingtin* indiscriminately.

For the remaining portion of HD patients (50–60%) who lack the disease-associated SNP or the $\Delta 2642$ mutation, SNP-specific RNAi is still possible. If there is a sufficient amount of heterozygosity within the *Huntingtin* gene, a panel of SNPs could be developed. The SLiC method (Liu et al., 2008) could then be used to determine SNP-CAG repeat linkage for heterozygous SNPs in individual patients, and the appropriate SNP-selective siRNA chosen

from a panel of therapeutically validated siRNAs. However, if each SNP covers only a few patients, the cost of developing such a panel of siRNAs would not only be prohibitive, but a meaningful phase III drug trial might be impossible. To address this question, the Aronin and Kaemmerer groups employed similar methods (Lombardi et al., 2009; Pfister et al., 2009). Aronin's group calculated the maximum percentage of patients with at least one heterozygous SNP for all combinations of one to seven SNP sites. The percentage of patients covered quickly reached a plateau at < 80 percent. Only three SNPs—corresponding to five siRNAs—were required to cover seventy-five percent of the HD population in the study.

Similarly, Lombardi et al. (Lombardi et al., 2009) selected 26 polymorphic sites, including 25 SNP sites and the $\Delta 2642$ mutation in exon 58 of the *Huntingtin* gene. Eighty-six percent of patients (282 out of 327) in their sample population of patients of European descent were heterozygous at least one site. To determine the number of SNP sites required to treat the maximum number of patients, Lombardi et al. (Lombardi et al., 2009) calculated the coverage for every possible subset of SNPs. Coverage reached a plateau of 86 percent using seven polymorphic sites. Again, seventy-five percent of patients were covered by only three polymorphisms; each additional SNP increased the percentage of patients covered by only a small amount. The small differences in maximum coverage reported in the two studies likely reflect differences in patient populations and in the SNPs evaluated.

There is sufficient heterozygosity at a reasonably small number of SNP sites in the *Huntingtin* gene to justify optimism that SNP targeting can be used therapeutically, but only if siRNAs can be designed to selectively target these SNP sites. The Aronin study reports a set of SNP-selective siRNAs targeting the top three sites, validating them using a luciferase reporter assay. Kaemmerer's group studied the subset of SNPs for which heterozygous patient fibroblasts are available. They designed siRNAs to target the isoform of the SNP associated with the expanded CAG repeat in their cell lines and, after an initial screening with a luciferase reporter assay, showed that targeting the appropriate isoform reduces the abundance of the mutant Huntingtin protein in the cells.

siRNAs bearing a purine:purine mismatch to the target mRNA at position 10 or position 16 of the guide strand are good candidates for single-nucleotide discrimination (Dykxhoorn et al., 2006; Schwarz et al., 2006). Kaemmerer's group gave preference to SNPs that would produce a purine:purine mismatch between siRNA and mRNA when selecting SNPs to study (Lombardi et al., 2009). When designing siRNAs both Kaemmerer and colleagues and Aronin's group first screened position 10 and position 16 mismatches (Lombardi et al., 2009; Pfister et al., 2009). In some cases, a single mismatch was insufficient. Paulson, Davidson and colleagues had previously shown that mutant Ataxin-3 could be preferentially reduced by targeting a SNP adjacent to the CAG repeat expansion. In this case, the most selective siRNA was mismatched to the wild-type allele for the SNP site at position 16 of the siRNA guide strand and also contained an additional mismatch to both wild-type and disease alleles at position 15 (Miller et al., 2003). Aronin's group found that a position 15 mismatch in one case destroyed the ability of the siRNA to target either isoform of a SNP. Combining a sensitizing mismatch at position 5 or 6 with a discriminatory mismatch at position 10 of the siRNA improved allele discrimination. In contrast, the nature of the $\Delta 2642$ mutation limited the strategies available to the Friedlander's group. By placing the micro-deletion in the center of the siRNA, they generated an siRNA that mismatched the wild-type *Huntingtin* mRNA at the 3' end of the guide strand. They found that the siRNA with greatest discrimination for the $\Delta 2642$ was mismatched to the wild-type target at both positions 16 and 19, whereas an siRNA with a third mismatch at position 13 was less effective at reducing either the mutant or the wild-type Huntingtin protein (Zhang et al., 2009).

What of the 10–20% of patients who lack the micro-deletion or appropriate SNPs in their *Huntingtin* genes? The consistent appearance of this set of patients across studies of different populations suggests that adding more polymorphisms outside the CAG repeat is unlikely to increase coverage to include these patients. To achieve allele-specific silencing for these patients another strategy will be required. Perhaps targeting the CAG repeat directly may no longer be impossible. Corey and colleagues recently reported that peptide nucleic acid conjugates (PNAs) targeting the CAG repeat region of the *Huntingtin* mRNA selectively down regulated the mutant Huntingtin allele (containing 47, 44, 41 or 69 CAG repeats) relative to the wild-type allele (15, 15, 20 or 17 repeats) in patient-derived fibroblasts with 2.1, 1.8, 1.2 or 3.5-fold selectivity and without affecting the expression of other CAG repeat containing proteins (Hu et al., 2009). While this selectivity is small compared to the selectivity achieved using siRNAs targeting SNPs, it may suffice to treat those patients for whom SNP-selective siRNAs are unavailable.

Although allele-specific silencing remains to be demonstrated in an animal model for *Huntingtin* disease, allele-specific silencing via the RNAi pathway has been demonstrated in animal models of dominant inherited ALS (Xia et al., 2006) and Machado-Joseph disease (Alves et al., 2008). Like HD, Machado-Joseph disease (also called spinocerebellar ataxia type 3) is an autosomal dominant disease caused by expansion of a CAG repeat—in the *ataxin-3* gene (Kawaguchi et al., 1994). A SNP 3' to the *ataxin-3* CAG repeat region is associated with the expansion and approximately seventy per cent of expanded CAG chromosomes carry the C variant (Gaspar et al., 2001). Lentiviral delivery of mutant *ataxin-3* to the adult rat brain recapitulates some of the features of the human disease (Alves et al., 2008); co-delivery of an shRNA targeting the C isoform of the associated SNP specifically reduced expression of mutant *ataxin-3* and protected against neurodegeneration, demonstrates the feasibility of allele-specific silencing in the central nervous system.

Before RNAi-based therapies reach the clinic, several safety issues must be addressed. These include off-target effects caused by unintended silencing of genes with sequences partially complementary to the therapeutic siRNA, induction of innate immune responses, and competition for the cellular RNAi machinery. In one study, targeting a sequence at the 5' end of human *Huntingtin* (siHUNT-2) caused a reduction in the levels of several other striatal mRNA transcripts in a mouse model of HD, whereas a second siRNA targeting a different region of *Huntingtin* (siHUNT-1) effectively reduced *Huntingtin* mRNA levels without these off-targeting effects (Denovan-Wright et al., 2008). Predicting sequence-specific off-target effects is currently not possible, as they can be caused by complementarity as short as six nucleotides and appear to be species-specific, preventing human off-target effects from being anticipated from pre-clinical rodent or non-human primate studies (Burchard et al., 2009). Immune stimulation, both sequence-dependant and sequence-independent has been observed with a number of different siRNA molecules (Kariko et al., 2004; Sioud, 2005; Hornung et al., 2006; Kleinman et al., 2008) but may be prevented by chemical modification of the siRNA (Judge et al., 2006; Eberle et al., 2008). Safety will also be of particular importance in selecting an appropriate siRNA delivery method.

To be useful, any HD treatment will need to be safe for prolonged use, but the long-term consequences of a reduction or loss of the normal Huntingtin protein are unknown. Conditional inactivation of the mouse *Huntingtin* homolog (*Hdh*) beginning early in postnatal development reduced Huntingtin levels by 84–90% in the forebrain and 30–50% in the cerebellum and caused a progressive motor defect, neurodegeneration, and reduced life span (Dragatsis et al., 2000). In contrast, a recent study (Drouet et al., 2009) used differences between the endogenous rat huntingtin and a lentiviral human huntingtin fragment (82Q) to examine the effects of allele-specific and non-specific silencing. After nine months, rats treated with either the human specific or the universal (rat and human) shRNA had similar numbers of surviving cells,

suggesting that silencing of wild-type *Huntingtin* does not cause or accelerate the HD-like pathology. The difference between the earlier and more recent findings may reflect differences in experimental timing, extent, or localization of *Huntingtin* reduction. Therefore, partial, localized reduction of both mutant and wild-type *Huntingtin* may, in fact, be a viable treatment strategy. Caution is still warranted: striatal-specific reduction of endogenous rat *Huntingtin* was associated with significant changes in gene expression that were not seen when only the human 82Q fragment was targeted, and these changes were specific to pathways associated with known Huntingtin functions (Drouet et al., 2009).

The results of this recent group of papers bring us closer to a clinically useful therapy for HD. A majority of HD patients may be treatable by a relatively small number of siRNAs, others may benefit from siRNA or other silencing molecules targeting common features of mutant and wild-type *Huntingtin* mRNA. The basic components of a strategy for allele-specific silencing are now in place: a testing method for selecting the appropriate target in patients (Liu et al., 2008), a list of SNP target candidates (Lombardi et al., 2009; Pfister et al., 2009), and a set of siRNAs with demonstrated selectivity in culture (Lombardi et al., 2009; Pfister et al., 2009; Zhang et al., 2009). Tempering our excitement however is the absence of a therapeutically relevant delivery method for introducing siRNAs or antisense drugs into the appropriate cells of the brain. This remaining challenge clearly demands our effort and will likely require close collaboration between academic and industrial scientists to solve.

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