Allele-specific silencing of mutant Huntington’s disease gene

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Abstract

Huntington’s disease (HD) is an autosomal-dominant neurodegenerative disorder caused by a poly-glutamine expansion in huntingtin, the protein encoded by the HD gene. PolyQ-expanded huntingtin is toxic to neurons, especially the medium spiny neurons (MSNs) of the striatum. At the same time, wild-type huntingtin has important -- indeed essential -- protective functions. Any effective molecular therapy must preserve the expression of wild-type huntingtin, while silencing the mutant allele. We hypothesized that an appropriate siRNA molecule would display the requisite specificity and efficacy. As RNA interference is incapable of distinguishing among alleles with varying numbers of CAG (glutamine) codons, another strategy is needed. We used HD fibroblasts in which the pathogenic mutation is linked to a polymorphic site: the Δ2642 deletion of one of four tandem GAG triplets. We silenced expression of the harmful Δ2642-marked polyQ-expanded huntingtin without compromising synthesis of its wild-type counterpart. Following this success in HD fibroblasts, we obtained similar results with neuroblastoma cells expressing both wild-type and mutant HD genes. As opposed to the effect of depleting wild-type huntingtin, specifically silencing the mutant species actually lowered caspase-3 activation and protected HD cells under stress conditions. These findings have therapeutic implications not only for HD, but also for other autosomal dominant diseases. This approach has great promise: it may lead to personalized genetic therapy, a holy grail in contemporary medicine.

Keywords

Huntington’s disease; polymorphism; siRNA; allele-specific
to be important for the production of BDNF (brain-derived neurotropic factor) and exerting an inhibitory effect on caspase-3 (Zuccato et al. 2001; Zhang et al. 2006). Therefore, preservation of wild-type huntingtin levels is of great benefit particularly to a neuron exposed to stressful conditions (Cattaneo et al. 2005; Zhang et al. 2006). Moreover, loss-of-function of wild-type huntingtin is likely to contribute to HD pathogenesis (Dragatsis et al. 2000; Zuccato et al. 2001; Cattaneo et al. 2005; Van Raamsdonk et al. 2005; Leavitt et al. 2006; Zhang et al. 2006). Significant toxicity is expected to result from indiscriminate silencing of both mutant and wild-type alleles. Consequently, a successful strategy for countering HD requires specific depletion of mutant huntingtin with preservation of its wild-type counterpart. Unfortunately, siRNAs cannot distinguish among HD alleles differing uniquely in the number of CAG repeats at their 5′ end. A different strategy is needed to specifically and effectively block synthesis of the troublesome protein.

A different sort of polymorphism among HD genes -- a functionally neutral one -- is the Δ2642 deletion of one among four tandem GAG repeats in exon 58 (Ambrose et al. 1994; Novelletto et al. 1994; Almqvist et al. 1995; Rubinsztein et al. 1995; Vuillaume et al. 1998). The deletion is found in 38% of mutant HD alleles and 7% of wild type HD ones (Ambrose et al. 1994). This polymorphism is a potential target for siRNA molecules which specifically silence expression of mutant HD.

Materials and methods

Cell culture

The HD fibroblast lines (GM00305, GM06274, and GM09197) were obtained from NIGMS Human Genetic Mutant Cell Repository of the Coriell Institute for Medical Research in Camden, NJ. Normal (control) human fibroblast cells CCD-25Lu were obtained from ATCC (ATCC number: CCL-215). Cells were grown in Minimum Essential Medium (MEM) supplemented with 2mM L-glutamine at final concentration, 1% penicillin-streptomycin and 15% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C with 5% CO2.

PCR

Total RNA was isolated from three HD fibroblast lines using the RNaseq kit (Qiagen). The SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen) was used with the following program: 30 min at 50°C, 5 min at 94 °C, 35 cycles of 15 sec at 94 °C, 1 min at 58 °C, 1 min at 72 °C, followed by 10 min at 72 °C. A sequence including the polymorphic site in exon 58 was amplified using 22mers 5′-GCTGGGAAAACATCACACCC-3′ and 5′-CCTGGAGTTGACTGGAGACGTG-3′ as forward and reverse primers, respectively. PCR products were denatured for three minutes at 70 °C and kept on ice. Following dilution in Novex® TBE-Urea sample buffer, DNA specimens were run on a 6% denaturing TBE-Urea polyacrylamide gel (Invitrogen). Gels were stained with SYBR® Gold (Invitrogen) in TBE buffer (1: 10,000) for 30 minutes and observed by transillumination with 302 nm UV light (ChemiDoc system, Bio-Rad). Following preliminary experiments with 10 pairs of PCR primers, the two most effective forward/backward couples were used for amplification of wild-type and mutant HD transcripts. Two sets of primers were selected to measure silencing of wild-type and mutant HD transcripts. Amplification by real time qRT-PCR of the wild-type sequence used 5′-AAGAGGAGGAGGAGGGCAGCCTCC-3′ and 5′-GCG TCA CAT ACA TCA GCT CAA ACT GGT-3′, as forward and reverse primers, respectively. In the equivalent procedure for the mutant sequence, the forward primer was 5′-AAGAGGAGGAGGAGGCACCCCT-3′ and the reverse one was 5′-GCG TCA CAT ACA TCA GCT CAA ACT GGT-3′. GAPDH mRNA was used as an internal control in all PCR amplifications. 20mers 5′-GAGTCAACGGATTTGGTCCG-3′ and 5′-
TTGATTGTGGAGGGATCTCG-3′ were used as forward and backwards primers, respectively. A one-step real time qRT-PCR program used cycles of 30 min at 50°C, 10 min at 94°C, 45 cycles of 30 sec at 95°C, 1 min at 55°C, 30 min at 72°C, followed by 1 min at 95°C, 30 min at 55°C, 30 min at 95°C to generate dissociation curves. Ct value of each sample was normalized to the Ct of their endogenous control GAPDH: ΔCt = Ct(test mRNA) − Ct(GAPDH mRNA). The relative quantity of huntingtin messenger in each siRNA transfected cells were calculated from ΔΔCt: ΔΔCt = ΔCt(test siRNA) − ΔCt average of controls. Relative expression level= power (2, −ΔΔCt). Fold difference are expressed as ratio to control sequence transfected group.

DNA constructs with luciferase as a reporter

Restriction enzymes and DNA ligase were obtained from New England Biolabs (Ipswich, MA). pMIR-REPORT™ Luciferase miRNA Expression Reporter Vector (Ambion) was cut with Spel and MluI restriction endonucleases. 50 bp or 47 bp deoxyoligonucleotides were synthesized (Invitrogen) and annealed to create 5′ and 3′ overhangs containing partial Spel and MluI flanking regions. DNA from selected colonies was sequenced to confirm the presence of the 50 bp insert in Lucwt2642 and the 47 bp insert in Lucmu2642.

Luciferase Reporter Assays

Cells were seeded at 1 ×10^5 cells per well in 12-well tissue culture plates the day before transfection. 1μg of each luciferase construct was co-transfected with various amount of siRNA using Lipofectamine 2000 and incubated at 37°C with 5% CO2 in culture medium. Forty hours after incubation, the medium was aspirated from off the cells. 200 μl of Glo lysis buffer (Promega, Madison, WI) was added to each sample well, and the plate was rocked slowly for 5 minutes. After transferring 50μl of lysates to the well of OptiPlate™-96F (PE, Waltham, MA), 50 μl of Bright-GloAssay Reagent (Promega) was added to measure the instantaneous level of luminescence using a Perkin Elmer 1420 Multilabel Counter (PE).

Transfection

The fibroblast cells were plated in 75 cm^2 culture flasks to 50% confluence. 20 μl of 40μM siRNA (Dharmacon, Inc.) were diluted in 1 ml culture medium without serum and added with 40 μl of HiPerfect transfection reagent (Qiagen). The above mixture was added to cells in 10 ml culture medium.

Western blotting

After washing the cells three times with PBS, total protein was extracted in NP-40 buffer with protease inhibitor cocktail set 1 (CALBIOCHEM). Lysates were boiled in loading buffer and separated in 4% or 4–20% SDS-polyacrylamide gel, and then transferred onto PVDF membrane. Antibodies were MAB2166 (mouse anti-huntingtin from Chemicon) at 1:2000, MAB1574 (mouse anti-polyglutamine from Chemicon) at 1:2000, mouse anti-actin (Sigma) at 1:5000, rabbit anti Bcl-2 (Santa Cruz biotech, Inc) at 1:3000, mouse anti-HSP-70 (Chemicon) at 1:1000 and horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham). Those samples run on 4–20% SDS-PAGE gradient gels were transferred to a membrane, the lower portion of which was cut off and probed with anti-actin antibody. For those samples run in 4% SDS-PAGE gel, a 12% SDS-PAGE gel was loaded with identical samples and run in parallel. Both gels were transferred to PVDF membranes, and the former was probed with anti-huntingtin antibody while the latter was probed with anti-actin.

Following staining with the respective primary antibodies, transfer membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ). The doubly-labeled blots were incubated with ECL.
reagents (Amersham Pharmacia Biotech) and the signal was captured on Kodak film. After being developed, the films were scanned. Band densities were analyzed using Quantity One software. The signal from huntingtin protein was normalized with respect to that from actin, yielding a ratio of huntingtin to actin. Signals obtained using test siRNAs were compared to one obtained with the control RI-htt siRNA (/control).

**Caspase activity assay**

Caspase-3-like activity was measured by the hydrolysis of DEVD-AFC according to the instruction in a fluorometric protease assay kit (Chemicon). The experimental protocols have been described in a previous study (Zhang et al., 2006)

**Immunocytochemistry**

HD or control fibroblasts were grown in cell culture chamber slides. 72 hours after transfection with RI-htt or s4, cells were washed in PBS and incubated with mouse anti-ubiquitin (Zymed) 1: 500 for 2h at room temperature. After washing with PBS, cells were incubated with Fluorescein conjugated anti-mouse IgG (Vector) 1: 500 for 1h. Slides were washed and counterstained with Hoechst 33342 (1: 100,000; Molecular Probe, Oregon) for 10 minutes. Cells with abnormally shaped or fragmented nuclei were counted from a total of seven slide chambers from four independent experiments. The percentage of positive cells per slide was obtained by averaging counts made in 6–7 different viewings each of which located ca. 200 cells.

**Statistical Analysis**

Data are presented as the mean ± SEM. Statistical comparisons were made using the Student’s t test.

**Results**

Obtaining an appropriate cell line is the first step towards testing RNAi as a way to silence an HD gene marked by the $\Delta^{2642}$ deletion. To this end, we evaluated human HD fibroblast cell lines GM00305, GM06274, and GM09197 obtained from the NIGMS Human Genetic Mutant Cell Repository of the Coriell Institute for Medical Research (Camden, NJ). Using RT-PCR we determined that the GM09197 cell line contained a pathogenic HD allele with three rather than four GAG triplets at the polymorphic site in exon 58 (Fig. 1a). The 5’ CAG repeat size of this line is 151/21 (Sathasivam et al. 2001). We designed and synthesized four siRNAs which spanned the polymorphic site (Fig. 1b) and used them to transfect GM09197 fibroblasts. 72 hours later, we lysed the cells and analyzed the extract by immunoblotting with the 1C2 antibody against the expanded polyglutamine repeat. HeLa cell lysate was used as a control, as that human cell line only expresses wild-type huntingtin. We found that siRNAs 2 and 4 (“s2” and “s4”) worked better than siRNA of s1 and s3. Analysis of the immunoreactive mutant huntingtin bands from five independent experiments revealed that siRNA of s1 reduced the level of mutant huntingtin 32% (P<0.01) and siRNA s4 causes a 43% reduction of mutant huntingtin levels relative to what is observed with the control RI-htt sequence (a siRNA with the reverse nucleotide sequence of I-htt) (p<0.001) (Fig. 2a). Blotting with an antibody (MAB2166) that recognizes both mutant and wild-type huntingtin, we demonstrated that sequence 4 specifically reduced the level of mutant huntingtin while preserving that of wild-type protein (Fig. 2b). As an additional control, we transfected fibroblasts with I-htt siRNA, a polynucleotide homologous to HD mRNA at a non-polymorphic site (Zhang et al. 2006). As expected for a siRNA that recognizes both alleles, I-htt reduced the level of both wild-type and mutant huntingtin. To preclude the possibility that RI-htt itself induces mutant huntingtin expression, we compared levels of wild-type and mutant huntingtin following treatment with transfection reagents but no siRNA. As is
apparent from figure 2c, this blank control is no different from the negative control (i.e., treatment with RI-htt) in that neither treatment significantly changes wild-type or mutant huntingtin levels (Fig. 2c). In addition to actin as a control for protein loading, Bcl-2 and HSP-70 (heat shock protein 70kDa) were blotted to check for the possibility of a non-specific effect on overall protein expression resulting from the siRNA transfection. As demonstrated in figure 2d, there is no difference between RI-htt and s4 transfected cells in Bcl-2 and HSP-70 protein expression. To evaluate the efficacy of different sequences in the suppression of mutant huntingtin and preservation of the wild-type protein, we tested the four siRNAs. At concentration of 50 nM, four sequences have no significant effect on wild-type huntingtin. In contrast to the effective and specific action of siRNAs of s2 and s4 on mutant huntingtin, siRNA of s1 and s3 were relatively if not entirely inert (Fig. 2e).

We followed up the immunoblotting experiments with the more precise technique of real-time qRT-PCR to measure the efficiency and specificity of gene silencing. Following transfection with each of the four siRNAs, total cellular RNA was isolated. cDNA sequences from mutant and wild-type HD messages were amplified using primers specific for one or the other allele (Fig. 2f). Transfection with s4 siRNA decreased the level of mutant HD mRNA by 51\% relative to what was observed with RT-htt. s2 siRNA lowered HD mRNA levels by 38\%, and s3 by 31\%. Sequence 1 did not alter the level of mutant HD mRNA to a significant extent. Just as was observed upon measuring the amount of polyQ-expanded huntingtin protein, sequence 4 proved to be the most effective at decreasing the concentration of the encoding HD mRNA. Additionally, silencing of mutant HD mRNA was specific, as there was no statistically-significant change in levels of wild-type HD mRNA.

A more efficient assay system was constructed in which the relevant sequences from wild-type HD allele (50 bp) or the mutant HD allele (47 bp) was inserted into the 3′-untranslated region of the firefly luciferase gene, thereby generating a fusion to this reporter gene (Fig. 3a). HeLa cells were doubly transfected with various concentrations of the s4 siRNA and the wild-type or mutant construct. (The insert in Lucwt2642 has four GAG triplets at the polymorphic locus; that in Lucmu2642 has only three.) When measured 40hrs after transfection, light emission from luciferase was found to decrease monotonically with increasing concentrations of siRNA. The dose-response curve was as follows: transfecting Lucmu2642 cells with 12.5, 25, 50, 100, and 200nM s4 siRNA caused 4\%, 48\%, 48\%, 50\%, and 70\% reductions in light emission relative to identically treated Lucwt2642-carrying cells. (A mock transfection had no effect on light emission from either system.) We chose to treat cells with 20nM of each siRNA, a concentration found to effectively silence the Δ2642 transcript while barely reducing expression of the wild-type (mismatched) mRNA. Using this concentration of each siRNA, sequence 4 and sequence 2 proved physiologically active: s4 reduced the luciferase signal by 51\%; s2 diminished it by 24\% (Fig. 3b). Sequence 4 consistently exhibited the highest specificity for the mutant over the wild-type HD allele. The s4 molecule’s effectiveness at silencing the Δ2642 transcript was roughly twice as great as its off-target reduction of the wild-type HD mRNA (data not shown).

We repeated these experiments using the SH-SY5Y line of human neuroblastoma cells. The result in this system resembled those from the HeLa-cell system: siRNAs of sequences 2, 3, and 4 reduced the luciferase activity from the mutant HD allele construct by 54\%, 41\% and 63\%, respectively. Again, none of the four test siRNAs silenced the wild-type HD construct (Fig. 3c). The extent of silencing as a function of siRNA concentration was different in the SH-SY5Y system than in the HeLa-based one. In both cases, however, it was sequence 4 which exhibited the highest degree of efficacy and specificity in silencing the Δ2642 transcript relative to its effect on the mismatched wild-type mRNA. These results suggest that gene silencing by siRNA is possible among a broad range of cell types including neurons.
Previous reports described abnormal nuclear morphology in fibroblasts derived from HD patients as compared to what’s seen in cells from healthy persons (Sathasivam et al. 2001). We speculated that this abnormal phenotype is caused by the polyQ-expanded huntingtin protein. To test this hypothesis, we determined whether silencing the mutant HD allele corrects this phenotype. Upon staining with a fluorescently-labeled anti-ubiquitin antibody, we observed that HD cells had brighter inclusion-like structures (Fig. 4e–j) than did normal human fibroblasts (Fig. 4a–d). In addition, HD fibroblasts exhibited such abnormalities as irregularly-shaped nuclei, a high degree of nuclear condensation, and a substantial frequency of multiple or fragmented nuclei (Fig 4f, h, j). Abnormal nuclear morphology was identified in 22.8 ± 2.5 % of HD cells as compared with in 5.7 ± 1.6 % of control cells. Seventy-two hours after transfection with s4 siRNA, Sequence 4 siRNA reduced the frequency of HD cells with nuclear fragmentation by 43 % (p=0.052) and nuclear irregularity by 39 % (P<0.05) (Fig. 4k). The frequency of HD cells with total abnormal nuclear morphology was 40% lower than in the same cells transfected with RI-htt (p<0.01).

We have previously reported that wild-type huntingtin inhibits the activation of caspase-3. In cells with a depressed level of huntingtin, we observed significant activation of caspase-3, as well as increased vulnerability to cell death. Mutant huntingtin also binds and inhibits caspase-3, although it does so to a lesser extent than the wild-type protein (Zhang et al. 2006). To determine the effect of specific silencing of mutant huntingtin on caspase-3 activity, we used a fluorogenic assay which measures the extent of DEVD-AFC cleavage. Human fibroblasts with both the mutant and wild-type HD allele were transfected with s4 or I-htt siRNA. As we reported for other cultured cells (Zhang et al. 2006), the simultaneous knockdown of both alleles by I-htt resulted in an increase of caspase-3-like activity. However, the specific knockdown of mutant huntingtin by s4 did not induce any activation of caspase-3 over what is observed in cells transfected with control siRNA of RI-htt (Fig. 4l). We also measured caspase-3 activity in physiologically stressed cells. Fifty six hours after their transfection with test or control siRNAs, HD fibroblasts were treated with H$_2$O$_2$. This procedure increased caspase-3 activity by 9.8-fold for RI-htt transfected HD cells. When cells transfected with s4 siRNA, the same stress caused a lesser 7.1-fold increase in caspase-3 activity. H$_2$O$_2$-treatment of fibroblasts transfected with I-htt siRNA caused a 12.9-fold increase caspase-3 activity. The latter result is not surprising in light of wild-type huntingtin’s property of inhibiting caspase-3. In summary, siRNAs which specifically silence expression of the mutant HD allele reduce H$_2$O$_2$’s toxicity towards the host fibroblasts.

**Discussion**

We demonstrated that RNA interference can be used to specifically knock down expression of mutant huntingtin while preserving that of its wild-type counterpart. These capacities of RNA interference -- **efficacy** and **specificity** -- suit it to be a therapy for HD. HD patients are heterozygous for a harmful polyQ-expanded allele of the HD gene and a vital wild-type allele. The results reported above show that siRNAs have the potential for suppressing synthesis of the pathogenic species while preserving levels of its essential counterpart.

Δ2642 is a deletion mutation in exon 58 of the HD gene, a change that occurs more frequently in polyQ-expanded alleles than in wild-type ones. It results in the deletion of one among four tandem GAG triplets. This in-frame mutation is silent; removal of a single glutamine residue from HD does not alter its function. Consequently, Δ2642 makes for a good tag by which to distinguish linked polymorphisms, *e.g.*, varying numbers of CAGs near the HD gene’s 5’ end.

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We determined the feasibility of specifically targeting a Δ2642-tagged gene for polyQ-expanded huntingtin in two model systems: transfected HeLa (non-neural) and SH-SY5Y (neuroblastoma) cell lines. These immortalized cells were transfected with plasmds encoding either 50 bases from wild-type exon 58 or 47 bases from its Δ2642 mutant counterpart. These cells were treated with four test siRNAs, each one designed to span the polymorphic site. All had perfect homology to the Δ2642 sequence but carried one or more mismatches to the wild-type one. (The sequences of these four siRNAs are shown in figure 1.) Among the candidate siRNAs, the s4 molecule (5′-GAGGAAAGAGGAGGAGCCGAC-3′) was particularly effective at silencing the Δ2642 allele while preserving expression of its wild-type counterpart. In a HeLa-based system, transfection with 20nM s4 siRNA lowered expression from the Lucmu2642 plasmid to just half the level observed with the Lucwt2642 control.

All four siRNAs were designed to have perfect homology with the wild-type (four GAGS) within their “seed region”. This portion of the siRNA is particularly important to the molecule’s ability to bind the target transcript. As one would expect, siRNA function is highly sensitive to mismatches in this portion of the sequence. Nevertheless, we found that mismatches outside the seed region are sufficient to eliminate most of the siRNAs’ effects on the wild-type mRNA. When aligned with the wild-type allele (i.e., the one without the Δ2642 deletion), sequence 1 has a U:G wobble at position 20, sequence 2 has a U:G wobble at position 19 and sequence 4 has a U:G wobble and a G:U wobble at positions 16 and 19. Sequence 3 has the greatest number of mismatches with the wild-type allele: a U:G wobble at position 13, a G:U wobble at position 16, and a C:G mismatch at position 19. It was therefore predicted that sequence 3 would have the greatest specificity in silencing the mutant allele over the wild-type. In fact, results from both the transfected neuroblastoma cells and HD fibroblast cells indicated that siRNA of sequence 4 has the highest ability to specifically silence the mutant mRNA as compared to the wild-type. It is unclear what molecular properties of sequence 3 siRNA prevent it from discriminating between the exact and imperfect targets. Sequence 4 has neither a seed-region mismatch nor a central mismatch relative to wild-type HD mRNA. Unexpectedly, mismatches 3′ to the seed region of sequence 4 (i.e., at position 16 and 19) seem to cause the siRNA’s preference for the mutant target. The specificity of these siRNAs for the target sequence fits with the documented effects of a mismatch at position 16 (Schwarz et al. 2006). The obvious conclusion is that the current computer algorithms only provide guidelines for the design of siRNA sequence which act both efficiently and specifically. The experimenter must synthesize a pool of candidate siRNAs and select physiologically useful molecules by a trial-and error approach.

The current literature includes studies in which RNA interference has been used to silence exogenous disease-associated genes (i.e., ones introduced by transfection) (Abdelgany et al. 2003; Miller et al. 2003; Schwarz et al. 2006). In addition, RNA interference has been shown to effectively silence exogenous mutant HD messages in vivo (Harper et al. 2005; Difiglia et al. 2007). These studies targeted human HD mRNA in transgenic mouse models for HD. In both situations, the siRNA distinguished between the human HD gene and its murine equivalent. There is far from perfect homology between the two sequences, so there is little difficulty designing siRNAs which act with both efficacy and specificity. The existence of the Δ2642 polymorphism associated with mutant huntingtin alleles (Ambrose et al. 1994; Novelletto et al. 1994; Almqvist et al. 1995; Rubinsztein et al. 1995; Vuillaume et al. 1998) enabled us to apply this technology to distinguish between two human HD alleles. Our experiments with HD fibroblasts demonstrate the feasibility of silencing an endogenous pathogenic allele while preserving expression of its vitaly important counterpart. The current work required that the allele encoding polyQ-expanded huntingtin must be marked by the Δ2642 deletion. Because many pathogenic HD alleles do not carry that mutation, we
are searching for other polymorphism that can be used to distinguish between the harmful and the vitally important HD alleles.

The technology of RNA interference holds great promise as a novel therapy for molecular diseases. There remains a major barrier to the usage of RNAi for treating ailments of the CNS: siRNAs are incapable of crossing the blood-brain barrier. Recent developments may overcome this problem. In particular, a siRNA molecule was covalently attached to a peptide derived from fused a rabies virus glycoprotein (RVG) via a nine D-arginine linker. Because the RVG interacts with the nicotinic acetylcholine receptor, the molecular complex is indeed taken up into the CNS (Kumar et al. 2007). An important part of our future experimental agenda is to use this molecular technique to introduce siRNAs that target mutant HD alleles.

This study provides proof of principle that a specific polymorphism may be exploited for the selective reduction of mutant huntingtin in HD while preserving the critical function of the normal protein. Not like other traditional drug development target on disease associated protein, the most promising of siRNA therapy is to block expression of the mutant protein at the RNA level, thereby preventing physiological changes at their source. With further chemical modifications to enhance the efficiency and delivery in vivo, this approach will meet the challenges to develop allele specific siRNA therapy for HD. Additionally, in HD patients not carrying this polymorphism, both alleles may be sequenced in order to, in a patient-specific manner, target alternate loci in the mutant gene. This approach may be viewed as a door to personalized genetic medical therapeutics. What’s more, our findings on therapies for HD may serve as prototypes in the study of other autosomal dominant diseases.

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Figure 1.
(a) Identification of the Δ2642 triplet deletion in one of four HD fibroblast cells. RT-PCR was conducted using primers spanning the Δ2642 polymorphic locus for three HD fibroblast lines. The reaction products were denatured and resolved in a 6% denaturing TBE-Urea polyacrylamide gel. Lane 1: HeLa cells as control; Lane 2: HD fibroblast-GM00305; Lane 3: HD fibroblast-GM06274; Lane 4: HD fibroblast-GM09197. A1 denotes the presence of codon 2642 (112-bp product); A2 denotes the absence of codon 2642 (109-bp product). (b) Sequences of four siRNAs designed to span the polymorphic locus (wild-type or Δ2642).
Allele-specific silencing of endogenous mutant huntingtin. (a) A western blot using 1C2 (MAB1574, Chemicon), an antibody that specifically recognizes mutant huntingtin protein, demonstrates the reduction of mutant huntingtin by siRNAs 2 and 4. (Lysates were prepared 72 hours after transfection. 10 μg/lane protein was resolved in 4–20% SDS-PAGE. Note that the MW of huntingtin is approximately 350kDa). A siRNA sequence targeting both the mutant and the wild-type allele (I-htt) was used as a positive control; its reverse sequence (RI-htt) was the negative control. Densitometric analyses of blots from five independent experiments demonstrate that transfection with siRNA of sequence 4 (s4) reduced the level of mutant huntingtin by 43%. The lower part of the membrane was probed with an anti-actin antibody to demonstrate equal loading. Data are presented as the mean ± SEM. Statistical comparisons were made by the Student’s t-test. HeLa cell lysate was used as a negative control, as these cells only express wild-type huntingtin. * P < 0.05, ** P < 0.01, and *** P < 0.005 as compared to cells transfected with RI-htt. (b) Western blotting using the MAB2166 antibody demonstrates both the reduction of mutant huntingtin and the preservation of wild-type protein in lysates of cells treated with test siRNA s4. An immunoblot of cells treated with I-htt serves as a positive control; one with RI-htt as a negative control. (c) An immunoblot probed with MAB2166 antibody demonstrates that RI-htt (50 nM) doesn’t change levels of either wild-type or mutant huntingtin protein in HD fibroblasts. Control is HD cell transfected without any siRNA. (d) Immunoblots probed with the MAB2166 antibody, and with anti-Bcl-2 and anti-HSP-70 antibodies demonstrated that these two proteins are not affected by siRNA of sequence 4 while there is a significant and specific reduction of mutant huntingtin. (e) A western blot of HD fibroblast lysates was probed with MAB2166 (Chemicon) to determine the effects of the four synthetic siRNAs. HeLa cell
lysate was used as a control for wild-type huntingtin. Actin was demonstrated that samples were loading equally. Densitometry was performed on gels from six independent experiments, and signals from huntingtin protein were normalized relative to those from actin. Values for huntingtin in cells transfected with test siRNAs were normalized against ones from cells treated with RI-htt. The data is plotted as the mean ± SEM. * P < 0.05, ** P < 0.01, and *** P < 0.005 as compared to control cells transfected with siRNA of the RI-htt sequence.

(f) Real-time qRT-PCR analysis of the endogenous wild-type HD mRNA (wt-allele) and mutant HD mRNA (mu-allele) after transfection with each of the four test siRNAs. HD fibroblasts were transfected with 50 nM of each siRNA for 72 hours and total RNA was harvested for subsequent qRT-PCR analysis. Measurements of the abundance of HD mRNA were normalized relative to signals from GAPDH mRNA in the same sample. Data are reported relative to samples treated with the control sequence. Following transfection with the four test siRNAs, we never observed a significant change in the level of wild-type HD mRNA. By contrast, siRNAs with sequence 2, 3, and 4 all reduced the level of mutant HD mRNA to an appreciable degree, and sequence 4 effected a remarkable 51% drop in the concentration of that message. Data is reported as the average of four independent experiments ± SEM, * P < 0.05, ** P < 0.01, *** P < 0.001 as compared to mutant HD mRNA level after transfection with the control sequence. n = 4.
Figure 3.
Further confirmation of the specificity of siRNA-mediated silencing of the mutant HD allele using a luciferase reporter assay. (a) Sequences of the inserts present in the luciferase reporter constructs. The first is a 50 bp fragment from the “wild-type” HD allele having codon 2642 and thus four tandem GAG triplets (Lucwt2642); the second is a 47 bp fragment from the “mutant” HD DNA lacking codon 2642 and thus having three GAGs (Lucmu2642). Like the immunoblotting techniques described above, the luciferase-based system was used to measure gene silencing by siRNAs. (b) The Lucwt2642 or Lucmu2642 DNA construct was transfected into HeLa cells together with 20 nM siRNA. Luciferase assays were performed 40 hours after transfection. The signal from luciferase fused to the control sequence in the Luc-wt2642 construct was not diminished by any of the four test siRNAs. By contrast, trasfection with siRNA of sequence 2 or 4 decreased the signal from cells with the Lucmu2642 plasmid. This change in light emission was statistically significant (**P<0.001, n= 6). Only sequence 4 siRNA showed allele-specific inhibition of luciferase expression, i.e., a statistically significant difference in the signals from the Lucwt2642 or Lucmu2642 systems. (# indicates this comparison. P<0.05 as compared to luciferase activity in the Lucwt2642 system transfected with the same siRNAs). Data from six distinct experiments are shown with bars indicating standard errors (± SEM). (c) SH-SY5Y.
neuroblastoma cells containing Lucwt2642 or Lucmu2642 were transfected with 10 nM each of the four test siRNAs. Forty hours after transfection, luciferase activity from the Lucwt2642 system was not diminished by any of the siRNAs. By contrast, when cells with the Lucmu2642 construct were transfected with s2, s3 or s4 siRNA, they showed significantly less luciferase activity than did the negative control (RT-htt). (*\( P<0.05 \), ***\( P<0.001 \), \( n=3 \)). Sequence 4 siRNA exhibited the greatest degree of selectivity in its inhibition of light emission from the Lucmu2642 system relative to that from Lucwt2642 (#\( P<0.05 \), ##\( P<0.01 \) as compared to luciferase activity from cells with Lucwt2642 that had been transfected with same siRNA sequence). The results from three independent experiments are plotted with bars indicating standard error mean (± SEM).
Figure 4.
Allele-specific silencing of endogenous mutant huntingtin by siRNA reduced the frequency of HD fibroblasts with abnormal nuclear morphology. It also reduced the extent to which treatment with H$_2$O$_2$ induced caspase-3 activation. (a–j) Control human fibroblasts and HD fibroblasts were stained with Hoechst 33342 dye to reveal nuclear morphology. In contrast to the regular nuclear shape in control cells (a–d), HD cells frequently exhibited nuclear atypia (e–f) and fragmentation (g–j). Sequence 4 siRNA reduced the fraction of HD cells with abnormally shaped and fragmented nuclei from 24 ± 2% to 14 ± 1% (k). The percentage of positive cells was obtained 6–7 different viewings of 200 cells. Cells were counted from a total of seven slides from four independent experiments, *p < 0.05, **p < 0.01 as compared to RI-htt transfected controls. (Counting was performed by an investigator blinded to the treatment conditions). (l) Caspase-3-like activity was measured in lysates of H$_2$O$_2$–treated or naïve cells by the hydrolysis of DEVD-AFC. Before exposure to H$_2$O$_2$ (or mock treatment), cells were transfected with siRNA molecules RI-htt, I-htt, or s4. Even when not exposed to 100 μM H$_2$O$_2$, caspase-3 activity increased following transfection with I-htt. (No such effect was observed following transfection with s4 siRNA.) Fifty-six hours after transfection with RI-htt, I-htt or s4, 100 μM of H$_2$O$_2$ was added for an additional 16 hours. In all cases, caspase-3 activity increased. The greatest increase was observed among HD fibroblasts transfected with I-htt; the least with cell treated with s4. * P < 0.05 as compared to cells transfected with RI-htt. n=6.