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Safe and Efficient Silencing with a Pol II, but Not a Pol III, Promoter Expressing an Artificial miRNA Targeting Human Huntingtin

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Huntington’s disease is a devastating, incurable neurodegenerative disease affecting up to 12 per 100,000 patients worldwide. The disease is caused by a mutation in the Huntington (Htt) gene. There is interest in reducing mutant Huntingtin by targeting it at the mRNA level, but the maximum tolerable dose and long-term effects of such a treatment are unknown. Using a self-complementary AAV9 vector, we delivered a mir-155-based artificial miRNA under the control of the chicken β-actin or human U6 promoter. In mouse brain, the artificial miRNA reduced the human huntingtin mRNA by 50%. The U6, but not the CβA promoter, produced the artificial miRNA at supraphysiologic levels. Embedding the antisense strand in a U6-mir-30 scaffold reduced expression of the antisense strand but increased the sense strand. In mice treated with scAAV9-U6-mir-155-HTT or scAAV9-CβA-mir-155-HTT, activated microglia were present around the injection site 1 month post-injection. Six months post-injection, mice treated with scAAV9-CβA-mir-155-HTT were indistinguishable from controls. Those that received scAAV9-U6-mir-155-HTT showed behavioral abnormalities and striatal damage. In conclusion, miRNA backbone and promoter can be used together to modulate expression levels and strand selection of artificial miRNAs, and in brain, the CβA promoter can provide an effective and safe dose of a human huntingtin miRNA.

INTRODUCTION

Huntington’s disease (HD) is a devastating inherited neurodegenerative disease caused by an expansion of the CAG repeat region in exon 1 of the huntingtin gene.1 Although huntingtin is expressed throughout the body,2 the polyglutamine expanded protein is especially toxic to medium spiny neurons in the striatum and their cortical connections.3 Patients struggle with emotional symptoms, including depression and anxiety, and with characteristic movement disturbances and chorea. Whereas the mutant protein exerts its toxic effects through myriad cellular pathways, elimination of huntingtin in the striatum has the potential to improve the lives of patients by treating some of the severe effects of the disease. Our goal is to reduce huntingtin in the brain using an artificial microRNA (miRNA) targeting human huntingtin mRNA, which can be delivered using a recombinant adeno-associated virus (AAV) vector and which will be safe and effective for long-term use.

RNAi-based therapy depends on successful delivery to striatal medium spiny neurons and to neurons in layers 5 and 6 of the cortex. Unfortunately, the blood-brain barrier limits the distribution of systemically delivered oligonucleotide therapeutics to the CNS. Following a single injection, antisense oligonucleotides targeting the human huntingtin mRNA can provide a sustained reduction in human huntingtin mRNA lasting up to 3 months.4 Nevertheless, therapeutics based on chemically synthesized oligonucleotides would necessitate repeated administration to maintain silencing. Recombinant AAV vectors can deliver an RNAi effector in the form of a short hairpin RNA (shRNA) or artificial miRNA and, in the non-dividing cells of the brain, a single dose is expected to last indefinitely.5 AAV vector-mediated RNAi has enormous potential for chronic, severe diseases such as HD.6,7

Initial studies using AAV-mediated RNAi focused on shRNAs as the effector molecules. shRNAs are transcribed from polymerase III promoters (usually the U6 or H1 promoter) and are designed to bypass Drosha/DGCR8 cleavage. After export from the nucleus by exportin 5, shRNAs are cleaved by Dicer to form a duplex that can be loaded into Argonaute-RISC complexes. shRNAs are simple and effective and, with care, off-target effects due to improper strand loading or imprecise Dicer cleavage can be minimized.8,9 However, shRNAs are often produced at extremely high levels, and toxicity due to
miRNA cleavage site.  Three of the nine arti
ture 1A). We have previously shown that probes upstream and down-
PCR probe targeting the boundary between exons 64 and 65 (Fig-
measured the levels of endogenous huntingtin mRNA using a qRT-
fected into HeLa cells, and 48 hr later, we harvested the cells and
a chicken beta-actin promoter. The resulting plasmids were trans-
of EGFP (Figure 1B, top), which was expressed under the control of
the safety of vector delivered small RNAs.8

arms reduces expression of the mature small RNA and improves
ery11, in practice the incorporation of endogenous miRNA
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the safety of vector delivered small RNAs.8

 oversaturation of the RNAi machinery has been reported in the liver,
heart, and CNS.10-11 In contrast to shRNAs, artificial miRNAs are de-
dsigned to undergo cleavage both by Drosophila and Dicer. They
can be transcribed from their own promoters, embedded in an intron,
or located in the 3'-UTR of a protein-coding gene. Although in theory
artificial miRNAs could also saturate the endogenous RNAi machin-
ery11, in practice the incorporation of endogenous miRNA flanking
arms reduces expression of the mature small RNA and improves

RESULTS

Design and Selection of Huntingtin Targeting Artificial miRNAs

We selected nine sequences targeting the human huntingtin mRNA
(Table 1; Figure 1A). These sequences were selected on the basis of
known targeting rules.16 We cloned two copies of the artificial
miRNA in tandem into a backbone on the basis of the endogenous
miRNA 155 and placed the entire artificial miRNA into the 3'-UTR of
EGFP (Figure 1B, top), which was expressed under the control of a
chicken beta-actin promoter. The resulting plasmids were trans-
ferred into HeLa cells, and 48 hr later, we harvested the cells and
measured the levels of endogenous huntingtin mRNA using a qRT-
PCR probe targeting the boundary between exons 64 and 65 (Fig-
ure 1A). We have previously shown that probes upstream and down-
stream of this target site report consistent levels of silencing of Yac128
mRNA using assays upstream and downstream of the artificial
miRNA cleavage site.1 Three of the nine artificial miRNAs reduced
huntingtin by >50% (Figure 2A).

We selected the three best sequences from our initial screen for in vivo
experiments. In addition, we included an artificial miRNA on the ba-
sis of a previously published small interfering RNA (siRNA) (E1.4).18
We packaged these candidates into a self-complementary AAV9
vector and injected it directly into the striatum of transgenic mice ex-
pressing human huntingtin with a stretch of approximately 128 poly-
glutamine encoding repeats (Yac128 mice).15 At a vector dose of 3.0 \times 10^8
vg/striatum, GFP staining was present throughout the striatum,
and human huntingtin mRNA was significantly reduced in mice
treated with either scAAV9-CBA-anti-HTT-6433 (p = 0.00077) (Fig-
ure 2B) compared with mice treated with an scAAV9-GFP.

Table 1. Predicted Antisense Sequences Targeting Human Huntingtin

| Name               | Predicted Antisense Sequence            |
|--------------------|----------------------------------------|
| miR-E14-anti-HTT    | 5'-UUCAUCAGCUUUUCCAGGGUC-3'            |
| miR-178-anti-HTT    | 5'-GUUGGACCGAGGGGUCGUUC-3'             |
| miR-1873-anti-HTT   | 5'-UAAUGUCCUGUUGAGGGG-3'               |
| miR-2029-anti-HTT   | 5'-AAGGUGCGAGAGUCUCAC-3'               |
| miR-473-anti-HTT    | 5'-UUCUGGAGCAUCAAACCAAU-3'             |
| miR-4448-anti-HTT   | 5'-UGACUGGGCAUCUCAUGUG-3'              |
| miR-5155-anti-HTT   | 5'-UAGGGUGAAGUCUGUCCCC-3'              |
| miR-6088-anti-HTT   | 5'-UUCUAAUGGGCAACUGGCAUA-3'            |
| miR-6433-anti-HTT   | 5'-UAAAGCAUGGACGACGCUCC-3'             |

Expressing the Artificial miRNA from the CBA Promoter Is
Sufficient for Maximal Silencing of Huntingtin mRNA

To investigate the possibility that increasing the expression further
would improve silencing, we cloned a single copy of the most potent
miRNA into an AAV9 vector under the control of the U6 promoter
(Figure 1B, bottom). Mice were injected unilaterally with the original
two-copy scAAV9-CBA-anti-HTT-6433, scAAV9-CBA-anti-HTT-
5155, scAAV9-U6-anti-HTT-6433, or scAAV9-U6-anti-HTT-5155.
One month later, we harvested the striatum and confirmed GFP
expression. We measured the levels of huntingtin mRNA by qRT-
PCR. There was a significant reduction in human huntingtin in
mice treated with scAAV9-U6-anti-HTT-5155, scAAV9-CBA-anti-
HTT-6433, or scAAV9-U6-anti-HTT-6433 but not in those treated
with scAAV9-CBA-anti-HTT-5155 compared with the contralateral
(non-injected) side (Figure 2C). We compared the relative quantity
of the mature artificial miRNA guide strand by qPCR. Expression
from scAAV9-U6-anti-HTT-6433 was about 150 times higher than
from scAAV9-CBA-anti-HTT-6433 (Figure S1).

Using the contralateral side as a control for each animal reduces the
inter-animal variability by controlling for animal to animal variation
in huntingtin expression. This approach assumes that there is no
spread to the contralateral side. Therefore, using the contralateral side as the
control may underestimate silencing. To eliminate this potential
confounding effect, we repeated the experiment using a group of animals
injected with PBS as the control. We confirmed that both scAAV9-
CBA-anti-HTT-6433 and scAAV9-U6-anti-HTT-6433 reduced hun-
ingtin mRNA by approximately 50% in the striatum (Figure 2D).
We did not observe a difference in silencing between the two studies, sug-
gest that spread to the contralateral side is insufficient to produce
silencing. To determine if we could achieve huntingtin silencing with
a lower vector dose, we injected mice (n = 3/group) with vector
diluted by 0.5 log (final dose 1.5 \times 10^9
vg/striatum) and 1 full log
(final dose 3.0 \times 10^8
vg/striatum). GFP is present in 89% of the stria-
um with the highest dose of the vector, but reducing the dose of the vector
results in reduced spread (Figures 3A and 3B) and decreased
silencing of human huntingtin mRNA (Figure 3C).

Expression of the Artificial miRNA Targeting Huntingtin from a
U6 Promoter Results in Expression of Multiple Small RNA
Species at Levels Comparable with Those of Endogenous
miRNAs

To examine whether the same small RNA species were produced
from processing of both the U6 and CBA promoter-driven artificial
miRNA, we injected groups of mice unilaterally with either
scAAV9-CBA-anti-HTT-6433 or scAAV9-U6-anti-HTT-6433. We
cloned and sequenced the total 18- to 30-nucleotide small RNAs at
2 weeks post-injection. We mapped the sequences back onto the pre-
dicted hairpin structure of the artificial miRNA (Figure 4A). In both
scAAV9-CBA-anti-HTT-6433- and scAAV9-U6-anti-HTT-6433-in-
jected groups, 96% of the sequences mapping to the AAV genome
were the expected small RNA product. Imprecise Dicer or Drosha cleavage of miRNA precursors can result in small RNAs with heterogeneous 5′ ends. These noncanonical small RNA isoforms (isomirs) can have a different target profile than the canonical isoform. Therefore, we looked at the distribution of 5′ ends along the sequence of the pre-miRNA. Fewer than 4% of the small RNAs produced from scAAV9-CbA-anti-HTT-6433 and scAAV9-U6-anti-HTT-6433 were noncanonical isomirs (Figure 4B; Table 2). However, because of the high levels of expression produced by the U6 promoter, it should be noted that these represent a much higher proportion of the total small RNA pool in the scAAV9-U6-anti-HTT-6433 group than in those mice injected with scAAV9-CbA-anti-HTT-6433. In the group injected with the U6 promoter-driven artificial miRNA, however, the huntingtin-targeting sequence dominated the sequencing results, accounting for half (50%) of the combined genome matching and AAV vector matching sequences, whereas in the mice injected with the CbA vector, only 5% of the sequences matched the vector-encoded small RNA (Figure 4B). This finding means that small RNAs with alternative seed sequences, including the sense strand, +1 and /C0 products, could be present at levels comparable with functional endogenous miRNAs.

Endogenous miRNA 30 sequences are commonly used as a scaffold for artificial miRNA.8,20 To determine if the isomir profiles derived from this scaffold were more favorable, we embedded the anti-HTT-6433 sequence in a miR-30 backbone and injected into 10-week-old Yac128 mice (Figure 4C). The miR-30 scaffold produces levels of the mature artificial miRNA comparable with those produced by the CbA promoter (Figure 4D) and also reduces human huntingtin by close to 50% (Figure S2). Unlike the mir-155 scaffold, the mir-30 scaffold produces the mature sense (passenger) strand at levels comparable with the antisense (guide) strand. When designing artificial miRNA, target selection can be critical to introducing the correct asymmetry. Here, miRNA backbone can be used as an additional method to control the asymmetry of artificial miRNA. For this huntingtin-targeting sequence, the combination of CbA promoter with mir-155 backbone is unique, as it is the only combination that produces only the intended antisense strand above background (Figure 4B).

**Long-Term Striatal Expression of mir-HTT-6433 from a U6 Promoter Is Toxic**

Having established that both scAAV9-U6-anti-HTT-6433 and scAAV9-CbA-anti-HTT-6433 silence human huntingtin in the short term, we wished to evaluate the duration of effect and long-term consequences of expression and overexpression of the huntingtin-targeting artificial miRNA. We injected scAAV9-U6-anti-HTT-6433 or scAAV9-CbA-anti-HTT-6433 unilaterally into the striatum of Yac128 mice. Six months after injection, we noticed that the mice injected with scAAV9-U6-anti-HTT-6433 appeared behaviorally abnormal. When a new nestlet is placed in the cage, normal mice will shred the material, producing a nest. The mice injected with scAAV9-U6-anti-HTT-6433 appeared to leave the bedding material untouched. To document this, we replaced the nestlets in each cage. Twenty-four hours later, the mice treated with scAAV9-U6-anti-HTT-6433 had not used the new nestlets, whereas the bedding of

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**Figure 1. Design of Artificial miRNAs Targeting Human Huntingtin**

(A) Position of target sites on the human huntingtin miRNA. Target sites are distributed throughout the coding region. The location of the qPCR assay, which spans the junction between exons 64 and 65, is also depicted. (B) AAV9 constructs expressing a miRNA from the CbA (polII) and U6 promoters. The CbA promoter-driven miRNA is located in the 3′-UTR of the GFP gene (top), whereas the construct containing the U6 promoter-driven artificial miRNA co-expresses GFP from a separate promoter (bottom).
and data are expressed relative to this group (n = 5 mice/group). Again, both scAAV9-C
independent replicates.

5155 reduce human huntingtin significantly in the striatum (p < 0.0001, p = 0.003, and p < 0.0001 respectively, two-way ANOVA). (D) Relative quantity of huntingtin mRNA in
PBS and scAAV9-CbA-anti-HTT-6433 mice looked as expected (Figure 5A). Using an automated home-cage monitoring system, we recorded the mice for 24 hr. This system produces unbiased tracking of the movements of the mice over the course 24 hr, without interference from the experimenter. Individual mice are placed in the cage, and the computer records the amount of time they spend moving versus remaining stationary. The mice treated with scAAV9-U6-anti-HTT-6433 spent significantly more time moving around their home cage than mice treated with PBS or with scAAV9-CbA-anti-HTT-6433 (Figure 5B). Finally, we measured the average time it took for the mice to cross an elevated beam. For this test, we require that the mice complete the beam crossing three times. Whereas both scAAV9-CbA-anti-HTT-6433-injected and PBS-injected mice crossed the beam easily, two of the four remaining mice in the scAAV9-U6-anti-HTT-6433 group were unable to successfully cross, either jumping or falling off the beam (Figure 5C). We repeated this experiment with a larger group of animals, testing them on the beam at regular intervals. Mice injected with scAAV6-U6-anti-HTT-6433 at 6 weeks of age were unable to cross the beam by 4 months post-injection (Figure S3A). Older Yac128 mice (injected at 7 months of age) exhibited an age-related increase in time to cross the beam. This increase was present in both naive mice and in mice treated with AAV9-CbA-anti-HTT-6433. Like the younger animals, older mice treated with scAAV9-U6-anti-HTT-6433 showed a deterioration of beam crossing 4 months post-injection (Figure S3B). By 6 months post-injection, three of five animals injected with AAV9-U6-anti-HTT-6433 failed to cross the beam in under 1 min, whereas all of the mice in the AAV9-CbA-anti-HTT-6433 and naive groups crossed within that time.

Neuropathological findings explained the behavioral outcomes. On the injected side, the scAAV9-U6-anti-HTT-6433 mice showed enlargement of the ventricle, loss of DARPP-32-positive neurons, and striatal shrinkage (Figure 6). It has previously been shown that Yac128 mice display increased numbers of activated microglia compared with wild-type mice.21 We expected, on the basis of the results in cell culture. Mice (n = 5/group) were injected unilaterally in the striatum. One month after injection, the striatum was harvested, and GFP expression was verified. Data are normalized to HPRT and expressed relative to the GFP control. Only scAAV9-CbA-anti-HTT-6433 human huntingtin significantly (p = 0.0007, one-way ANOVA) relative to the GFP control. (C) Relative quantity of huntingtin mRNA in the injected striatum 1 month after injection of the U6 and CbA promoter-driven artificial miRNAs targeting sites

Figure 2. Artificial miRNAs Targeting Human Huntingtin Reduce the Huntingtin mRNA in Cell Culture and In Vivo

Expressing an artificial miRNA from the U6 promoter does not improve silencing of huntingtin in the mouse striatum. (A) HeLa cells were transfected with plasmids expressing artificial miRNAs targeting human huntingtin, and the huntingtin mRNA levels were measured after 48 hr by qPCR. Huntingtin expression was normalized to HPRT to account for well-to-well variation in cell number and is expressed relative to the naive cells. Error bars represent SE of three independent experiments. The negative control is an anti-SOD1 miRNA, and the positive control is an siRNA (E1-4) targeting exon 1 of the huntingtin mRNA. Data were analyzed by one-way ANOVA with Bonferroni comparison. Three artificial miRNAs, 5155 (p = 0.03), 1873 (p = 0.04), and 6433 (p = 0.06), significantly reduce the native huntingtin mRNA relative to untreated HeLa cells. (B) Top candidates were selected for in vivo testing on the basis of the results in cell culture. Mice (n = 5/group) were injected unilaterally in the striatum. One month post-injection, the striatum was harvested, and GFP expression was verified. Data are normalized to HPRT and expressed relative to the GFP-only control. Only scAAV9-CbA-anti-HTT-6433 reduced human huntingtin significantly (p = 0.0007, one-way ANOVA) relative to the GFP control. (D) Relative quantity of huntingtin mRNA in the injected striatum 1 month after injection of the U6 and CbA promoter-driven artificial miRNAs targeting sites

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Neuropathological findings explained the behavioral outcomes. On the injected side, the scAAV9-U6-anti-HTT-6433 mice showed enlargement of the ventricle, loss of DARPP-32-positive neurons, and striatal shrinkage (Figure 6). It has previously been shown that Yac128 mice display increased numbers of activated microglia compared with wild-type mice.21 We expected, on the basis of the results in cell culture. Mice (n = 5/group) were injected unilaterally in the striatum. One month after injection, the striatum was harvested, and GFP expression was verified. Data are normalized to HPRT and expressed relative to the GFP control. Only scAAV9-CbA-anti-HTT-6433 reduced human huntingtin significantly (p = 0.0007, one-way ANOVA) relative to the GFP control. (C) Relative quantity of huntingtin mRNA in the injected striatum 1 month after injection of the U6 and CbA promoter-driven artificial miRNAs targeting sites
striatum, the scAAV9-U6-anti-HTT-6433 mice exhibited increased Iba1 staining (Figure 7A, bottom), an increase in total and activated microglia, and a decrease in the number of resting microglia (Figures 7B–7D). This suggests an ongoing innate immune response, which could be a result of or participate in striatal cell death. In contrast, by 6 months post-injection, the mice treated with scAAV9-CbA-anti-HTT-6433 do not show an increase in microglial activation.

We considered the possibility that Yac128 mice, which may have a “primed” immune system, may be more susceptible to toxicity by miRNA overexpression than wild-type mice. To determine if toxicity was dependent on the presence of mutant huntingtin, we injected wild-type C57BL/6 mice and FVB mice with the same vectors and assessed the consequences of the U6-driven miRNA in that context. In FVB mice, the effect was similar to that in Yac128 mice with rapid degeneration on the beam and severely enlarged ventricles (data not shown). In C57BL6 mice, the effect was present but less pronounced (Figure S3). Although there was an initial increase in time to cross the beam in the U6 cohort, at the study endpoint, there was no significant difference between groups (Figure S4A). Striatal shrinkage was also less severe in the C57BL6 mice (Figure S4B).

We examined the small RNA sequencing for clues to explain the toxicity. At 1 week post-injection, the mice treated with CbA-anti-HTT-6433 produced 32 endogenous miRNAs whose expression was changed greater than 2-fold compared with PBS-injected mice (Table S1). Of these, 18 (56%) were downregulated, whereas 14 (44%) were upregulated. In the mice treated with U6-anti-HTT-6433, 58 miRNAs were significantly altered, with 33 being downregulated (57%) and 25 (43%) being upregulated. We also performed RNA sequencing (RNA-seq) on total RNA. In the mice injected with AAV9-U6-anti-HTT-6433, 44 transcripts were significantly downregulated, while 30 were significantly upregulated compared with the naive control. In the AAV-CbA-anti-HTT-6433 group, 12 transcripts were downregulated and 4 were upregulated. Overall, transcripts containing a seed sequence target are downregulated in the scAAV-U6-anti-HTT-6433 group but not the scAAV9-CbA-anti-HTT-6433 group (Figures S5B and S5F). Those originating from the +1 and −1 positions and the sense strand (Figures S5A, S5C, S5D, S5E, S5G, and S5H) were not altered. This result suggests that off-target effects are exacerbated by overexpression and are likely to be due to overexpression of the intended huntingtin-targeting artificial guide strand rather than to the presence of other small RNAs with alternative seed sequences.

DISCUSSION

We have achieved a higher level of expression of the vector-encoded small RNA than has been previously described for artificial miRNAs.
We show that the mir-155 backbone produces mostly the intended small RNA species, reducing the chance of off-target effects due to improper strand loading or imprecise processing. We also show that the mir-155-based artificial miRNA using a CβA promoter reduces human huntingtin by 50% at 1 month and causes no overt toxicity up to 6 months. Using a U6 promoter increases the levels of this artificial miRNA guide strand but does not result in additional huntingtin lowering. Expression of the anti-HTT artificial miRNA from a CβA promoter in the context of the scAAV9 and the mir-155 backbone is sufficient to achieve maximum silencing from a direct injection. We have previously reported that the same artificial miRNA reduces human huntingtin by up to 80% in liver.17 Unlike the liver, the striatum contains a highly heterogeneous population of cell types, including medium spiny neurons, cholinergic and GABAergic interneurons, astrocytes, oligodendrocytes, and microglia. In homozygous Q140 knockin mice, individual medium spiny neurons show variable levels of huntingtin mRNA expression.23 Treatment with an artificial miRNA targeting mouse huntingtin shifts the distribution, but does not eliminate huntingtin mRNA from DARPP32-positive medium spiny neurons.23 This suggests that a relatively small amount of huntingtin mRNA is present in non-neuronal cell types and hints at the presence of a miRNA-inaccessible pool of huntingtin mRNA in medium spiny neurons. Additional delivery routes, AAV optimization, improved formulation, and additional therapeutic moi-

eties could improve silencing by improving delivery to other cell types, increasing distribution and targeting inaccessible huntingtin mRNA.

There are several possible explanations for the observed toxicity of the AAV9-U6-anti-HTT-6433. First, overexpression of the miR-155-based hairpin results not only in overexpression of the intended mature artificial miRNA strand, but it also reveals additional processing products. These additional products are expressed at levels comparable with functional endogenous and artificial miRNAs and increase the potential for sequence-specific off-target effects. However, our results indicate that only the artificial miRNA guide strand is expressed at a high enough level to produce a detectable global pattern of seed-mediated silencing. So, although we have been unable to find a single off-target mRNA that would explain the observed toxicity, overexpression of the guide strand may in fact be revealing an otherwise hidden off-target effect. However, species-specific toxicities due to seed-mediated off-target effects have been reported for other huntingtin-targeting artificial miRNAs.24 Second, overexpression of an exogenous miRNA might disrupt the balance of endogenous miRNAs. In liver, expression of an shRNA causes a reduction in the predominant liver miRNA, mir-122.15 In brain, there is no corresponding dominant miRNA; nonetheless, it is conceivable that overexpression of an artificial miRNA causes a disruption in
endogenous miRNA in brain. Additional explanations for the observed toxicity include saturation of various components of the cellular RNAi machinery (Argonaute2, exportin-5) and an innate immune response. We do see evidence for a long-lived immune response in the form of activated microglia that persist up to 1 month post-injection, even in mice treated with AAV9-C5A-anti-HTT-6433. These toxicities may be exacerbated by ongoing disease processes or the presence of a toxic disease-related transgene. The fact that some strains of mice exhibit accelerated pathology should encourage us to consider factors that might influence susceptibility to such toxicity and caution when transitioning vectors from one animal species or model to another.

In non-human primates, partial silencing of huntingtin with a U6 promoter-driven, mir-30-based artificial miRNA appears safe up to 6 weeks, and intrathecal delivery of a U6 promoter-driven artificial miRNA targeting SOD1 is safe in non-human primates. It is possible that the toxicity of the vector carrying the U6 promoter is exacerbated by the method of delivery, particularly in the small brain of a mouse. When we deliver an AAV vector by direct injection to the brain of a mouse. When we deliver an AAV vector by direct injection to the brain, the sense and antisense strands were produced at very similar levels. In contrast, in the context of the mir-155 backbone, there was strong asymmetry, and the antisense strand predominated. Asymmetric strand selection of miRNA is due primarily to thermodynamic instability at the 5’ end of the guide/antisense strand. When a different, luciferase-targeting sequence is embedded in the mir-155 backbone, both strands were produced in roughly equal proportions. However, components of the pri-miRNA may also influence asymmetry. Slight differences in the construction of the miRNA backbone are common. These differences likely affect processing efficiency and fidelity of artificial miRNAs, while interactions between the targeting sequence and backbone determine strand selection. A comprehensive analysis of pri-miRNA features was used to design from scratch a miRNA scaffold that is processed as efficiently as the most commonly used endogenously derived miRNA scaffolds. This should allow further refinement and improvement of artificial miRNA.

MATERIALS AND METHODS

Cell Culture and Screening Assays

HeLa cells were maintained in DMEM, high glucose with 10% heat-inactivated fetal bovine serum (FBS), and 1% penicillin streptomycin (ThermoFisher). Twenty-four hours before transfection, cells were seeded onto six-well plates at 0.8 × 10^6 cells/well. On the day of transfection, we first replaced the growth medium with 1.6 mL of Opti-MEM (ThermoFisher). Plasmids were transfected in triplicate using 2 μL/well of DharmaFECT Duo (Dharmacon). Each well received 0.5 μg of plasmid DNA. Forty-eight hours after transfection, the cells were harvested, and total RNA was extracted using the MirVana RNA isolation kit. We made cDNA using 1 μg of RNA per reaction using oligo-dT and Superscript III (Invitrogen). Htt mRNA was measured using TaqMan assay # (ThermoFisher). Relative levels of Htt mRNA were calculated using the ΔΔC(T) method, with human hypoxanthine-guanine phosphoribosyltransferase (Hprt) as the housekeeping gene. Each experiment was performed three times using three independent cell passages.

Mouse Housing, Injections, and Maintenance

YAC128 and wild-type FVB mice were purchased from The Jackson Laboratory. They were bred on the FVB background by mating wild-type male mice with YAC128 females. The resulting heterozygous YAC128 and wild-type mice were maintained on a 12:12 light schedule and were given access to food and water ad libitum. All animals were maintained and used according to the Institutional Animal

| Table 2. Percentage of Total Hairpin Mapping Reads for Each Seed Sequence Represented in the Small RNA Sequencing Results |
| Seed Sequence (2-8) | U6-Anti-HTT-6433 | C5A-Anti-HTT-6433 |
|---------------------|-----------------|-----------------|
| GUAAGCA (2-2)       | 0.01            | 0.01            |
| UAAAGCAU (1-1)      | 0.05            | 0.57            |
| AAGCAUG (antisense) | 96.43           | 96.68           |
| AGCAUGG (+1)        | 0.97            | 0.85            |
| GCAUGGA (+2)        | 0.03            | 0.04            |
| CAUGGAG (+3)        | 0.03            | 0.02            |
| AUGGAGC (+4)        | 0.09            | 0.07            |
| UGGAGCU (+5)        | 0.53            | 0.42            |
| GGAGCUA (+6)        | 0.23            | 0.15            |
| GAGCUAG (2-2)       | 0.06            | 0.09            |
| AGCUAGC (+1)        | 0.04            | 0.04            |
| AGCCUGC (sense)     | 1.49            | 0.97            |
| GCCUGCU (+1)        | 0.03            | 0.02            |
| CUGCUCU (+2)        | 0.00            | 0.03            |
| UGCUCUC (+3)        | 0.00            | 0.01            |
| Total               | 99.99           | 99.97           |
Care and Use Committee guidelines of University of Massachusetts Medical School (docket A978-12 or A978-15). Genotypes were verified by PCR of DNA extracted from tail snips or ear punches. Mice were injected with selected AAV directly into the striatum by means of a small animal stereotax SAS-4100 (ASI Instruments) aided by UMPC3 or UMPC4 microinjectors (World Precision Instruments). Mice were anesthetized with 284 mg/kg of tribromoethanol and placed in the stereotax. Surgery was performed using the bregma as the zero point, measuring anterior 1.0 mm, lateral 2.0 mm, and lowering a 33 gauge needle 3.0 mm into the striatum. The pumps were set to deliver $3.0 \mu$L at a rate of 125 nL/min. After the injections the mice were allowed to recover on a warming pad and then placed back in their cages in the housing area. Females were housed together in groups of three and males were housed separately to prevent fighting.

**Figure 5. Long-Term Striatal Expression of mir-HTT-6433 from a U6 Promoter Causes Behavioral Abnormalities**

(A) Six months post-injection, mice injected with the U6 promoter-driven mir-HTT-6433 failed to make nests. When new nestlets are placed in their cages, mice injected intrastrially with PBS (left) or with CβA-mir-HTT-6433 (center) shred the bedding, making a nest. In contrast, mice injected with U6-mir-HTT-6433 do not. Pictures were taken 24 hr after placing new nestlets in the cage. (B) Home cage monitoring of Yac128 mice treated with PBS, CBA-mir-HTT-6433, or U6-mir-HTT-6433. Animals were individually placed in an automated home cage monitoring system. The amount of time spent moving around the cage was recorded for 24–27 hr. Average time per hour was calculated by dividing the total amount of time by the number of hours of recording ($n = 4$ U6-mir-HTT-6433, $n = 5$ PBS and CβA-mir-HTT-6433). (C) Animals were trained to cross a round beam and enter a darkened box. For each mouse, the average time to cross from one end was calculated for three recorded trials. If a mouse failed to cross within 60 s, we recorded the time as >60 s. Error bars in (B) and (C) represent SE of independent replicates.

**Mouse Behaviors**

**Beam Walking**

Mice were trained to cross a beam 2 cm in diameter. After training, we recorded the mice as they crossed from one end of the beam to the other. We recorded three trials per mouse. From the recording we measured the amount of time it took for the mice to cross from mark on one end of the beam to the other. The investigator was not blinded to the treatment group of each mouse.
ment. We then divided the total time spent walking by the total time. One hour of data, during which the mouse acclimates to the new environment, was then removed from the analysis.

Immunohistochemistry and Quantification

To calculate the average active time per hour, we first calculated the activity of the mouse during both the active and inactive periods. The percentage of active time was determined for each hour. The activity was defined as the number of movements divided by the total number of movements during the hour. The active period was defined as the time during which the mouse was moving. The inactive period was defined as the time during which the mouse was not moving.

Immunohistochemistry was performed using Vector Laboratories Elite ABC kits. Fixed tissue slices were blocked with 3% hydrogen peroxide for 3 min. Immunohistochemistry was performed using the Metal DAB Substrate Kit (Pierce). The sections were then incubated with 0.5% triton x for 20 min. Immunocytochemistry was performed using Vector Laboratories Elite ABC kit reagents.

Cloning of Artificial miRNA and AAV Packaging

Artificial miRNAs were designed and cloned according to the protocol detailed by Toro Cabrera and Mueller. AAVs were packaged by the University of Massachusetts vector core, according to published protocols.

Small RNA Library Cloning and Analysis

Total RNA was extracted using the MirVana RNA isolation kit, according to the manufacturer’s protocol. Size selection of the 18- to 30-nucleotide RNAs was performed using 5 μg of total RNA on a 15% denaturing polyacrylamide gel. Following size selection, the small RNAs were ethanol precipitated and ligated to a pre-adenylated 3′-adaptor (5′-rAppTGGAATTCTCGGGTGCCAAGG/ddC/-3′). The ligated products were annealed to the RT primer (5′-CCTTGACACGGAGAATTCCA-3′) and ligated to a 5′-adaptor (RNA: 5′-GUUCAGAGUUC UACAGUCGACGAUC-3′). Reverse transcription was performed using AMV Reverse transcriptase mix (NEB) and PCR-amplified using AccuPrime Pfx DNA Polymerase (Invitrogen) with one universal primer (5′-AATGATACGGCGACCACCGAGATCTACACGTTCA GAGTCTACAGTCCGA-3′) and one barcoded primer (5′-CAAGC AGAAGACGGGATCCACGAGTNNNNNTGACTAGTTCTGGTGCACCCGAGATTTCCA-3′). Libraries were sequenced on the Illumina HiSeq at the University of Massachusetts Deep Sequencing Core. Libraries were mapped to the mm9 genome and to the AAV genome using bowtie. We classified miRNA species on the basis of the position of the 5′ end mapping on the miRNA hairpin; therefore each species consists of all the small RNAs with shared seed sequences.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.omtn.2017.04.011.
AUTHOR CONTRIBUTIONS
Conceptualization, E.L.P., N.A., and C.M.; Methodology, E.L.P. and C.M.; Investigation, K.O.C., L.A.K., R.M., F.C., E.J., F.B.; Formal Analysis, H.S.; Writing—Original Draft, E.L.P.; Writing—Review and Editing, N.A. and C.M.; Project Management, E.L.P.; Funding Acquisition, N.A. and C.M.; Supervision, N.A. and C.M.

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