AAV TREATMENT OF HUNTINGTON'S DISEASE

RELATED APPLICATIONS

This Application claims the benefit under 35 U.S.C. 119(e) of the filing date of U.S. Provisional Application Serial Number 62/398,487, filed September 22, 2016, entitled "AAV TREATMENT OF HUNTINGTON'S DISEASE", the entire contents of which are incorporated herein by reference.

FEDERALLY SPONSORED RESEARCH

This invention was made with government support under NS038194 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

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. While the Huntingtin protein (HTT) is expressed throughout the body, the polyglutamine expanded protein is especially toxic to medium spiny neurons in the striatum and their cortical connections. Patients struggle with emotional symptoms including depression and anxiety and with characteristic movement disturbances and chorea. There is currently no cure for Huntington's disease; therapeutic options are limited to ameliorating disease symptoms.

SUMMARY

Aspects of the disclosure relate to compositions and methods useful for treating Huntington's disease (HD). In some embodiments, inhibitory nucleic acids (*e.g.*, miRNAs, such as artificial miRNAs) are provided that hybridize specifically to and inhibit expression of human huntingtin (HTT).

Accordingly, in some aspects, the disclosure provides an isolated nucleic acid comprising or encoding the sequence set forth in any one of SEQ ID NO: 2-10 or 21-22. In some embodiments, human huntingtin comprises a sequence as set forth in SEQ ID NO: 1. In some embodiments, the disclosure provides an nucleic acid (*e.g.*, a miRNA) that is complementary to at least two (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25) continuous bases of SEQ ID NO: 1.

In some aspects, the disclosure provides an isolated nucleic acid comprising: a first region comprising a first adeno-associated virus (AAV) inverted terminal repeat (ITR), or a variant thereof; and, a second region comprising a transgene encoding one or more miRNAs. In some embodiments, the sequence encoding each miRNA comprises a sequence set forth in any one of SEQ ID NOs: 2-10 flanked by sequence encoding a miRNA backbone sequence.

In some embodiments, each miRNA backbone sequence is a mir-155 backbone sequence, a mir-30 backbone sequence, or a mir-64 backbone sequence.

In some embodiments, the transgene further comprises a nucleic acid sequence encoding a promoter. In some embodiments, the promoter is a chicken beta-actin (CBA) promoter or a U6 promoter.

In some embodiments, the transgene further comprises a nucleic acid sequence encoding a protein. In some embodiments, the protein is a therapeutic protein (*e.g.*, non-mutant huntingtin) or a reporter protein (*e.g.*, a fluorescent protein, such as GFP).

In some embodiments, the one or more miRNAs is located in an untranslated portion of the transgene. In some embodiments, the untranslated portion is an intron. In some embodiments, the untranslated portion is between the last codon of the nucleic acid sequence encoding a protein and a poly-A tail sequence. In some embodiments, the untranslated portion is between the last nucleic acid base of a promoter sequence and the first base of a poly-A tail sequence.

In some embodiments, the isolated nucleic acid further comprises a third region that comprises a second adeno-associated virus (AAV) inverted terminal repeat (ITR), or a variant thereof.

In some embodiments, the first or second ITR variant lacks a functional terminal resolution site (TRS), optionally wherein the ITR variant is a ΔTRS ITR.

In some embodiments, at least one of the miRNAs hybridizes with and inhibits expression of human huntingtin.

In some aspects, the disclosure provides a vector comprising an isolated nucleic acid as described by the disclosure. In some embodiments, the vector is a plasmid.

In some aspects, the disclosure provides a host cell comprising an isolated nucleic acid or a vector as described by the disclosure.

In some aspects, the disclosure provides a recombinant AAV (rAAV) comprising: a capsid protein; and, an isolated nucleic acid as described by the disclosure.

In some embodiments, the capsid protein is an AAV9 capsid protein. In some embodiments, the capsid protein comprises the sequence set forth in SEQ ID NO: 20.

In some embodiments, the rAAV is a self-complementary AAV (scAAV).

In some embodiments, the rAAV is formulated for delivery to the central nervous system (CNS).

Aspects of the disclosure relate to isolated nucleic acids capable of reducing (*e.g.*, inhibiting) expression of pathogenic huntingtin and thus may be useful for the treatment of Huntington's disease. Accordingly, in some aspects, the disclosure provides a method for treating Huntington's disease in a subject in need thereof, the method comprising administering to a subject having or at risk of developing Huntington's disease a therapeutically effective amount of an isolated nucleic acid or rAAV as described by the disclosure.

In some embodiments, the subject comprises a huntingtin gene having more than 36 CAG repeats, more than 40 repeats, or more than 100 repeats. In some embodiments, the subject is less than 20 years of age, or is diagnosed as having juvenile HD.

In some embodiments, the administration results in delivery of the isolated nucleic acid or rAAV to the central nervous system (CNS) of the subject. In some embodiments, the administration is via injection, optionally intravenous injection or intrastriatal injection.

In some aspects, the disclosure provides an isolated nucleic acid comprising a transgene encoding one or more miRNAs, wherein the sequence of the transgene encoding each miRNA comprises a sequence set forth in SEQ ID NO: 7 or 8 flanked by a miRNA backbone sequence.

In some embodiments, each miRNA backbone sequence is a mir-155 backbone sequence, a mir-30 backbone sequence, or a mir-64 backbone sequence.

In some embodiments, the transgene comprises a promoter. In some embodiments, the promoter is a chicken beta-actin (CBA) promoter or a U6 promoter.

In some embodiments, the transgene comprises the sequence set forth in SEQ ID NO: 21 or 22.

In some embodiments, the transgene is flanked by adeno-associated virus (AAV) inverted terminal repeats (ITRs), or variants thereof. In some embodiments, the ITR variant lacks a functional terminal resolution site (TRS). In some embodiments, the ITR variant lacking a TRS is a ΔTRS ITR.

In some aspects, the disclosure provides a vector comprising an isolated nucleic acid comprising a transgene encoding one or more miRNAs, wherein the sequence of the transgene

encoding each miRNA comprises a sequence set forth in SEQ ID NO: 7 or 8 flanked by a miRNA backbone sequence. In some embodiments, the vector is a plasmid.

In some embodiments, each miRNA backbone sequence of the transgene is a mir-155 backbone sequence, a mir-30 backbone sequence, or a mir-64 backbone sequence.

In some embodiments, the transgene comprises a promoter. In some embodiments, the promoter is a chicken beta-actin (CBA) promoter or a U6 promoter.

In some embodiments, the transgene comprises the sequence set forth in SEQ ID NO: 21 or 22.

In some embodiments, the transgene is flanked by adeno-associated virus (AAV) inverted terminal repeats (ITRs), or variants thereof. In some embodiments, the ITR variant lacks a functional terminal resolution site (TRS). In some embodiments, the ITR variant lacking a TRS is a ΔTRS ITR.

In some aspects, the disclosure provides a recombinant AAV (rAAV) comprising a capsid protein; and, an isolated nucleic acid comprising a transgene encoding one or more miRNAs, wherein the sequence of the transgene encoding each miRNA comprises a sequence set forth in SEQ ID NO: 7 or 8 flanked by a miRNA backbone sequence.

In some embodiments, the transgene is flanked by full-length AAV ITR sequences. In some embodiments, the transgene is flanked by a full-length AAV ITR and a ΔTRS ITR.

In some embodiments, the capsid protein is an AAV9 capsid protein. In some embodiments, the capsid protein comprises the sequence set forth in SEQ ID NO: 20.

In some aspects, the disclosure provides a method for treating Huntington's disease in a subject in need thereof, the method comprising administering to a subject having or at risk of developing Huntington's disease a therapeutically effective amount of an rAAV as described herein (*e.g.*, an rAAV comprising a transgene encoding one or more miRNAs, wherein the sequence of the transgene encoding each miRNA comprises a sequence set forth in SEQ ID NO: 7 or 8 flanked by a miRNA backbone sequence.

In some embodiments, the transgene of the isolated nucleic acid is flanked by full-length AAV ITR sequences. In some embodiments, the transgene is flanked by a full-length AAV ITR and a ΔTRS ITR.

In some embodiments, the rAAV comprises an AAV9 capsid protein.

In some embodiments, the subject comprises a huntingtin gene having more than 36 CAG repeats, more than 40 repeats, or more than 100 repeats. In some embodiments, the subject is less than 20 years of age.

In some embodiments, the administration is via injection. In some embodiments, the injection is intravenous injection or intrastriatal injection.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 shows HeLa cells transfected with a plasmid expressing mir-HTT-6433, targeting human huntingtin. 48 hours after transfection, cells were harvested and RNA was extracted for quantitative RT-PCR (qRT-PCR). Results indicate that mir-HTT-6433 reduces the endogenous human huntingtin by up to 50%.

FIG. 2 shows mir-HTT-6433 packaged into an AAV9 vector and injected directly into the striatum of transgenic mice expressing mutant human huntingtin (Yac128 mice). One month post-injection, levels of human huntingtin mRNA were measured by qRT-PCR. In one set of animals (n=5) levels of human huntingtin were compared on the injected side with levels on the non-injected side. A significant (p=0.0017) reduction of huntingtin mRNA was observed on the injected side. In a second set of animals (n=5/group) levels of huntingtin mRNA were compared in animals injected with mir-HTT-6433 to animals who received an injection of vehicle only. A significant reduction (p=0.0004) of huntingtin was observed in these animals as well. In a third set of animals (n=5/group) the levels of huntingtin mRNA were compared in animals injected with mir-HTT-6433 to those injected with an AAV9-GFP. There was a significant (p=0.0064) reduction in huntingtin mRNA in these animals as well. In sum, data indicate that mir-HTT-6433 reduces huntingtin mRNA in vivo in the brain by 50%.

FIG. 3 shows injection of transgenic mice expressing mutant huntingtin (human) unilaterally with AAV9-mir-HTT-6433 or PBS. Six months post-injection, mice were tested on a balance beam. Data indicate that mice treated with mir-HTT-6433 show a decrease in the amount of time it took to cross the beam when compared to HD mice treated with PBS.

FIGs. 4A-4C show artificial miRNAs targeting human huntingtin reduce the huntingtin mRNA in cell culture and in vivo. FIG. 4A shows positions of target sites on the human huntingtin mRNA. FIG. 4B shows HeLa cells transfected with plasmids expressing artificial miRNAs targeting human huntingtin; the huntingtin mRNA levels were measured after 48 hours by qPCR. Huntingtin expression was normalized to HPRT to account for well to well variation in cell number and are expressed relative to the untreated/naïve control. Error bars represent standard error. FIG. 4C shows candidate miRNAs selected for *in vivo* testing based on the results in cell culture. Mice were injected unilaterally in the striatum. One month post-injection, the striatum was harvested and GFP positive tissue was dissected out. Data are normalized to HPRT and expressed relative to the GFP-only control.

FIGs. 5A-5C show expressing an artificial miRNA from the U6 promoter does not improve silencing of huntingtin in the mouse striatum. FIG. 5A shows data relating to 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, whereas the construct containing the U6 promoter driven artificial miRNA co-expresses GFP from a separate promoter. FIG. 5B shows relative quantity of huntingtin mRNA in the injected striatum following injection of the U6 and CBA-promoter driven artificial miRNAs targeting sites 5155 (left) and 6433 (right). Data are expressed relative to the non-injected side. FIG. 5C shows relative quantity of huntingtin mRNA in mice injected unilaterally with the U6 and CBA-promoter driven miRNA targeting site 6433. Data are expressed relative to the group of mice injected with a GFP-expressing control vector.

FIGs. 6A-6B show long-term striatal expression of mir-HTT-6433 from a U6 promoter causes behavioral abnormalities. FIG. 6A shows six months post-injection, mice injected with the U6-promoter driven mir-HTT-6433 failed to make nests. Pictures were taken 24 hours after placing new nestlets in the cage. FIG. 6B shows cage monitoring of Yac128 mice treated with PBS, CBA-mir-HTT-6433 or U6-mir-HTT-6433. The amount of time spent moving around the cage was recorded for 24-27 hours. Average time per hour was calculated by dividing the total amount of time by the number of hours of recording.

FIGs. 7A-7B show long-term expression of mir-HTT-6433 from a U6 promoter causes striatal shrinkage. FIG. 7A shows representative images of DARPP-32 staining on the injected side in Yac128 mice at 1 (top) and 6 (bottom) months post-injection. FIG. 7B shows quantification of DARPP-32 positive area 6 months post-injection.

FIGs. 8A-8D show long-term expression of mir-HTT-6433 from a U6 promoter causes persistent activation of microglia. FIG. 8A shows representative images of Iba1 staining on the injected side in Yac128 mice at 1 (top) and 6 (bottom) months post-injection. Images were taken at the site of injection. Quantification of total (FIG. 8B), activated (FIG. 8C) and resting (FIG. 8D) microglia at 6 months post injection are shown.

FIGs. 9A-9C show expression of the mir155 based artificial miRNA from a U6 promoter results in overexpression of the huntingtin targeting guide strand and other sequences. FIG. 9A shows start positions of reads mapping to the huntingtin targeting artificial miRNA hairpin (mir-155 backbone). Positions are reported relative to the mature strand and reads are normalized to the total number of endogenous miRNA mapped in each sample. The horizontal line represents the background levels of the artificial miRNA found in control samples. FIG. 9B shows relative quantification of mature miR-HTT (from mir-155 backbone) by quantitative RT-PCR. FIG. 9C shows start portions of reads mapping to the huntingtin targeting artificial miRNA embedded in a mir-30 backbone and expressed from a U6 promoter.

FIGs. 10A-10B show expression of mir-HTT-6433 preferentially decreases mRNAs with target sites. FIG. 10A shows mRNAs were divided into those containing canonical miRNA binding matching the artificial miRNA sites (legend) and those without. In the group of mice injected with AAV-CbA-mir-HTT-6433, there is no difference between mRNAs with and without such sites. FIG. 10B shows in contrast, in the AAV-U6-mir-HTT-6433 group there is a shift toward repression of mRNAs with perfect 8mer sites.

FIGs. 11A-11C show reducing the vector dose results in reduced spread and knockdown in the mouse striatum. FIG. 11A shows GFP staining in the striatum of mice were injected with a vector encoding both the huntingtin targeting artificial miRNA and EGFP. ImageJ was used to measure the percent of the striatum that was GFP positive. FIG. 11B shows quantitative RT-PCR

measuring human huntingtin mRNA in the striatum of Yac128 mice. FIG. 11C shows representative photographs of mice injected with a vector encoding both the huntingtin targeting miRNA and EGFP at three different doses. Data indicate reducing vector dose results in reduced spread and knockdown.

FIGs. 12A-12B show data indicating that Yac128 mice show a decline in ability to cross the beam following injection of the U6-promoter driven huntingtin targeting artificial miRNA. FIG. 12A shows mice injected at 2-3 months show a clear increase in time to cross the beam and some of them fail to cross altogether. FIG. 12B shows Yac128 mice injected with either PBS or CBA-mir-HTT-6433 at 7 months of age show an age-related decline in behavior on the beam. Injection with U6-mir-HTT-6433 (red dots) accelerates this decline.

FIGs. 13A-13B show C57BL/6 mice show an initial deterioration in behavior on the beam following injection of the U6-promoter driven huntingtin targeting artificial miRNA. FIG. 13A shows the amount of time taken to cross the beam for control (naïve) mice and mice injected with AAV-U6-mir-HTT-6433 and AAV-CbA-mir-HTT-6433. FIG. 13B shows quantification of DARPP-32 positive striatal area in control (naïve) mice and mice injected with AAV-U6-mir-HTT-6433 and AAV-CbA-mir-HTT-6433.

FIGs. 14A-14B show the distribution of endogenous miRNAs is largely unaffected following injection with mir-HTT-6433. FIG. 14A shows distribution in mice injected with AAV-CbA-mir-HTT-6433; levels of the mir-HTT-6433 guide and passenger strands are shown in green, in red are all endogenous miRNA species showing significant changes in the mice injected with AAV-CbA-mir-HTT-6433. FIG. 14B shows distribution in mice injected with AAV-U6-mir-HTT-6433.

FIGs. 15A-15C show mRNA profiles in mice treated with mir-HTT-6433. FIG. 15A shows mice injected with AAV-CbA-mir-HTT-6433 show few changes in mRNA expression; in green are all genes which show significant p-values, in blue are those that remain significant after adjustment for multiple comparison. FIG. 15B shows mice injected with AAV-U6-mir-HTT-6433 show more changes in mRNA expression compared to the CBA. FIG. 15C shows

mRNA profiles in mice treated with mir-HTT-6433; 7 RNAs are significantly differentially expressed between the U6 and CbA treated groups.

FIG. 16 shows data relating to relative expression of human huntingtin (human htt) RNA in the middle caudate of a sheep model of Huntington's disease one month after intrastriatal injection of either scAAV9 CBA-mir-HTT ("CBA Promoter"), scAAV9 U6-mir-HTT ("U6 Promoter"), or empty scAAV9 control vector. Data for htt expression level in un-injected control sheep is also shown. Relative htt expression levels were normalized to sheep calnexin. Note: a mir155 backbone was used in each of the CBA and U6 constructs.

FIG. 17 shows data relating to relative expression of human huntingtin (human htt) RNA in the middle putamen of a sheep model of Huntington's disease one month after intrastriatal injection of either scAAV9 CBA-mir-HTT ("CBA Promoter"), scAAV9 U6-mir-HTT ("U6 Promoter"), or empty scAAV9 control vector. Data for expression level in un-injected control sheep is also shown. Relative htt expression levels were normalized to sheep calnexin.

FIG. 18 shows data relating to relative expression of human huntingtin (human htt) RNA in the medial side of the middle caudate of a sheep model of Huntington's disease six months after intrastriatal injection of either scAAV9 CBA-mir-HTT ("CBA Promoter") or empty scAAV9 control vector. Data for expression level in the non-injected side and the injected side are shown. Relative htt expression levels were normalized to sheep calnexin.

FIG. 19 shows data relating to relative expression of human huntingtin (human htt) RNA in the lateral side of the middle caudate of a sheep model of Huntington's disease six months after intrastriatal injection of either scAAV9 CBA-mir-HTT ("CBA Promoter") or empty scAAV9 control vector. Data for expression level in the non-injected side and the injected side are shown. Relative htt expression levels were normalized to sheep calnexin.

FIG. 20 shows data relating to relative expression of human huntingtin (human htt) RNA in the middle caudate of a sheep model of Huntington's disease six months after intrastriatal injection of either scAAV9 CBA-mir-HTT ("CBA Promoter"), scAAV9 U6-mir-HTT ("U6

Promoter"), or empty scAAV9 control vector. Data for htt expression level in un-injected control sheep is also shown. Relative htt expression levels were normalized to sheep calnexin.

FIG. 21 shows data relating to relative expression of human huntingtin (human htt) RNA in the lateral side of the middle putamen of a sheep model of Huntington's disease six months after intrastriatal injection of either scAAV9 CBA-mir-HTT ("CBA Promoter") or empty scAAV9 control vector. Data for expression level in the non-injected side and the injected side are shown. Relative htt expression levels were normalized to sheep calnexin.

FIG. 22 shows data relating to relative expression of human huntingtin (human htt) RNA in the medial side of the middle putamen of a sheep model of Huntington's disease six months after intrastriatal injection of either scAAV9 CBA-mir-HTT ("CBA Promoter") or empty scAAV9 control vector. Data for expression level in the non-injected side and the injected side are shown. Relative htt expression levels were normalized to sheep calnexin.

FIG. 23 shows data relating to relative expression of human huntingtin (human htt) RNA in the middle putamen of a sheep model of Huntington's disease six months after intrastriatal injection of either scAAV9 CBA-mir-HTT ("CBA Promoter"), scAAV9 U6-mir-HTT ("U6 Promoter"), or empty scAAV9 control vector. Data for htt expression level in un-injected control sheep is also shown. Relative htt expression levels were normalized to sheep calnexin.

FIG. 24 shows data relating to relative expression of human huntingtin (human htt) RNA in the anterior striatum of a sheep model of Huntington's disease six months after intrastriatal injection of either scAAV9 CBA-mir-HTT ("CBA Promoter"), scAAV9 U6-mir-HTT ("U6 Promoter"), or empty scAAV9 control vector. Data for htt expression level in un-injected control sheep is also shown. Relative htt expression levels were normalized to sheep calnexin.

FIGs. 25A-25B show predicted hairpin structures of artificial miRNA targeting human huntingtin. FIG. 25A shows the predicted hairpin structure of mir-155-6433 (SEQ ID NO: 23). FIG. 25B shows the predicted hairpin structure of mir-30-6433 (SEQ ID NO: 24).

FIGs. 26A-26B show delivery of AAV vectors to sheep brain. FIG. 26A shows a schematic overview of a sheep brain dissected in the coronal plane (top), such that the entire striatum was contained within 4, 6mm blocks. The anterior block contains the anterior portion of the striatum which is not divided by the internal capsule (middle). The medial blocks, to which the injection is targeted have a defined putamen and caudate are shown on the bottom. FIG. 26B shows AAV vector genomes in control (AAV9) and treated (AAV9miRHTT) treated animals. Vector genomes were measured by digital droplet PCR using genomic HPRT as the reference gene. The values are plotted on a log scale.

FIG. 27 shows the artificial miRNA guide strand was quantified by digital droplet PCR. Relative miRNA levels were calculated by normalizing to let-7e*, and this value was plotted on a log scale. Samples with RQN < 5 were excluded. The number reported is the total number of samples that survived this quality threshold and were used in the miRNA analysis. P values were calculated using 2 -way ANOVA with Tukey's correction for multiple testing.

FIG. 28 shows scAAV9-anti-HTT-6433 reduces human mutant huntingtin mRNA in the striatum. Data shown are the signal for HTT mRNA normalized to sheep calnexin. Asterisks indicate significant differences in means between treatment groups (AAV9 or AAV9miRHTT) at p,0.03 or less with unpaired t-tests. The U6-promoter driven artificial miRNA significantly lowers human mutant HTT mRNA caudate and putamen at 1 month post-injection and in putamen at 6 months post-injection. The CBA-promoter driven artificial miRNA lowers the HTT mRNA in the caudate, putamen and anterior striatum at 1 month post-injection and in the caudate and putamen at 6 month post-injection. The medial region of the caudate, lateral putamen and anterior striatum were examined in the analysis.

FIGs. 29A-29B show levels of endogenous sheep htt mRNA and protein in AAV9 and AAV9miRHTT treated sheep. FIG. 29A shows sheep htt mRNA levels were determined as described in methods and are expressed relative to sheep calnexin (Canx). Shown are results from Study 2. There is no difference in endogenous sheep htt mRNA levels between AAV9 and AAV9miRHTT treated groups. FIG. 29B shows levels of endogenous sheep huntingtin and human mHTT protein detected with anti-htt1-17 antibody (Ab1) in putamen from Study 2, 6 months post-injection. Sample Western blot shows signal for wt htt (arrow) and human mutant

htt (arrowhead) for the injected and non-injected sides of the brain in 4 different sheep injected on one side with AAV9miRHTT. Graph shows mean wt sheep htt and human mHTT signals determined from the densitometry as percent injected side to non-injected side. Note that treatment with AAV9miRHTT does not affect levels of endogenous sheep huntingtin protein but significantly reduces levels of human mHTT. Asterisk indicates p=0.005 with unpaired t-test.

FIG. 30 shows that AAV9-miRHTT reduces the human mutant huntingtin protein in the striatum. Sample Western blots of putamen from Studies 1 and 2 show mutant HTT detected with antibody 3B5H10 and actin as loading control (top). A graph shows distribution of individual values and mean (horizontal bar) for sheep treated with either AAV9 (control) or AAV9-miRHTT (bottom). Shown are results for different striatal regions (caudate, putamen and anterior striatum) in studies 1 and 2 and 1 and 6 months post-injection. In study 2, 6 months post-injection two areas (Area 1 and 2) were examined in each region. Asterisks indicate significant difference on the injected side between AAV9 and AAV9-miRHTT at $p<0.05$ or less based on unpaired t-tests.

FIG. 31 shows human mutant HTT levels detected by MSD assay at 1 and 6 months post-injection in study 1 (U6 promoter) and study 2 (CBA promoter). Graph shows distribution of individual values and means (horizontal bars) for sheep treated with either AAV9 (control) or AAV9-miRHTT. Results are shown for different striatal regions (caudate, putamen and anterior striatum). Asterisks * indicate significant difference on the injected side between AAV9 and AAV9-miRHTT at $p<0.05$ or less based on unpaired t-tests.

FIGs. 32A-32B show mHTT levels are unchanged in the ipsilateral cortex and contralateral caudate putamen of miRHTT injected sheep striatum. FIG. 32A shows a graph indicating mean levels of mHTT normalized to actin in the cortex ipsilateral to the miRHTT injected striatum. Data are from study 1, 1 and 6 months post-injection, NS, based on unpaired t-test. FIG. 32B shows a bar graph indicating levels of mHTT normalized to actin in the caudate and putamen contralateral to the miRHTT-injected striatum. Data are from study 1, 1 and 6 months post-injection, NS, based on unpaired t-test.

DETAILED DESCRIPTION

Aspects of the invention relate to certain interfering RNAs (*e.g.*, miRNAs, such as artificial miRNAs) that when delivered to a subject are effective for reducing the expression of pathogenic huntingtin protein (HTT) in the subject. Accordingly, methods and compositions described by the disclosure are useful, in some embodiments, for the treatment of Huntington's disease.

Methods for treating Huntington's disease

Methods for delivering a transgene (*e.g.*, an inhibitory RNA, such as a miRNA) to a subject are provided by the disclosure. The methods typically involve administering to a subject an effective amount of an isolated nucleic acid encoding an interfering RNA capable of reducing expression of huntingtin (htt) protein, or a rAAV comprising a nucleic acid for expressing an inhibitory RNA capable of reducing expression of huntingtin protein.

In some aspects, the disclosure provides inhibitory miRNA that specifically binds to (*e.g.*, hybridizes with) at least two (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more) continuous bases of human huntingtin (*e.g..*, SEQ ID NO: 1). As used herein "continuous bases" refers to two or more nucleotide bases that are covalently bound (*e.g.*, by one or more phosphodiester bond, *etc.*) to each other (*e.g.* as part of a nucleic acid molecule). In some embodiments, the at least one miRNA is about 50%, about 60% about 70% about 80% about 90%, about 95%, about 99% or about 100% identical to the two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more) continuous nucleotide bases of SEQ ID NO: 1. In some embodiments, the inhibitory RNA is a miRNA which is comprises or is encoded by the sequence set forth in any one of SEQ ID NOs: 2-10.

As used herein, "Huntington's disease", or "HD", refers to a neurodegenerative disease characterized by progressively worsening movement, cognitive and behavioral changes caused by a tri-nucleotide repeat expansion (*e.g.*, CAG, which is translated into a poly-Glutamine, or PolyQ, tract) in the *HTT* gene that results in production of pathogenic mutant huntingtin protein (HTT, or mHTT). In some embodiments, mutant huntingtin protein accelerates the rate of neuronal cell death in certain regions of the brain. Generally, the severity of HD is correlated to the size of the tri-nucleotide repeat expansion in a subject. For example, a subject having a CAG repeat region comprising between 36 and 39 repeats is characterized as having "reduced

penetrance" HD, whereas a subject having greater than 40 repeats is characterized as having "full penetrance" HD. Thus, in some embodiments, a subject having or at risk of having HD has a *HTT* gene comprising between about 36 and about 39 CAG repeats (*e.g.*, 36, 37, 38 or 39 repeats). In some embodiments, a subject having or at risk of having HD has a *HTT* gene comprising 40 or more (*e.g.*, 40, 45, 50, 60, 70, 80, 90, 100, 200, or more) CAG repeats. In some embodiments, a subject having a *HTT* gene comprising more than 100 CAG repeats develops HD earlier than a subject having fewer than 100 CAG repeats. In some embodiments, a subject having a *HTT* gene comprising more than 100 CAG repeats may develop HD symptoms before the age of about 20 years, and is referred to as having juvenile HD (also referred to as akinetic-rigid HD, or Westphal variant HD). The number of CAG repeats in a *HTT* gene allele of a subject can be determined by any suitable modality known in the art. For example, nucleic acids (*e.g.*, DNA) can be isolated from a biological sample (*e.g.*, blood) of a subject and the number of CAG repeats of a *HTT* allele can be determined by a hybridization-based method, such as PCR or nucleic acid sequencing (*e.g.*, Illumina sequencing, Sanger sequencing, SMRT sequencing, *etc.*).

An "effective amount" of a substance is an amount sufficient to produce a desired effect. In some embodiments, an effective amount of an isolated nucleic acid is an amount sufficient to transfect (or infect in the context of rAAV mediated delivery) a sufficient number of target cells of a target tissue of a subject. In some embodiments, a target tissue is central nervous system (CNS) tissue (*e.g.*, brain tissue, spinal cord tissue, cerebrospinal fluid (CSF), *etc.*). In some embodiments, an effective amount of an isolated nucleic acid (*e.g.*, which may be delivered via an rAAV) may be an amount sufficient to have a therapeutic benefit in a subject, *e.g.*, to reduce the expression of a pathogenic gene or protein (*e.g.*, HTT), to extend the lifespan of a subject, to improve in the subject one or more symptoms of disease (*e.g.*, a symptom of Huntington's disease), *etc.* The effective amount will depend on a variety of factors such as, for example, the species, age, weight, health of the subject, and the tissue to be targeted, and may thus vary among subject and tissue as described elsewhere in the disclosure.

Isolated nucleic acids

In some aspects, the disclosure provides isolated nucleic acids that are useful for reducing (*e.g.*, inhibiting) expression of human huntingtin (HTT). A "nucleic acid" sequence refers to a DNA or RNA sequence. In some embodiments, proteins and nucleic acids of the

disclosure are isolated. As used herein, the term "isolated" means artificially produced. As used herein with respect to nucleic acids, the term "isolated" means: (i) amplified *in vitro* by, for example, polymerase chain reaction (PCR); (ii) recombinantly produced by cloning; (iii) purified, as by cleavage and gel separation; or (iv) synthesized by, for example, chemical synthesis. An isolated nucleic acid is one which is readily manipulable by recombinant DNA techniques well known in the art. Thus, a nucleotide sequence contained in a vector in which 5' and 3' restriction sites are known or for which polymerase chain reaction (PCR) primer sequences have been disclosed is considered isolated but a nucleic acid sequence existing in its native state in its natural host is not. An isolated nucleic acid may be substantially purified, but need not be. For example, a nucleic acid that is isolated within a cloning or expression vector is not pure in that it may comprise only a tiny percentage of the material in the cell in which it resides. Such a nucleic acid is isolated, however, as the term is used herein because it is readily manipulable by standard techniques known to those of ordinary skill in the art. As used herein with respect to proteins or peptides, the term "isolated" refers to a protein or peptide that has been isolated from its natural environment or artificially produced (*e.g.*, by chemical synthesis, by recombinant DNA technology, *etc.*).

The skilled artisan will also realize that conservative amino acid substitutions may be made to provide functionally equivalent variants, or homologs of the capsid proteins. In some aspects the disclosure embraces sequence alterations that result in conservative amino acid substitutions. As used herein, a conservative amino acid substitution refers to an amino acid substitution that does not alter the relative charge or size characteristics of the protein in which the amino acid substitution is made. Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references that compile such methods, *e.g.,* Molecular Cloning: A Laboratory Manual, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or Current Protocols in Molecular Biology, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. Conservative substitutions of amino acids include substitutions made among amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D. Therefore, one can make conservative amino acid substitutions to the amino acid sequence of the proteins and polypeptides disclosed herein.

The isolated nucleic acids of the invention may be recombinant adeno-associated virus (AAV) vectors (rAAV vectors). In some embodiments, an isolated nucleic acid as described by

the disclosure comprises a region (*e.g.*, a first region) comprising a first adeno-associated virus (AAV) inverted terminal repeat (ITR), or a variant thereof. The isolated nucleic acid (*e.g.*, the recombinant AAV vector) may be packaged into a capsid protein and administered to a subject and/or delivered to a selected target cell. "Recombinant AAV (rAAV) vectors" are typically composed of, at a minimum, a transgene and its regulatory sequences, and 5' and 3' AAV inverted terminal repeats (ITRs). The transgene may comprise, as disclosed elsewhere herein, one or more regions that encode one or more inhibitory RNAs (*e.g.*, miRNAs) comprising a nucleic acid that targets an endogenous mRNA of a subject. The transgene may also comprise a region encoding, for example, a protein and/or an expression control sequence (*e.g.*, a poly-A tail), as described elsewhere in the disclosure.

Generally, ITR sequences are about 145 bp in length. Preferably, substantially the entire sequences encoding the ITRs are used in the molecule, although some degree of minor modification of these sequences is permissible. The ability to modify these ITR sequences is within the skill of the art. (See, *e.g.*, texts such as Sambrook et al., "Molecular Cloning. A Laboratory Manual", 2d ed., Cold Spring Harbor Laboratory, New York (1989); and K. Fisher et al., J Virol., 70:520 532 (1996)). An example of such a molecule employed in the present invention is a "cis-acting" plasmid containing the transgene, in which the selected transgene sequence and associated regulatory elements are flanked by the 5' and 3' AAV ITR sequences. The AAV ITR sequences may be obtained from any known AAV, including presently identified mammalian AAV types. In some embodiments, the isolated nucleic acid (*e.g.*, the rAAV vector) comprises at least one ITR having a serotype selected from AAV1, AAV2, AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAV10, AAV11, and variants thereof. In some embodiments, the isolated nucleic acid comprises a region (*e.g.*, a first region) encoding an AAV2 ITR.

In some embodiments, the isolated nucleic acid further comprises a region (*e.g.*, a second region, a third region, a fourth region, *etc.*) comprising a second AAV ITR. In some embodiments, the second AAV ITR has a serotype selected from AAV1, AAV2, AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAV10, AAV11, and variants thereof. In some embodiments, the second ITR is a mutant ITR that lacks a functional terminal resolution site (TRS). The term "lacking a terminal resolution site" can refer to an AAV ITR that comprises a mutation (*e.g.*, a sense mutation such as a non-synonymous mutation, or missense mutation) that abrogates the function of the terminal resolution site (TRS) of the ITR, or to a truncated AAV ITR that lacks a nucleic acid sequence encoding a functional TRS (*e.g.*, a ΔTRS ITR). Without wishing to be

bound by any particular theory, a rAAV vector comprising an ITR lacking a functional TRS produces a self-complementary rAAV vector, for example as described by McCarthy (2008) *Molecular Therapy* 16(10):1648-1656.

In addition to the major elements identified above for the recombinant AAV vector, the vector also includes conventional control elements which are operably linked with elements of the transgene in a manner that permits its transcription, translation and/or expression in a cell transfected with the vector or infected with the virus produced by the invention. As used herein, "operably linked" sequences include both expression control sequences that are contiguous with the gene of interest and expression control sequences that act in trans or at a distance to control the gene of interest. Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation (polyA) signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (*i.e.*, Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. A number of expression control sequences, including promoters which are native, constitutive, inducible and/or tissue-specific, are known in the art and may be utilized.

As used herein, a nucleic acid sequence (*e.g.*, coding sequence) and regulatory sequences are said to be operably linked when they are covalently linked in such a way as to place the expression or transcription of the nucleic acid sequence under the influence or control of the regulatory sequences. If it is desired that the nucleic acid sequences be translated into a functional protein, two DNA sequences are said to be operably linked if induction of a promoter in the 5' regulatory sequences results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region would be operably linked to a nucleic acid sequence if the promoter region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide. Similarly two or more coding regions are operably linked when they are linked in such a way that their transcription from a common promoter results in the expression of two or more proteins having been translated in frame. In some embodiments, operably linked coding

sequences yield a fusion protein. In some embodiments, operably linked coding sequences yield a functional RNA (*e.g.*, miRNA).

In some aspects, the disclosure provides an isolated nucleic acid comprising a transgene, wherein the transgene comprises a nucleic acid sequence encoding one or more microRNAs (*e.g.*, miRNAs). A "microRNA" or "miRNA" is a small non-coding RNA molecule capable of mediating transcriptional or post-translational gene silencing. Typically, miRNA is transcribed as a hairpin or stem-loop (*e.g.*, having a self-complementarity, single-stranded backbone) duplex structure, referred to as a primary miRNA (pri-miRNA), which is enzymatically processed (*e.g.*, by Drosha, DGCR8, Pasha, *etc.*) into a pre-miRNA. The length of a pri-miRNA can vary. In some embodiments, a pri-miRNA ranges from about 100 to about 5000 base pairs (*e.g.*, about 100, about 200, about 500, about 1000, about 1200, about 1500, about 1800, or about 2000 base pairs) in length. In some embodiments, a pri-miRNA is greater than 200 base pairs in length (*e.g.*, 2500, 5000, 7000, 9000, or more base pairs in length.

Pre-miRNA, which is also characterized by a hairpin or stem-loop duplex structure, can also vary in length. In some embodiments, pre-miRNA ranges in size from about 40 base pairs in length to about 500 base pairs in length. In some embodiments, pre-miRNA ranges in size from about 50 to 100 base pairs in length. In some embodiments, pre-miRNA ranges in size from about 50 to about 90 base pairs in length (*e.g.*, about 50, about 52, about 54, about 56, about 58, about 60, about 62, about 64, about 66, about 68, about 70, about 72, about 74, about 76, about 78, about 80, about 82, about 84, about 86, about 88, or about 90 base pairs in length).

Generally, pre-miRNA is exported into the cytoplasm, and enzymatically processed by Dicer to first produce an imperfect miRNA/miRNA* duplex and then a single-stranded mature miRNA molecule, which is subsequently loaded into the RNA-induced silencing complex (RISC). Typically, a mature miRNA molecule ranges in size from about 19 to about 30 base pairs in length. In some embodiments, a mature miRNA molecule is about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, or 30 base pairs in length. In some embodiments, an isolated nucleic acid of the disclosure comprises a sequence encoding a pri-miRNA, a pre-miRNA, or a mature miRNA comprising a sequence set forth in any one of SEQ ID NOs: 2-10 or 21-22.

It should be appreciated that an isolated nucleic acid or vector (*e.g.*, rAAV vector), in some embodiments comprises a nucleic acid sequence encoding more than one (*e.g.*, a plurality, such as 2, 3, 4, 5, 10, or more) miRNAs. In some embodiments, each of the more than one

miRNAs targets (*e.g.*, hybridizes or binds specifically to) the same target gene (*e.g.*, an isolated nucleic acid encoding three unique miRNAs, where each miRNA targets the HTT gene). In some embodiments, each of the more than one miRNAs targets (*e.g.*, hybridizes or binds specifically to) a different target gene.

In some aspects, the disclosure provides isolated nucleic acids and vectors (*e.g.*, rAAV vectors) that encode one or more artificial miRNAs. As used herein "artificial miRNA" or "amiRNA" refers to an endogenous pri-miRNA or pre-miRNA (*e.g.*, a miRNA backbone, which is a precursor miRNA capable of producing a functional mature miRNA), in which the miRNA and miRNA* (*e.g.*, passenger strand of the miRNA duplex) sequences have been replaced with corresponding amiRNA/amiRNA* sequences that direct highly efficient RNA silencing of the targeted gene, for example as described by Eamens et al. (2014), *Methods Mol. Biol.* 1062:211-224. For example, in some embodiments an artificial miRNA comprises a miR-155 pri-miRNA backbone into which a sequence encoding a mature HTT-specific miRNA (*e.g.*, any one of SEQ ID NOs: 2-10) has been inserted in place of the endogenous miR-155 mature miRNA-encoding sequence. In some embodiments, miRNA (*e.g.*, an artificial miRNA) as described by the disclosure comprises a miR-155 backbone sequence, a miR-30 backbone sequence, a mir-64 backbone sequence, or a miR-122 backbone sequence.

A region comprising a transgene (*e.g.*, a second region, third region, fourth region, *etc.*) may be positioned at any suitable location of the isolated nucleic acid. The region may be positioned in any untranslated portion of the nucleic acid, including, for example, an intron, a 5' or 3' untranslated region, *etc.*

In some cases, it may be desirable to position the region (*e.g.*, the second region, third region, fourth region, *etc.*) upstream of the first codon of a nucleic acid sequence encoding a protein (*e.g.*, a protein coding sequence). For example, the region may be positioned between the first codon of a protein coding sequence) and 2000 nucleotides upstream of the first codon. The region may be positioned between the first codon of a protein coding sequence and 1000 nucleotides upstream of the first codon. The region may be positioned between the first codon of a protein coding sequence and 500 nucleotides upstream of the first codon. The region may be positioned between the first codon of a protein coding sequence and 250 nucleotides upstream of the first codon. The region may be positioned between the first codon of a protein coding sequence and 150 nucleotides upstream of the first codon.

In some cases (*e.g.*, when a transgene lacks a protein coding sequence), it may be desirable to position the region (*e.g.*, the second region, third region, fourth region, *etc.*) upstream of the poly-A tail of a transgene. For example, the region may be positioned between the first base of the poly-A tail and 2000 nucleotides upstream of the first base. The region may be positioned between the first base of the poly-A tail and 1000 nucleotides upstream of the first base. The region may be positioned between the first base of the poly-A tail and 500 nucleotides upstream of the first base. The region may be positioned between the first base of the poly-A tail and 250 nucleotides upstream of the first base. The region may be positioned between the first base of the poly-A tail and 150 nucleotides upstream of the first base. The region may be positioned between the first base of the poly-A tail and 100 nucleotides upstream of the first base. The region may be positioned between the first base of the poly-A tail and 50 nucleotides upstream of the first base. The region may be positioned between the first base of the poly-A tail and 20 nucleotides upstream of the first base. In some embodiments, the region is positioned between the last nucleotide base of a promoter sequence and the first nucleotide base of a poly-A tail sequence.

In some cases, the region may be positioned downstream of the last base of the poly-A tail of a transgene. The region may be between the last base of the poly-A tail and a position 2000 nucleotides downstream of the last base. The region may be between the last base of the poly-A tail and a position 1000 nucleotides downstream of the last base. The region may be between the last base of the poly-A tail and a position 500 nucleotides downstream of the last base. The region may be between the last base of the poly-A tail and a position 250 nucleotides downstream of the last base. The region may be between the last base of the poly-A tail and a position 150 nucleotides downstream of the last base.

It should be appreciated that in cases where a transgene encodes more than one miRNA, each miRNA may be positioned in any suitable location within the transgene. For example, a nucleic acid encoding a first miRNA may be positioned in an intron of the transgene and a nucleic acid sequence encoding a second miRNA may be positioned in another untranslated region (*e.g.*, between the last codon of a protein coding sequence and the first base of the poly-A tail of the transgene).

In some embodiments, the transgene further comprises a nucleic acid sequence encoding one or more expression control sequences (*e.g.*, a promoter, *etc.*). Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences;

efficient RNA processing signals such as splicing and polyadenylation (polyA) signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (*i.e.*, Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. A great number of expression control sequences, including promoters which are native, constitutive, inducible and/or tissue-specific, are known in the art and may be utilized.

A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrases "operatively positioned," "under control" or "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

For nucleic acids encoding proteins, a polyadenylation sequence generally is inserted following the transgene sequences and before the 3' AAV ITR sequence. A rAAV construct useful in the present disclosure may also contain an intron, desirably located between the promoter/enhancer sequence and the transgene. One possible intron sequence is derived from SV-40, and is referred to as the SV-40 T intron sequence. Another vector element that may be used is an internal ribosome entry site (IRES). An IRES sequence is used to produce more than one polypeptide from a single gene transcript. An IRES sequence would be used to produce a protein that contain more than one polypeptide chains. Selection of these and other common vector elements are conventional and many such sequences are available [see, *e.g.*, Sambrook et al., and references cited therein at, for example, pages 3.18 3.26 and 16.17 16.27 and Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1989]. In some embodiments, a Foot and Mouth Disease Virus 2A sequence is included in polyprotein; this is a small peptide (approximately 18 amino acids in length) that has been shown to mediate the cleavage of polyproteins (Ryan, M D et al., EMBO, 1994; 4: 928-933; Mattion, N M et al., J Virology, November 1996; p. 8124-8127; Furler, S et al., Gene Therapy, 2001; 8: 864-873; and Halpin, C et al., The Plant Journal, 1999; 4: 453-459). The cleavage activity of the 2A sequence has previously been demonstrated in artificial systems including plasmids and gene therapy vectors (AAV and retroviruses) (Ryan, M D et al., EMBO, 1994; 4: 928-933; Mattion, N M et al., J Virology, November 1996; p. 8124-8127; Furler, S et al., Gene Therapy, 2001; 8: 864-873; and Halpin, C et al., The Plant Journal, 1999; 4: 453-459; de Felipe, P et al., Gene Therapy,

1999; 6: 198-208; de Felipe, P et al., Human Gene Therapy, 2000; 11: 1921-1931.; and Klump, H et al., Gene Therapy, 2001; 8: 811-817).

Examples of constitutive promoters include, without limitation, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, *e.g.*, Boshart et al., Cell, 41:521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the β-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1α promoter [Invitrogen]. In some embodiments, a promoter is an enhanced chicken β-actin promoter. In some embodiments, a promoter is a U6 promoter.

Inducible promoters allow regulation of gene expression and can be regulated by exogenously supplied compounds, environmental factors such as temperature, or the presence of a specific physiological state, *e.g.*, acute phase, a particular differentiation state of the cell, or in replicating cells only. Inducible promoters and inducible systems are available from a variety of commercial sources, including, without limitation, Invitrogen, Clontech and Ariad. Many other systems have been described and can be readily selected by one of skill in the art. Examples of inducible promoters regulated by exogenously supplied promoters include the zinc-inducible sheep metallothionine (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter, the T7 polymerase promoter system (WO 98/10088); the ecdysone insect promoter (No et al., Proc. Natl. Acad. Sci. USA, 93:3346-3351 (1996)), the tetracycline-repressible system (Gossen et al., Proc. Natl. Acad. Sci. USA, 89:5547-5551 (1992)), the tetracycline-inducible system (Gossen et al., Science, 268:1766-1769 (1995), see also Harvey et al., Curr. Opin. Chem. Biol., 2:512-518 (1998)), the RU486-inducible system (Wang et al., Nat. Biotech., 15:239-243 (1997) and Wang et al., Gene Ther., 4:432-441 (1997)) and the rapamycin-inducible system (Magari et al., J. Clin. Invest., 100:2865-2872 (1997)). Still other types of inducible promoters which may be useful in this context are those which are regulated by a specific physiological state, *e.g.*, temperature, acute phase, a particular differentiation state of the cell, or in replicating cells only.

In another embodiment, the native promoter for the transgene will be used. The native promoter may be preferred when it is desired that expression of the transgene should mimic the native expression. The native promoter may be used when expression of the transgene must be regulated temporally or developmentally, or in a tissue-specific manner, or in response to specific transcriptional stimuli. In a further embodiment, other native expression control

elements, such as enhancer elements, polyadenylation sites or Kozak consensus sequences may also be used to mimic the native expression.

In some embodiments, the regulatory sequences impart tissue-specific gene expression capabilities. In some cases, the tissue-specific regulatory sequences bind tissue-specific transcription factors that induce transcription in a tissue specific manner. Such tissue-specific regulatory sequences (*e.g.*, promoters, enhancers, *etc.*.) are well known in the art. Exemplary tissue-specific regulatory sequences include, but are not limited to the following tissue specific promoters: a liver-specific thyroxin binding globulin (TBG) promoter, an insulin promoter, a glucagon promoter, a somatostatin promoter, a pancreatic polypeptide (PPY) promoter, a synapsin-1 (Syn) promoter, a creatine kinase (MCK) promoter, a mammalian desmin (DES) promoter, a α-myosin heavy chain (a-MHC) promoter, or a cardiac Troponin T (cTnT) promoter. Other exemplary promoters include Beta-actin promoter, hepatitis B virus core promoter, Sandig et al., Gene Ther., 3:1002-9 (1996); alpha-fetoprotein (AFP) promoter, Arbuthnot et al., Hum. Gene Ther., 7:1503-14 (1996)), bone osteocalcin promoter (Stein et al., Mol. Biol. Rep., 24:185-96 (1997)); bone sialoprotein promoter (Chen et al., J. Bone Miner. Res., 11:654-64 (1996)), CD2 promoter (Hansal et al., J. Immunol., 161:1063-8 (1998); immunoglobulin heavy chain promoter; T cell receptor α-chain promoter, neuronal such as neuron-specific enolase (NSE) promoter (Andersen et al., Cell. Mol. Neurobiol., 13:503-15 (1993)), neurofilament light-chain gene promoter (Piccioli et al., Proc. Natl. Acad. Sci. USA, 88:5611-5 (1991)), and the neuron-specific vgf gene promoter (Piccioli et al., Neuron, 15:373-84 (1995)), among others which will be apparent to the skilled artisan.

Aspects of the disclosure relate to an isolated nucleic acid comprising more than one promoter (*e.g.*, 2, 3, 4, 5, or more promoters). For example, in the context of a construct having a transgene comprising a first region encoding a protein and an second region encoding an inhibitory RNA (*e.g.*, miRNA), it may be desirable to drive expression of the protein coding region using a first promoter sequence (*e.g.*, a first promoter sequence operably linked to the protein coding region), and to drive expression of the inhibitory RNA encoding region with a second promoter sequence (*e.g.*, a second promoter sequence operably linked to the inhibitory RNA encoding region). Generally, the first promoter sequence and the second promoter sequence can be the same promoter sequence or different promoter sequences. In some embodiments, the first promoter sequence (*e.g.*, the promoter driving expression of the protein coding region) is a RNA polymerase III (polIII) promoter sequence. Non-limiting examples of

polIII promoter sequences include U6 and H1 promoter sequences. In some embodiments, the second promoter sequence (*e.g.*, the promoter sequence driving expression of the inhibitory RNA) is a RNA polymerase II (polII) promoter sequence. Non-limiting examples of polII promoter sequences include T7, T3, SP6, RSV, and cytomegalovirus promoter sequences. In some embodiments, a polIII promoter sequence drives expression of an inhibitory RNA (*e.g.*, miRNA) encoding region. In some embodiments, a polII promoter sequence drives expression of a protein coding region.

In some embodiments, the nucleic acid comprises a transgene that encodes a protein. The protein can be a therapeutic protein (*e.g.*, a peptide, protein, or polypeptide useful for the treatment or prevention of disease states in a mammalian subject) or a reporter protein. In some embodiments, the therapeutic protein is useful for treatment or prevention of Huntington's disease, for example Polyglutamine binding peptide 1 (QBP1), PTD-QBP1, ED11, C4 intrabody, V_L12.3 intrabody, MW7 intrabody, Happ1 antibodies, Happ3 antibodies, mEM48 intrabody, certain monoclonal antibodies (*e.g.*, 1C2), and peptide P42 and variants thereof, as described in Marelli et al. (2016) *Orphanet Journal of Rare Disease* 11:24; doi:10.1186/s13023-016-0405-3. In some embodiments, the therapeutic protein is wild-type huntingtin protein (*e.g.*, huntingtin protein having a PolyQ repeat region comprising less than 36 repeats).

Without wishing to be bound by any particular theory, allele-specific silencing of mutant huntingtin (HTT) may provide an improved safety profile in a subject compared to non-allele specific silencing (*e.g.*, silencing of both wild-type and mutant HTT alleles) because wild-type HTT expression and function is preserved in the cells. Aspects of the invention relate to the inventors' recognition and appreciation that isolated nucleic acids and vectors that incorporate one or more inhibitory RNA (*e.g.*, miRNA) sequences targeting the HTT gene in a non-allele-specific manner while driving the expression of hardened wild-type HTT gene (a wild-type HTT gene that is not targeted by the miRNA) are capable of achieving concomitant mutant HTT knockdown *e.g.*, in the CNS tissue, with increased expression of wildtype HTT. Generally, the sequence of the nucleic acid encoding endogenous wild-type and mutant HTT mRNAs, and the nucleic acid of the transgene encoding the "hardened" wild-type HTT mRNA are sufficiently different such that the "hardened" wild-type HTT transgene mRNA is not targeted by the one or more inhibitory RNAs (*e.g.*, miRNAs). This may be accomplished, for example, by introducing one or more silent mutations into the HTT transgene sequence such that it encodes the same protein as the endogenous wild-type HTT gene but has a different nucleic acid sequence. In this

case, the exogenous mRNA may be referred to as "hardened." Alternatively, the inhibitory RNA (*e.g.*, miRNA) can target the 5' and/or 3' untranslated regions of the endogenous wild-type HTT mRNA. These 5' and/or 3' regions can then be removed or replaced in the transgene mRNA such that the transgene mRNA is not targeted by the one or more inhibitory RNAs.

Reporter sequences (*e.g.*, nucleic acid sequences encoding a reporter protein) that may be provided in a transgene include, without limitation, DNA sequences encoding β-lactamase, β -galactosidase (LacZ), alkaline phosphatase, thymidine kinase, green fluorescent protein (GFP), chloramphenicol acetyltransferase (CAT), luciferase, and others well known in the art. When associated with regulatory elements which drive their expression, the reporter sequences, provide signals detectable by conventional means, including enzymatic, radiographic, colorimetric, fluorescence or other spectrographic assays, fluorescent activating cell sorting assays and immunological assays, including enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and immunohistochemistry. For example, where the marker sequence is the LacZ gene, the presence of the vector carrying the signal is detected by assays for β-galactosidase activity. Where the transgene is green fluorescent protein or luciferase, the vector carrying the signal may be measured visually by color or light production in a luminometer. Such reporters can, for example, be useful in verifying the tissue-specific targeting capabilities and tissue specific promoter regulatory activity of a nucleic acid.

Recombinant adeno-associated viruses (rAAVs)

In some aspects, the disclosure provides isolated AAVs. As used herein with respect to AAVs, the term "isolated" refers to an AAV that has been artificially produced or obtained. Isolated AAVs may be produced using recombinant methods. Such AAVs are referred to herein as "recombinant AAVs". Recombinant AAVs (rAAVs) preferably have tissue-specific targeting capabilities, such that a nuclease and/or transgene of the rAAV will be delivered specifically to one or more predetermined tissue(s). The AAV capsid is an important element in determining these tissue-specific targeting capabilities. Thus, an rAAV having a capsid appropriate for the tissue being targeted can be selected.

Methods for obtaining recombinant AAVs having a desired capsid protein are well known in the art. (See, for example, US 2003/0138772), the contents of which are incorporated herein by reference in their entirety). Typically the methods involve culturing a host cell which contains a nucleic acid sequence encoding an AAV capsid protein; a functional *rep* gene; a

recombinant AAV vector composed of, AAV inverted terminal repeats (ITRs) and a transgene; and sufficient helper functions to permit packaging of the recombinant AAV vector into the AAV capsid proteins. In some embodiments, capsid proteins are structural proteins encoded by the cap gene of an AAV. AAVs comprise three capsid proteins, virion proteins 1 to 3 (named VP1, VP2 and VP3), all of which are transcribed from a single cap gene via alternative splicing. In some embodiments, the molecular weights of VP1, VP2 and VP3 are respectively about 87 kDa, about 72 kDa and about 62 kDa. In some embodiments, upon translation, capsid proteins form a spherical 60-mer protein shell around the viral genome. In some embodiments, the functions of the capsid proteins are to protect the viral genome, deliver the genome and interact with the host. In some aspects, capsid proteins deliver the viral genome to a host in a tissue specific manner.

In some embodiments, an AAV capsid protein is of an AAV serotype selected from the group consisting of AAV2, AAV3, AAV4, AAV5, AAV6, AAV8, AAVrh8, AAV9, and AAV10. In some embodiments, an AAV capsid protein is of a serotype derived from a non-human primate, for example AAVrh8 serotype. In some embodiments, an AAV capsid protein is of an AAV9 serotype. In some embodiments, the AAV capsid protein comprises the sequence set forth in SEQ ID NO: 20.

The components to be cultured in the host cell to package a rAAV vector in an AAV capsid may be provided to the host cell in *trans*. Alternatively, any one or more of the required components (*e.g.*, recombinant AAV vector, rep sequences, cap sequences, and/or helper functions) may be provided by a stable host cell which has been engineered to contain one or more of the required components using methods known to those of skill in the art. Most suitably, such a stable host cell will contain the required component(s) under the control of an inducible promoter. However, the required component(s) may be under the control of a constitutive promoter. Examples of suitable inducible and constitutive promoters are provided herein, in the discussion of regulatory elements suitable for use with the transgene. In still another alternative, a selected stable host cell may contain selected component(s) under the control of a constitutive promoter and other selected component(s) under the control of one or more inducible promoters. For example, a stable host cell may be generated which is derived from 293 cells (which contain E1 helper functions under the control of a constitutive promoter), but which contain the rep and/or cap proteins under the control of inducible promoters. Still other stable host cells may be generated by one of skill in the art.

In some embodiments, the instant disclosure relates to a host cell containing a nucleic acid that comprises a coding sequence encoding a protein (*e.g.*, wild-type huntingtin protein, optionally "hardened" wild-type huntingtin protein). In some embodiments, the instant disclosure relates to a composition comprising the host cell described above. In some embodiments, the composition comprising the host cell above further comprises a cryopreservative.

The recombinant AAV vector, rep sequences, cap sequences, and helper functions required for producing the rAAV of the disclosure may be delivered to the packaging host cell using any appropriate genetic element (vector). The selected genetic element may be delivered by any suitable method, including those described herein. The methods used to construct any embodiment of this disclosure are known to those with skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic techniques. See, *e.g.*, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. Similarly, methods of generating rAAV virions are well known and the selection of a suitable method is not a limitation on the present disclosure. See, *e.g.*, K. Fisher et al., J. Virol., 70:520-532 (1993) and U.S. Pat. No. 5,478,745.

In some embodiments, recombinant AAVs may be produced using the triple transfection method (described in detail in U.S. Pat. No. 6,001,650). Typically, the recombinant AAVs are produced by transfecting a host cell with an recombinant AAV vector (comprising a transgene) to be packaged into AAV particles, an AAV helper function vector, and an accessory function vector. An AAV helper function vector encodes the "AAV helper function" sequences (*i.e.*, rep and cap), which function in *trans* for productive AAV replication and encapsidation. Preferably, the AAV helper function vector supports efficient AAV vector production without generating any detectable wild-type AAV virions (*i.e.*, AAV virions containing functional rep and cap genes). Non-limiting examples of vectors suitable for use with the present disclosure include pHLP19, described in U.S. Pat. No. 6,001,650 and pRep6cap6 vector, described in U.S. Pat. No. 6,156,303, the entirety of both incorporated by reference herein. The accessory function vector encodes nucleotide sequences for non-AAV derived viral and/or cellular functions upon which AAV is dependent for replication (*i.e.*, "accessory functions"). The accessory functions include those functions required for AAV replication, including, without limitation, those moieties involved in activation of AAV gene transcription, stage specific AAV mRNA splicing, AAV DNA replication, synthesis of cap expression products, and AAV capsid assembly. Viral-based

accessory functions can be derived from any of the known helper viruses such as adenovirus, herpesvirus (other than herpes simplex virus type-1), and vaccinia virus.

In some aspects, the disclosure provides transfected host cells. The term "transfection" is used to refer to the uptake of foreign DNA by a cell, and a cell has been "transfected" when exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are generally known in the art. See, *e.g.*, Graham et al. (1973) Virology, 52:456, Sambrook et al. (1989) Molecular Cloning, a laboratory manual, Cold Spring Harbor Laboratories, New York, Davis et al. (1986) Basic Methods in Molecular Biology, Elsevier, and Chu et al. (1981) Gene 13:197. Such techniques can be used to introduce one or more exogenous nucleic acids, such as a nucleotide integration vector and other nucleic acid molecules, into suitable host cells.

A "host cell" refers to any cell that harbors, or is capable of harboring, a substance of interest. Often a host cell is a mammalian cell. A host cell may be used as a recipient of an AAV helper construct, an AAV minigene plasmid, an accessory function vector, or other transfer DNA associated with the production of recombinant AAVs. The term includes the progeny of the original cell which has been transfected. Thus, a "host cell" as used herein may refer to a cell which has been transfected with an exogenous DNA sequence. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation.

As used herein, the term "cell line" refers to a population of cells capable of continuous or prolonged growth and division *in vitro*. Often, cell lines are clonal populations derived from a single progenitor cell. It is further known in the art that spontaneous or induced changes can occur in karyotype during storage or transfer of such clonal populations. Therefore, cells derived from the cell line referred to may not be precisely identical to the ancestral cells or cultures, and the cell line referred to includes such variants.

As used herein, the terms "recombinant cell" refers to a cell into which an exogenous DNA segment, such as DNA segment that leads to the transcription of a biologically-active polypeptide or production of a biologically active nucleic acid such as an RNA, has been introduced.

As used herein, the term "vector" includes any genetic element, such as a plasmid, phage, transposon, cosmid, chromosome, artificial chromosome, virus, virion, *etc.*, which is capable of

replication when associated with the proper control elements and which can transfer gene sequences between cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors. In some embodiments, useful vectors are contemplated to be those vectors in which the nucleic acid segment to be transcribed is positioned under the transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrases "operatively positioned," "under control" or "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene. The term "expression vector or construct" means any type of genetic construct containing a nucleic acid in which part or all of the nucleic acid encoding sequence is capable of being transcribed. In some embodiments, expression includes transcription of the nucleic acid, for example, to generate a biologically-active polypeptide product or functional RNA (*e.g.*, guide RNA) from a transcribed gene. The foregoing methods for packaging recombinant vectors in desired AAV capsids to produce the rAAVs of the disclosure are not meant to be limiting and other suitable methods will be apparent to the skilled artisan.

In some embodiments, any one or more thymidine (T) nucleotides or uridine (U) nucleotides in a sequence provided herein, including a sequence provided in the sequence listing, may be replaced with any other nucleotide suitable for base pairing (*e.g.*, via a Watson-Crick base pair) with an adenosine nucleotide. For example, in some embodiments, any one or more thymidine (T) nucleotides in a sequence provided herein, including a sequence provided in the sequence listing, may be suitably replaced with a uridine (U) nucleotide or vice versa.

Modes of Administration

The rAAVs of the disclosure may be delivered to a subject in compositions according to any appropriate methods known in the art. For example, an rAAV, preferably suspended in a physiologically compatible carrier (*i.e.*, in a composition), may be administered to a subject, *i.e.* host animal, such as a human, mouse, rat, cat, dog, sheep, rabbit, horse, cow, goat, pig, guinea pig, hamster, chicken, turkey, or a non-human primate (*e.g.,* Macaque). In some embodiments a host animal does not include a human.

Delivery of the rAAVs to a mammalian subject may be by, for example, intramuscular injection or by administration into the bloodstream of the mammalian subject. Administration

into the bloodstream may be by injection into a vein, an artery, or any other vascular conduit. In some embodiments, the rAAVs are administered into the bloodstream by way of isolated limb perfusion, a technique well known in the surgical arts, the method essentially enabling the artisan to isolate a limb from the systemic circulation prior to administration of the rAAV virions. A variant of the isolated limb perfusion technique, described in U.S. Pat. No. 6,177,403, can also be employed by the skilled artisan to administer the virions into the vasculature of an isolated limb to potentially enhance transduction into muscle cells or tissue. Moreover, in certain instances, it may be desirable to deliver the virions to the CNS of a subject. By "CNS" is meant all cells and tissue of the brain and spinal cord of a vertebrate. Thus, the term includes, but is not limited to, neuronal cells, glial cells, astrocytes, cerebrospinal fluid (CSF), interstitial spaces, bone, cartilage and the like. Recombinant AAVs may be delivered directly to the CNS or brain by injection into, *e.g.*, the ventricular region, as well as to the striatum (*e.g.*, the caudate nucleus or putamen of the striatum), spinal cord and neuromuscular junction, or cerebellar lobule, with a needle, catheter or related device, using neurosurgical techniques known in the art, such as by stereotactic injection (see, *e.g.*, Stein et al., J Virol 73:3424-3429, 1999; Davidson et al., PNAS 97:3428-3432, 2000; Davidson et al., Nat. Genet. 3:219-223, 1993; and Alisky and Davidson, Hum. Gene Ther. 11:2315-2329, 2000). In some embodiments, rAAV as described in the disclosure are administered by intravenous injection. In some embodiments, the rAAV are administered by intracerebral injection. In some embodiments, the rAAV are administered by intrathecal injection. In some embodiments, the rAAV are administered by intrastriatal injection. In some embodiments, the rAAV are delivered by intracranial injection. In some embodiments, the rAAV are delivered by cisterna magna injection. In some embodiments, the rAAV are delivered by cerebral lateral ventricle injection.

Aspects of the instant disclosure relate to compositions comprising a recombinant AAV comprising a capsid protein and a nucleic acid encoding a transgene, wherein the transgene comprises a nucleic acid sequence encoding one or more miRNAs. In some embodiments, each miRNA comprises a sequence set forth in any one of SEQ ID NOs: 2-10. In some embodiments, the nucleic acid further comprises AAV ITRs. In some embodiments, the rAAV comprises an rAAV vector represented by the sequence set forth in any one of SEQ ID NO: 16-19, or a portion thereof. In some embodiments, a composition further comprises a pharmaceutically acceptable carrier.

The compositions of the disclosure may comprise an rAAV alone, or in combination with one or more other viruses (*e.g.*, a second rAAV encoding having one or more different transgenes). In some embodiments, a composition comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more different rAAVs each having one or more different transgenes.

Suitable carriers may be readily selected by one of skill in the art in view of the indication for which the rAAV is directed. For example, one suitable carrier includes saline, which may be formulated with a variety of buffering solutions (*e.g.*, phosphate buffered saline). Other exemplary carriers include sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil, sesame oil, and water. The selection of the carrier is not a limitation of the present disclosure.

Optionally, the compositions of the disclosure may contain, in addition to the rAAV and carrier(s), other conventional pharmaceutical ingredients, such as preservatives, or chemical stabilizers. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable chemical stabilizers include gelatin and albumin.

The rAAVs are administered in sufficient amounts to transfect the cells of a desired tissue and to provide sufficient levels of gene transfer and expression without undue adverse effects. Conventional and pharmaceutically acceptable routes of administration include, but are not limited to, direct delivery to the selected organ (*e.g.*, intraportal delivery to the liver), oral, inhalation (including intranasal and intratracheal delivery), intraocular, intravenous, intramuscular, subcutaneous, intradermal, intratumoral, and other parental routes of administration. Routes of administration may be combined, if desired.

The dose of rAAV virions required to achieve a particular "therapeutic effect," *e.g.*, the units of dose in genome copies/per kilogram of body weight (GC/kg), will vary based on several factors including, but not limited to: the route of rAAV virion administration, the level of gene or RNA expression required to achieve a therapeutic effect, the specific disease or disorder being treated, and the stability of the gene or RNA product. One of skill in the art can readily determine a rAAV virion dose range to treat a patient having a particular disease or disorder based on the aforementioned factors, as well as other factors that are well known in the art.

An effective amount of an rAAV is an amount sufficient to target infect an animal, target a desired tissue. In some embodiments, an effective amount of an rAAV is an amount sufficient to produce a stable somatic transgenic animal model. The effective amount will depend

primarily on factors such as the species, age, weight, health of the subject, and the tissue to be targeted, and may thus vary among animal and tissue. For example, an effective amount of the rAAV is generally in the range of from about 1 ml to about 100 ml of solution containing from about 10^9 to 10^{16} genome copies. In some cases, a dosage between about 10^{11} to 10^{13} rAAV genome copies is appropriate. In certain embodiments, 10^{12} or 10^{13} rAAV genome copies is effective to target CNS tissue. In some cases, stable transgenic animals are produced by multiple doses of an rAAV.

In some embodiments, a dose of rAAV is administered to a subject no more than once per calendar day (*e.g.*, a 24-hour period). In some embodiments, a dose of rAAV is administered to a subject no more than once per 2, 3, 4, 5, 6, or 7 calendar days. In some embodiments, a dose of rAAV is administered to a subject no more than once per calendar week (*e.g.*, 7 calendar days). In some embodiments, a dose of rAAV is administered to a subject no more than bi-weekly (*e.g.*, once in a two calendar week period). In some embodiments, a dose of rAAV is administered to a subject no more than once per calendar month (*e.g.*, once in 30 calendar days). In some embodiments, a dose of rAAV is administered to a subject no more than once per six calendar months. In some embodiments, a dose of rAAV is administered to a subject no more than once per calendar year (*e.g.*, 365 days or 366 days in a leap year).

In some embodiments, rAAV compositions are formulated to reduce aggregation of AAV particles in the composition, particularly where high rAAV concentrations are present (*e.g.*, ~10^{13} GC/ml or more). Methods for reducing aggregation of rAAVs are well known in the art and, include, for example, addition of surfactants, pH adjustment, salt concentration adjustment, *etc*. (See, *e.g.*, Wright FR, et al., Molecular Therapy (2005) 12, 171–178, the contents of which are incorporated herein by reference.)

Formulation of pharmaceutically-acceptable excipients and carrier solutions is well-known to those of skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens.

Typically, these formulations may contain at least about 0.1% of the active compound or more, although the percentage of the active ingredient(s) may, of course, be varied and may conveniently be between about 1 or 2% and about 70% or 80% or more of the weight or volume of the total formulation. Naturally, the amount of active compound in each therapeutically-useful composition may be prepared is such a way that a suitable dosage will be obtained in any

given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

In certain circumstances it will be desirable to deliver the rAAV-based therapeutic constructs in suitably formulated pharmaceutical compositions disclosed herein either subcutaneously, intraopancreatically, intranasally, parenterally, intravenously, intramuscularly, intrathecally, or orally, intraperitoneally, or by inhalation. In some embodiments, the administration modalities as described in U.S. Pat. Nos. 5,543,158; 5,641,515 and 5,399,363 (each specifically incorporated herein by reference in its entirety) may be used to deliver rAAVs. In some embodiments, a preferred mode of administration is by portal vein injection.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. In many cases the form is sterile and fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

For administration of an injectable aqueous solution, for example, the solution may be suitably buffered, if necessary, and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous,

intramuscular, subcutaneous and intraperitoneal administration. In this connection, a sterile aqueous medium that can be employed will be known to those of skill in the art. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the host. The person responsible for administration will, in any event, determine the appropriate dose for the individual host.

Sterile injectable solutions are prepared by incorporating the active rAAV in the required amount in the appropriate solvent with various of the other ingredients enumerated herein, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The rAAV compositions disclosed herein may also be formulated in a neutral or salt form. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug-release capsules, and the like.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Supplementary active ingredients can also be incorporated into the compositions. The phrase "pharmaceutically-acceptable" refers

to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a host.

Delivery vehicles such as liposomes, nanocapsules, microparticles, microspheres, lipid particles, vesicles, and the like, may be used for the introduction of the compositions of the present disclosure into suitable host cells. In particular, the rAAV vector delivered transgenes may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like.

Such formulations may be preferred for the introduction of pharmaceutically acceptable formulations of the nucleic acids or the rAAV constructs disclosed herein. The formation and use of liposomes is generally known to those of skill in the art. Recently, liposomes were developed with improved serum stability and circulation half-times (U.S. Pat. No. 5,741,516). Further, various methods of liposome and liposome like preparations as potential drug carriers have been described (U.S. Pat. Nos. 5,567,434; 5,552,157; 5,565,213; 5,738,868 and 5,795,587).

Liposomes have been used successfully with a number of cell types that are normally resistant to transfection by other procedures. In addition, liposomes are free of the DNA length constraints that are typical of viral-based delivery systems. Liposomes have been used effectively to introduce genes, drugs, radiotherapeutic agents, viruses, transcription factors and allosteric effectors into a variety of cultured cell lines and animals. In addition, several successful clinical trials examining the effectiveness of liposome-mediated drug delivery have been completed.

Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs). MLVs generally have diameters of from 25 nm to 4 μm. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 Å, containing an aqueous solution in the core.

Alternatively, nanocapsule formulations of the rAAV may be used. Nanocapsules can generally entrap substances in a stable and reproducible way. To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) should be designed using polymers able to be degraded *in vivo*. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use.

In addition to the methods of delivery described above, the following techniques are also contemplated as alternative methods of delivering the rAAV compositions to a host.

Sonophoresis (*i.e.*, ultrasound) has been used and described in U.S. Pat. No. 5,656,016 as a device for enhancing the rate and efficacy of drug permeation into and through the circulatory system. Other drug delivery alternatives contemplated are intraosseous injection (U.S. Pat. No. 5,779,708), microchip devices (U.S. Pat. No. 5,797,898), ophthalmic formulations (Bourlais et al., 1998), transdermal matrices (U.S. Pat. Nos. 5,770,219 and 5,783,208) and feedback-controlled delivery (U.S. Pat. No. 5,697,899).

Kits and Related Compositions

The agents described herein may, in some embodiments, be assembled into pharmaceutical or diagnostic or research kits to facilitate their use in therapeutic, diagnostic or research applications. A kit may include one or more containers housing the components of the disclosure and instructions for use. Specifically, such kits may include one or more agents described herein, along with instructions describing the intended application and the proper use of these agents. In certain embodiments agents in a kit may be in a pharmaceutical formulation and dosage suitable for a particular application and for a method of administration of the agents. Kits for research purposes may contain the components in appropriate concentrations or quantities for running various experiments.

In some embodiments, the instant disclosure relates to a kit for producing a rAAV, the kit comprising a container housing an isolated nucleic acid comprising an miRNA comprising or encoded by the sequence set forth in any one of SEQ ID NOs: 2-10. In some embodiments, the kit further comprises a container housing an isolated nucleic acid encoding an AAV capsid protein, for example an AAV9 capsid protein.

The kit may be designed to facilitate use of the methods described herein by researchers and can take many forms. Each of the compositions of the kit, where applicable, may be provided in liquid form (*e.g.*, in solution), or in solid form, (*e.g.*, a dry powder). In certain cases, some of the compositions may be constitutable or otherwise processable (*e.g.*, to an active form), for example, by the addition of a suitable solvent or other species (for example, water or a cell culture medium), which may or may not be provided with the kit. As used herein, "instructions" can define a component of instruction and/or promotion, and typically involve written instructions on or associated with packaging of the disclosure. Instructions also can include any oral or electronic instructions provided in any manner such that a user will clearly recognize that the instructions are to be associated with the kit, for example, audiovisual (*e.g.*,

videotape, DVD, *etc.*), Internet, and/or web-based communications, *etc.* The written instructions may be in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which instructions can also reflects approval by the agency of manufacture, use or sale for animal administration.

The kit may contain any one or more of the components described herein in one or more containers. As an example, in one embodiment, the kit may include instructions for mixing one or more components of the kit and/or isolating and mixing a sample and applying to a subject. The kit may include a container housing agents described herein. The agents may be in the form of a liquid, gel or solid (powder). The agents may be prepared sterilely, packaged in syringe and shipped refrigerated. Alternatively it may be housed in a vial or other container for storage. A second container may have other agents prepared sterilely. Alternatively the kit may include the active agents premixed and shipped in a syringe, vial, tube, or other container.

Exemplary embodiments of the invention will be described in more detail by the following examples. These embodiments are exemplary of the invention, which one skilled in the art will recognize is not limited to the exemplary embodiments.

EXAMPLES

Example 1: Materials and Methods

Cell culture and screening assays

HeLa cells were maintained in DMEM, high glucose with 10% heat inactivated FBS and 1% Penicillin Streptomycin (ThermoFisher). Twenty-four hours before transfection, cells were seeded onto 6-well plates at 0.8-1.0x10^6 cells/well. On the day of transfection, growth medium was replaced with 1.6ml of Opti-MEM (ThermoFisher). Plasmids were transfected using 2µl/well of DharmaFECT Duo (Dharmacon). Each well received 0.6µg of plasmid DNA. Forty-eight hours after transfection, the cells were harvested and total RNA was extracted using the MirVana RNA isolation kit. cDNA was produced using 1µg of RNA/reaction using oligo-dT and Superscript III (Invitrogen). Huntingtin mRNA was measured using a TaqMan assay (ThermoFisher). Relative levels of huntingtin mRNA were calculated using the ΔΔC(T) method

with human Hypoxanthine-guanine phosphoribosyltransferase (HPRT) as the housekeeping gene.

Mouse housing, injections and maintenance

YAC128 and wild type FVB mice were obtained. Mice were bred on the FVB background by mating wildtype 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*. 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, Warren, MI) aided by UMPC3 or UMPC4 microinjectors (World Precision Instruments, Sarasota, FL). Mice were anesthetized with 284mg/kg of tribromoethanol and placed in the stereotax. Surgery was performed using the bregma as the zero point, measuring anterior 1.0mm, lateral 2.0mm, and lowering a 33guage needle 3.0mm into the striatum. The pumps were set to deliver 3.0ul at a rate of 125nl/minute. After the injections the mice were allowed to recover on a warming pad and then placed back in their cages in the housing area.

Tissue Extraction

At the appropriate time-point, mice were sacrificed and tissue extracted for RNA analysis or immunohistochemistry. For RNA extraction, mice were anesthetized and killed by cervical dislocation. Brains were removed and the striatum was dissected out. When available, GFP expression was used to guide the dissection so that only GFP positive tissue was analyzed. Tissue was placed immediately in RNALater (Ambion). Subsequently they were stored frozen at -80° C. At the end of the experiment the mice meant for immunocytochemistry were deeply anesthetized and perfused intracardially with saline followed by 4% paraformaldehyde. Samples were post fixed overnight in cold 2% paraformaldehyde and then stored in phosphate buffered saline at 4° C. Coronal sections were made by slicing 40 micron sections on the Leica VT1000s vibratome.

Mouse Behaviors

Beam walking: Mice were trained to cross a (size of beam) beam. After training, the mice were recorded as they crossed from one end of the beam to the other. Three trials per mouse were recorded. Based on the recording, the amount of time it took for the mice to cross from mark on one end of the beam to the other was measured.

Home cage activity: Mice were placed singly in an automated home cage phenotyping scanning system (Clever Sys, Inc., Reston VA) for 26 hours. To calculate the average active time per hour, the first hour of data during which the mouse acclimates to the new environment was removed; then the total time spent walking by the total recorded time, minus one hour, was calculated.

Immunohistochemistry and Quantification

Fixed tissue slices were blocked with 3% hydrogen peroxide for three minutes and then incubated with 0.5% triton x for 20 minutes. Immunocytochemistry was performed using Vector Laboratories Elite ABC kit reagents for rabbit or mouse derived antibodies against DARPP32 (Abcam ab40801; 1:10,000 dilution), Iba1 (Wako 019-19741; 1:1,000 dilution), GFP (Life Technologies G10362; 1:1000 dilution) and NeuN (EMD Millipore MAB377; 1:1000 dilution). Sections were stained for 2 minutes with diaminobenzidine using the Metal Enhanced DAB Substrate Kit (Pierce).

Small RNA library cloning and analysis

Total RNA was extracted using the MirVana RNA isolation kit. Size selection of the 18-30 nucleotide RNAs was performed using 5µg 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′-adapter (5′-rAppTGGAATTCTCGGGTGCCAAGG/ddC/-3′; SEQ ID NO: 11). The ligated products were annealed to the RT primer (5′-CCTTGGCACCCGAGAATTCCA-3′; SEQ ID NO: 12) and ligated to a 5′-adapter (RNA: 5′-GUUCAGAGUUCUACAGUCCGACGAUC-3′; SEQ ID NO: 13). Reverse transcription was performed using AMV Reverse transcriptase mix (NEB) and PCR amplified using AccuPrime *Pfx* DNA Polymerase (Invitrogen) with one universal primer (5′-AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA-3′; SEQ ID NO: 14) and one barcoded primer (5′-CAAGCAGAAGACGGCATACGAGATNNNNNNGTGACTGGAGTTCCTTGGCACCCGAG

AATTCCA-3′; SEQ ID NO: 15). Libraries were sequenced and mapped to the mm9 genome and to the AAV genome. miRNA species were classified based on the position of the 5′-end mapping on the miRNA hairpin, therefore each species consists of all the small RNAs with shared seed sequences. The 3′-end was not considered in species assignment. Differential expression of endogenous miRNAs was analyzed using the edgeR package.

mRNA library cloning and analysis

RNA was extracted as above. Libraries were constructed by standard methods. Reads were mapped using topHat2 and differential expression was calculated using the deseq2 package.

Sheep Experiments

A transgenic sheep model of Huntington's disease (*e.g.*, transgenic sheep expressing pathogenic human huntingtin protein) were injected with either scAAV9-CBA-mir-HTT (comprising miRNA 6433 in a mir-155 backbone), scAAV-U6-mir-HTT (comprising miRNA 6433 in a mir-155 backbone), or empty scAAV9 control vector. Sheep were sacrificed at either one month or six months post-injection. Tissue and nucleic acid samples were prepared and analyzed by quantitative PCR and immunohistochemistry.

Example 2: Mouse *in vivo* experiments

Design and selection of huntingtin targeting artificial miRNAs

Nine sequences targeting the human huntingtin mRNA (Table 1, FIGs. 1) were tested. A schematic depicting the locations of the nine targeting sequences is provided in FIG. 4A. Two copies of the artificial miRNA were cloned in tandem into a backbone based on the endogenous miRNA-155 or miR-30 and the entire artificial miRNA was inserted into the 3′-UTR of EGFP (FIG. 5A, top). The predicted hairpins structures of mir-155-based and mir-30-based anti-HTT amiRNAs are shown in FIGs. 25A-25B, respectively. The resulting plasmids were transfected into Hela cells. Forty-eight hours later, the cells were harvested and levels of endogenous huntingtin mRNA were measured by quantitative RT-PCR (qRT-PCR). Three out of the nine artificial miRNAs reduced huntingtin by greater than 50% (FIGs. 1-2 and 4B-4C).

Table 1: Huntingtin mir targets

Name	Mature miRNA Sequences	SEQ ID NO:
miR-1873-anti-HTT	5'-TAAATGTGCCTGTTGAAGGGC-3'	2
miR-2029-anti-HTT	5'-AAGAGGTGCAGAGTCATCATC-3'	3
miR-4173-anti-HTT	5'-TTCTGGAGGACATCAAACCAT-3'	4
miR-4448-anti-HTT	5'-TGAACTGGCCCACTTCAATGT-3'	5
mIR-6088-anti-HTT	5'-TTCCATTGGCAACTGGGCCAT-3'	6
miR-6433-anti-HTT	5'-TAAGCATGGAGCTAGCAGGCT-3'	7
miR-TS1-anti-HTT	5'-TAGCGTTGAAGTACTGTCCCC-3'	8
miR-TS2-anti-HTT	5'-TTGAGGCAGCAGCGGCTGTGC-3'	9
miR-E14-anti-HTT	5'-TTCATCAGCTTTTCCAGGGTC-3'	10

Three sequences from the initial screen were selected for *in vivo* experiments. An artificial miRNA based on a known siRNA (E1.4) was also tested. Candidate sequences were packaged into a self-complementary AAV9 vector and injected it directly into the striatum of transgenic mice expressing human huntingtin with a stretch of approximately 128 polyglutamine encoding repeats (Yac128 mice). One month later, distribution of AAV9 and expression of the GFP reporter were evaluated at three different doses. At the highest dose, GFP staining was present throughout the striatum (FIG. 11A) and human huntingtin mRNA was significantly reduced in mice treated with either AAV9-CβA-anti-HTT-6433 (p=0.006) or AAV9-CβA-anti-HTT-5155 (p=0.013, FIG. 4C; mir-155 backbone was used). Reducing the dose of the vector resulted in reduced GFP expression (FIG. 11A) and at a 1:10 dilution, no significant reduction in human huntingtin mRNA was achieved (FIG. 11B). FIG. 11C provides representative photos of mice injected with a vector encoding both the huntingtin targeting miRNA and EGFP at three different doses.

Expressing an artificial miRNA from the U6 promoter does not improve silencing of huntingtin

A single copy of the most potent miRNA (HTT-6433) was cloned into an AAV9 vector under the control of the U6 promoter (FIG. 5A, bottom). Mice were injected unilaterally with either the original two copy AAV9-CBA-anti-HTT-6433 (comprising SEQ ID NO: XX), AAV9-CBA-anti-HTT-5155, AAV9-U6-anti-HTT-6433 (comprising SEQ ID NO: XX or AAV9-U6-anti-HTT-5155. One month later, the striatum was harvested and GFP expression

was confirmed and the level of huntingtin mRNA was measured by qRT-PCR. Regardless of the sequence of the artificial miRNA, no significant difference in knockdown between mice treated with AAV9-U6-anti-HTT and AAV9-CBA-anti-HTT was observed. In both the mice treated with the AAV9-U6-anti-HTT-6433 and AAV9-CBA-anti-HTT-6433, the quantity of huntingtin mRNA on the injected side was approximately 50% of that on the non-injected side (FIG. 5B). Note that using the contralateral (non-injected) side as a control for each animal reduces the animal to animal variability. In mice injected with AAV9-GFP unilaterally, a small number of GFP positive neurons on the contralateral side are occasionally observed, indicating that some virus spreads to the un-injected side. Therefore, using the contralateral side as the control may underestimate knockdown. To eliminate the potential confounding effects of using the contralateral side as a control, the experiment was repeated using a group of animals injected with PBS only as the control. Data indicate that both AAV9-U6-anti-HTT-6433 and AAV9-CBA-mir-HTT-6433 reduced huntingtin mRNA by approximately 50% (FIG. 5C).

Long-term striatal expression of mir-HTT-6433 from a U6 promoter is toxic in mice

AAV9-U6-anti-HTT-6433 or AAV9-CBA-anti-HTT-6433 were unilaterally injected directly into the striatum of Yac128 mice. Six months after injection, it was observed that the mice injected with AAV9-U6-anti-HTT-6433 were not nesting and some exhibited a hyperactive phenotype. To document these abnormalities, the nestlets in each cage were replaced. Twenty-four hours later, the new nestlets of the AAV9-U6-anti-HTT-6433 were unused whereas PBS and AAV9-CBA-anti-HTT-6433 injected mice made nests as expected (FIG. 6A). Using a home-cage monitoring system, the mice were recorded for twenty-four hours. Mice treated with AAV9-U6-anti-HTT-6433 spent significantly more time moving around their home cage than mice treated with PBS or with AAV9-CBA-anti-HTT-6433 (FIG. 6B). The average time it took for the mice to cross the beam was also measured. For this test, the mice are required to complete the beam crossing three times. On average, AAV9-CBA-anti-HTT-6433 treated mice trended to cross faster than mice that received only a PBS injection (FIGs. 3 and 12A). Of the four remaining mice in the AAV9-U6-anti-HTT-6433 group, two were unable to successfully cross, either jumping or falling off the beam (FIG. 12A). In a second experiment carried out on older Yac128 mice (7 months of age), an age related increase in time to cross the beam was observed. This increase was present in both naïve mice and in mice treated with AAV9-CBA-anti-HTT-6433 and was accelerated in mice treated with AAV9-U6-anti-HTT-6433 (FIG. 12B).

Neuropathological findings correlated with the behavioral outcomes described above. On the injected side, the AAV9-U6-anti-HTT-6433 mice showed enlargement of the ventricle, loss of DARPP-32 positive neurons and striatal shrinkage (FIGs. 7A-7B). In the remaining striatum, they exhibited increased Iba1 staining (FIG. 8A, bottom), an increase in total and activated microglia and a decrease in the number of resting microglia (FIGs. 8B-8D). Wild-type C57Bl/6 mice and FVB mice were injected with the same vectors and the consequences of the U6 driven miR were assessed to determine if the toxicity was dependent on the presence of mutant Huntington 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. However, in C57BL6 mice, the effect was present but less pronounced. While 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 (FIG. 13A). Striatal shrinkage was also less severe in the C57BL6 mice (Figure FIG. 13B).

Expression of the artificial miRNA targeting huntingtin from a U6 promoter results in overexpression of the huntingtin targeting small RNA

Groups of mice were injected unilaterally with scAAV9 vectors expressing the artificial miRNA 6433 from the U6 and CβA promoters. The small RNAs produced at two weeks post-injection were cloned and sequenced. In both groups, ninety-six percent of the sequences mapping to the AAV genome mapped to the expected small RNA product, with only a small percentage representing imprecise Dicer or Drosha cleavage. In the group injected with the U6 promoter driven artificial miRNA, the huntingtin targeting sequence dominated the sequencing results, accounting for half (50%) of all mappable sequences whereas in the mice injected with the CBA vector, only 5% of the sequences matched the vector encoded small RNA (FIG. 9A). Thus, potentially, small RNAs with alternative seed sequences, including the sense strand and slippage products, may be present at levels comparable to those of functional endogenous miRNAs (FIG. 9A). The relative quantity of the huntingtin targeting small RNA was measured by qPCR to confirm the overexpression of the huntingtin targeting sequence. It was observed that expression of the small RNA was 150 to 250 times higher in the mice injected with the U6 promoter driven construct (FIG. 9B).

Endogenous miRNA 30 sequences are commonly used as a scaffold for artificial miRNA. To determine if the isomir profiles derived from this scaffold were more favorable, we

embedded theanti-HTT-6433 sequence in a miR-30 backbone and injected into 10 week old Yac128 mice (FIG. 9C). The mir-30 scaffold produces levels of the mature artificial miRNA which are comparable to those produced by the CβA promoter (FIG.9A) and reduces human huntingtin by close to 50%. Unlike the mir-155 scaffold, the mir-30 scaffold produces the mature sense (passenger) strand at levels comparable to the antisense (guide) strand. The combination of CβA promoter with mir-155 backbone produces only the intended antisense strand above background (FIG. 9A).

Although over half of the reads in the sample could be mapped to the AAV-encoded artificial miRNA, overexpression of the artificial miRNA targeting huntingtin had minimal effects on the distribution of endogenous miRNAs (FIGs. 14A-14B). In fact, the largest differences were observed when the injected groups were compared to the non-injected (contralateral) side, suggesting that the injection itself produces a local change in miRNA profiles. This may reflect a local inflammatory response to injury which could resolve over time.

Expression of the artificial miRNA targeting huntingtin from a U6 promoter disrupts the expression of multiple mRNAs

RNAseq analysis of the striatum of mice treated with either the AAV9-U6-anti-HTT-6433 or AAV9-CBA-anti-HTT-6433 was performed to investigate the consequences of overexpression of the huntingtin targeting miRNA. Two weeks post-injection, striatal mRNA profiles on the injected and non-injected sides were compared. Data indicate that there were few significant differences in mice treated with the CBA-mirHTT-6433 (FIG. 14A). In mice treated with the AAV9-U6-anti-HTT-6433, both mRNAs that were increased and those that were decreased in response to treatment were observed (FIG. 14B). When the profiles of AAV9-U6-anti-HTT-6433 and AAV9-CBA-anti-HTT-6433 were compared at two weeks, it was observed that only 8 mRNAs were significantly differentially expressed between these two groups. RNAs were sorted according to presence or absence of predicted target sites for the artificial miRNA and plotted the cumulative distribution of changes in mRNAs where the target sites were present or absent. In the mice injected with the AAV-U6-6433, a small shift toward downregulation of genes containing in their 3′-UTRs, perfect 8mer target sites matching the most abundant AAV-derived small RNA species were observed (FIG. 10B). This shift was not apparent in the mice

injected with AAV-CBA-6433 (FIG. 10A) nor with any of the other AAV-derived small RNA species (FIGs. 15A-15C).

Example 3: Sheep *in vivo* experiments

A sheep model of human Huntington's disease was used in this example. Briefly, transgenic sheep that express human huntingtin (human htt) protein were produced. Sheep were injected intrastriatally with either scAAV9 CBA-mir-HTT ("CBA Promoter"), scAAV9 U6-mir-HTT ("U6 Promoter"), or empty scAAV9 control vector. Each construct comprises a single copy of the anti-huntingtin mir-6433 sequence (SEQ ID NO: 7) inserted into a mir-155 backbone located within an intron that is between the CBA promoter or the U6 promoter, respectively, and a β-globulin polyadenylation sequence. Constructs used to produce rAAVs administered in this experiment are set forth in SEQ ID NOs: 18 (scAAV9 CBA-mir-HTT) and 19 (scAAV9 U6-mir-HTT).

Sheep were sacrificed at either one month or six months post-injection. Tissue and nucleic acid samples were prepared and analyzed by quantitative PCR and immunohistochemistry.

Data indicate that at the one month time point, injection of mir-HTT expressed under the U6 promoter resulted in a reduction of htt expression in both the middle caudate and middle putamen of sheep when compared to un-injected and empty-scAAV9-injected control mice. FIG. 16 shows data relating to reduction of human htt expression in the middle caudate of sheep one month-post injection of scAAV9 U6-mir-HTT. FIG. 17 shows data relating to reduction of human huntingtin expression in the middle putamen of a sheep model one month post-injection of scAAV9 U6-mir-HTT. Note that unlike the mouse model described in the previous example, the mir-HTT expressed from the U6 promoter was not toxic in the sheep model of Huntington's disease.

Data indicate that at the six month time point, injection of mir-HTT expressed under the CBA promoter resulted in a reduction of htt expression in both the middle caudate and middle putamen of sheep when compared to un-injected and empty scAAV9-injected control mice.

FIGs. 18 and 19 show data relating to reduction of human htt expression in the medial (FIG. 18) and lateral (FIG. 19) sides of the middle caudate of the sheep. Data indicate mir-HTT expressed from a CBA promoter causes a reduction of human htt expression in the injected side of the brain when compared to the non-injected side of the brain and empty scAAV9-injected

control mice. The effects of expression of mir-HTT from the CBA and U6 promoters on silencing of htt in the middle caudate was also compared six months post-injection. Data indicates that mir-HTT expressed from either U6 promoter or CBA promoter results in a reduction of human htt expression in the middle caudate when compared to non-injected and empty scAAV9-injected control animals (FIG. 20).

FIGs. 21 and 22 show data relating to reduction of human htt expression in the lateral (FIG. 21) and medial (FIG. 22) sides of the middle putamen of the sheep six months post-injection. Data indicate mir-HTT expressed from a CBA promoter causes a reduction of human htt expression in the injected side of the brain when compared to the non-injected side of the brain and empty scAAV9-injected control mice. The effects of expression of mir-HTT from the CBA and U6 promoters on silencing of htt in the middle caudate was also compared six months post-injection. Data indicates that mir-HTT expressed from either U6 promoter or CBA promoter results in a reduction of human htt expression in the middle putamen when compared to non-injected and empty scAAV9-injected control animals (FIG. 23).

FIG. 24 shows data relating to relative expression of human huntingtin (human htt) RNA in the anterior striatum of a sheep model of Huntington's disease six months after intrastriatal injection of either scAAV9 CBA-mir-HTT ("CBA Promoter"), scAAV9 U6-mir-HTT ("U6 Promoter"), or empty scAAV9 control vector.

Example 4: Artificial miRNAs reduce human mutant Huntingtin throughout the striatum in a transgenic sheep model of Huntington's disease

Animals and animal procedures

Merino sheep were used in this example. Prior to the administration of anesthetic, the animals were fasted overnight for approximately 8 hours. Animals were given a pre-operative physical including heart rate, respiratory rate, temperature and weight. Baseline samples of serum (5ml) and CSF were collected.

The study was conducted in two parts with two different cohorts of sheep. For the first study, forty-one transgenic animals (21 Wethers, 20 Ewes), aged approximately 8 months were injected unilaterally with 300µl of self-complementary AAV9 (scAAV9) vector at a titer of $1x10^{13}$ gc/ml for a total of $3x10^{12}$ genome copies. For the second study, fourteen animals aged 14 months were injected with this vector and fourteen with the control vector. Gadolinium was

added to the vector formulation to allow post-surgical imaging of the injection spread. The animals were moved to the operating room and prepped for surgery. They were rested in the sphinx position on a foam cushion on folded extremities or with extremities dangling. A stereotactic frame (Kopf, large animal) was used to hold the animal's head in place. Cerebrospinal fluid was collected via lumbar puncture using a 19 gauge spinal tap cannula. The rAAV was delivered directly to the striatum, targeting the internal capsule. The animal's head was shaved, prepped with betadine, and draped with clear plastic. A curvilinear incision was made using a #15 scalpel to expose the bregma. Once the bregma was identified, a 3 – 4mm burr hole was placed 10mm rostral to the bregma and 11mm lateral of the midline using an electric drill. The convection enhanced delivery (CED) cannula (MRI Interventions, Irvine, CA) was secured in the manipulator and primed with agent to be injected to remove air from the line. The dura was opened with a 1.5mm incision using a #11 scalpel and the CED cannula was advanced 25mm from dural surface to the target depth. The outer cannula (1.65mm) sealed the dural incision to prevent CSF leakage during the infusion. The infusion began 5 minutes after cannula insertion to allow for tissue around the tip to stabilize. The infusion rate was set at 3.33µl/minute until a total volume of 300µl was injected. Ten minutes after infusion was completed the cannula was slowly withdrawn and a bone wax plug was used to repair skull and prevent CSF leakage. The wound was cleansed with saline and closed using a 3.0 vicryl suture. Standard anesthesia wake-up and recovery procedure was followed. Post-surgery MRI was performed to determine the spread of gadolinium. One animal from the first study was excluded following surgery because no gadolinium was visible upon imaging and a second animal from the second study was excluded because the gadolinium appeared to be primarily in the ventricle. After the surgery, the animals were kept under observation for three days and housed indoors for 5. They were then transferred outdoors and house outdoors in paddocks for the remainder of the study. Animals were monitored visually for signs of distress and changes in behavior throughout the study. Two animals suffered surgical complications, resulting in partial limb paralysis. This was thought to be due to the positioning of the animals under anesthesia. One was anesthetized early and one was moved from the six month to the one-month cohort. Animals were weighed periodically throughout the post-injection period and samples of cerebrospinal fluid, blood and serum were taken and saved for further analysis.

For cell counts and differentials, blood was collected via jugular venipuncture into a potassium EDTA blood collection tube (Lavender top; LT) and a complete blood examination

with differential (CBE differential) was performed. For clinical chemistry, blood was collected via jugular venipuncture into a serum collection tube (red top; RT). The samples were submitted for multiple biochemical analysis (MBA).

At one and six-months post-injection animals were harvested, with animals being used for either histology or biochemical analysis. Animals were transported to operating table and placed in ventral recumbency while approximately 6mL of CSF was collected. The animal was repositioned in dorsal recumbency. The carotid arteries were exposed and cannulated at a depth of 4cm from the tip of the cannula. The jugular veins were exposed and 200-500 U Heparin/kg were injected into the jugular vein. Five minutes after administering the Heparin, sheep were euthanized by intravenous injection of Lethabarb (325mg pentabarbitone sodium/ml) at 1 ml/2kg of body weight. The infusion pump was primed with cold 9% NaCl and connected to the carotid cannulas. The animal was perfused with approximately 8L of cold 9% NaCl at a pressure of 500mmHg. For histology, the infusion was switched to 8L 4% paraformaldehyde at a pressure of 500mmHg. The brain and liver were extracted. The tissues were post-fixed in 4% paraformaldehyde for 24 hours at 4˚C and transferred to 30% sucrose in 1X phosphate buffered saline for a minimum of 14 days at 4˚C

For RNA, protein, and DNA assays, sheep were perfused with cold 9% NaCl as described above. Collection of the peripheral tissue was performed in the following order: liver, adrenal gland, ovaries (if applicable), muscle, and heart. Cross contamination was prevented by the use of different instruments and washing necropsy surfaces with 10% bleach and 70% ethanol. The organ was removed from the body and a 3mm biopsy punch was used to collect samples. A total of ten samples were collected from each organ; two samples were snap frozen in liquid nitrogen and eight samples were stored in RNA later at 4˚C for 24 hours (300µl of RNA later for liver, muscle and heart samples, 500µl of RNA later for adrenal gland and ovary samples).

The brain was removed from the skull using a circular saw and bone forceps. After extraction, the brain was weighed and placed ventrally in a custom made plexiglass brain matrix. Nine cuts were made to the brain to fully contain the striatum in 4 6mm blocks. The first cut was made posterior to the olfactory bulb attachment (approximately 18mm from the beginning of the matrix) and the subsequent four cuts were made at 6mm intervals. The striatum was divided into four 6 mm blocks from posterior to anterior: 2p (posterior), 2m1 (medial 1), 2m2 (medial 2) and 2a (anterior). The striatal dissection was performed in the following order: 2p,

2m1, 2a. The striatum in the right (non-injected) hemisphere was dissected first in all blocks and scalpel blade was changed between hemispheres. The dissection was performed in a petri dish on dry ice and care was taken to remove as much white matter from the striatal tissue as possible. Once dissected out, the striatal pieces (caudate and putamen) were split in half; with the medial piece (closest to midline of block) was stored in 1ml of RNA later at 4˚C and the lateral piece was snap frozen in liquid nitrogen. The striatal dissection for the 6 month cohort in the CBA study was done in a manner to produce four striatal samples from both the caudate and putamen. The dorsal sections (both medial and lateral) were snap frozen in liquid nitrogen and the ventral sections (both medial and lateral) were stored in 1ml of RNA later at 4˚C. RNA later was removed after twenty four hours and samples were stored at -80˚C.

The 2m2 block was generously covered with OCT and frozen in a 2-methylbutane and dry ice bath. The remainder of the 2a, 2m1, and 2p block was frozen in the same manner. Ten cortex samples were taken from each block in a dorsal to ventral manner; two were snap frozen in liquid nitrogen and eight were stored in 1ml RNA later at 4˚C.

Sectioning of tissue for histological analysis

Prior to tissue sectioning for histological analysis the striatum was isolated from the brain, generously covered with OCT, and stored at -20˚C for twenty four hours. Coronal sections measuring 40µm thick were cut with a sliding microtome (Reichert-Jung Tetrander sliding microtome) through the entire striatum. The sections were stored in 0.01% sodium azide in 1X phosphate buffered saline at 4˚C.

Vector cloning and rAAV9 Production

For the first study, the test vector contained a U6 promoter driving an artificial miRNA based on the endogenous mir155 backbone (AAV9-U6-miR^{HTT}). The artificial miRNA targets human, but not the sheep huntingtin. A chimeric cytomegalovirus enhancer/chicken β-actin (CBA) promoter driving a chimeric intron was included to improve AAV packaging. The control vector (AAV9) contained only the empty CBA promoter and the intron. For the second study, the test vector contained the CMV enhancer and CBA promoter, the intron and the miRNA-155 based artificial miRNA (AAV9-CBA-miR^{HTT}).

For packaging, the rAAV vector plasmid, a packaging plasmid and an adenovirus helper plasmid are co-transfected into HEK 293 cells. The packaging plasmid expresses the regulatory

and AAV9 capsid proteins leading to excision, replication and packaging of the recombinant genome from the rAAV vector plasmid into AAV virions. The recombinant viruses are purified by standard CsCl gradient sedimentation and desalted by dialysis.

Analysis of Huntingtin mRNA Levels

The RNA levels in the RNA later preserved samples were analyzed using a branched DNA assay (bDNA). Samples were processed according to the manufacturer's guidelines for preparation of tissue homogenates from tissues stored in RNA later (Affymetrix eBioscience, Quantigene® Sample Processing Kit). The homogenized samples were analyzed according to the manufacturer's guidelines for the bDNA assay (QuantiGene® 2.0 Reagent System). The samples were analyzed with a probe to detect human huntingtin (Human HD, SA-50339 from Quantigene), ovine huntingtin (Sheep Huntingtin, SF-10586 from Quantigene), and ovine calnexin as a housekeeping gene (Sheep Calnexin, SF-10622 from Quantigene). The assay results were measured with a Tecan Infinite M1000 PRO luminometer (integration time set at 200ms).

Analysis of miR-Htt levels

Biopsy punches (2mm) were sampled from the lateral caudate and the medial putamen, from frozen blocks. The RNA extractions were performed using the TRIzol manufacturer's guidelines (Ambion) with some modifications made. After the phase separation in the TRIzol extraction, the aqueous phase was transferred to RNA Clean & Concentrator (Zymo) column and that protocol was followed. RNA was stored at -80°C until analysis. RNA quality and concentration were determined on a Fragment Analyzer (Advanced Analytical Technologies Inc.). Immediately prior to analysis, the RNA was diluted to 20ng/µl. Artificial miRNA guide strands were retro-transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Cat# 4366596, Thermo Scientific), 2µl of RNA and guide strand specific stem-loop primers (ThermoFisher custom assay targeting UAAGCAUGGAGCUAGCAGGCU (SEQ ID NO: 25) or assay id 002407, let7e*), according to the manufacturer's instructions. ddPCR reactions were setup using 5µl of RT products, a 1x concentration of the miR-Htt assay and a 0.3x concentration of the let-7e* assay to allow for multiplexing. Droplets were generated with a QX200 Droplet Generator (Cat# 1864002, Biorad), and monitored for positive signal following

endpoint PCR amplification (40 cycles). Relative expression of miR^{HTT} was determined by calculating the ratio between absolute concentrations of miR^{HTT} and let-7e*.

Vector Genome Distribution

Genomic DNA was extracted from samples that had been snap frozen in liquid nitrogen using the Gentra Puregene Tissue kit (Qiagen). The genomic DNA concentrations were measured using the NanoDrop ONE^c spectrophotometer. Droplet Digital PCR (ddPCR, Biorad) was performed according to the manufacturer's recommendations, using 50ng of DNA as input and TaqMan assays detecting the vector-specific CB and U6 promoter and the HPRT reference gene. Results are expressed as vector genome per diploid genome (vg/dg).

Analysis of Huntingtin Protein Levels--Mesoscale Detection Assay (MSD) for mHTT

Striatal samples were homogenized in buffer composed of 10 mM HEPES, 250 mM sucrose, 1 mM EDTA and protease inhibitors (Roche) and sonicated 10s at 10% amplitude. Protein concentration was measured using Bradford assay. A 96-well QuicPlex standard plate (MSD) was coated with rabbit monoclonal anti-HTT proline 1220 region antibody (D7F7, Cell Signaling, 1:250) in PBS, overnight at 4°C. The plate was washed 3x 10 min with PBST (PBS + 0.05% Tween20) and blocked with 3% bovine serum albumin (BSA) in PBS for 2 hours at RT. After washing 3x 10 min with PBST, technical duplicates of samples with 20 µg of protein in 25 µL of homogenization buffer or blanks (homogenization buffer) were distributed into the plate and incubated overnight at 4°C on an orbital shaker. The plate was washed 3x 10 min in PBST and incubated in secondary/detection antibody mix as follows: For detection of mHTT, mouse monoclonal anti-polyQ antibody MW1 (DSHB) was mixed with anti-mouse SulfoTag detection antibody (MSD) at 1 µg/mL of each antibody in 1% BSA in PBS. 30 µL of detection antibody mix was applied per well and incubated for 3 hours at RT on an orbital shaker. The plate was washed 3x 10 min in PBST and 150 µL of 2x Read Buffer (MSD) was applied per well right before readout on QuickPlex SQ120 (MSD).

Western Blotting

Small pieces of tissue were removed from frozen blocks and homogenized on ice in 200 µl 10mM HEPES pH7.2, 250mM sucrose, 1mM EDTA + protease inhibitor tablet (mini, complete, EDTA-free Roche #11836170001). Samples were sonicated for 10 seconds and

protein concentration was determined using the Bradford method (BioRad #500-0006). Equal concentrations of protein (25 μg) were separated by SDS-PAGE on 3-8%Tris-Acetate gels (Life Technologies #EA03785BOX) and transferred to nitrocellulose using TransBlot Turbo (BioRad). Blots were blocked in 5% non-fat dry milk in Tris-buffered saline + 0.1% Tween-20 (TBST) for 1 hour and incubated overnight in primary antibody at 4°C diluted in blocking solution. Primary antibodies used were: anti-poly-Q (MW1, Coriell, 1:500 or 3B5H10, Sigma, 1:1000), anti-huntingtin (MAB2166, EMD Millipore, 1:1000 or Ab1, DiFiglia et al., 1995, 1:1000), anti-DARPP32 (#ab40801, Abcam, 1:10,000), anti-actin (A4700, Sigma, 1:1000), and anti-spectrin (MAB1622, EMD Milliopore, 1:4000). Blots were washed in TBST, incubated in peroxidase labeled secondary antibodies diluted 1:5000 in blocking solution for 1 hour at room temperature, washed in TBST and incubated in SuperSignal West Pico Chemiluminescent Substrate (Pierce #34080). Images were obtained with a CCD imaging system (Alpha Innotech) and Hyperfilm ECL (GE Healthcare). Densitometry was performed on the digital images using ImageJ software (NIH). Statistical analysis was performed using upaired t-tests and results were expressed as mean value for the injected side.

Immunohistochemistry for DARPP32, NeuN, and Iba1

To quantify the DARPP32 positive cells, every twentieth section was incubated for three minutes in 3% hydrogen peroxide in 1X PBS, twenty minutes in 0.5% Triton-X-100, and then four hours in 1.5% normal goat serum (Vector Labs, S-1000) in 1X PBS. Sections were incubated in anti-DARPP32 (AbCam, ab40801, 1:1,000 dilution) in 1.5% normal goat serum overnight at 4˚C. Sections were then incubated in biotinylated goat, anti-rabbit IgG antibody (Vector Labs, AP-1000, 1:200 dilution) in 1X PBS for 10 minutes. The sections were incubated with 2% Elite A and 2% Elite B reagent from the Vectastain Elite ABC Kit (Vector Labs, PK-6100) in 1X PBS for five minutes. The Metal Enhanced DAB kit (ThermoFisher Scientific, 34065) was used to visualize the DARPP32 positive cells. The sections were incubated in 1X 3, 3'-diaminobenzidine in stable peroxide buffer.

To quantify the NeuN positive cells, every twentieth section was incubated for three minutes in 3% hydrogen peroxide in 1X PBS, twenty minutes in 0.5% Triton-X-100, and then overnight in 1.5% normal goat serum (Vector Labs, S-1000) in 1X PBS at 4˚C overnight. The sections were incubated in anti-NeuN (Chemicon, MAB377, 1:1,000 dilution) in 1.5% normal goat serum for one hour at 4˚C. The sections were then incubated for 40 minutes in a

fluorescent AF594 goat, anti-mouse IgG (ThermoFisher Scientific, A-11005, 1:2,000 dilution) to visualize the NeuN positive cells.

To quantify the Iba1 positive cells, every twentieth section was incubated for one hour in a solution of 5% normal goat serum (Vector Labs, S-1000), 1% bovine serum albumin (Sigma, A-3059), 0.2% Triton-X-100, and 0.03% hydrogen peroxide in 1X PBS. The sections were incubated in anti-Iba1 (Wako Chemicals, 019-19741, 1:1,000 dilution) in 5% normal goat serum (Vector Labs, S-1000) and 1% bovine serum albumin (Sigma, A-3059) at 4°C overnight. Sections were incubated biotinylated goat, anti-rabbit IgG antibody (Vector Labs, AP-1000, 1:200 dilution) in 1X PBS for ten minutes. The sections were incubated with 2% Elite A and 2% Elite B reagent from the Vectastain Elite ABC Kit (Vector Labs, PK-6100) in 1X PBS for five minutes. The Metal Enhanced DAB kit (ThermoFisher Scientific, 34605) was used to visualize the reaction by incubating section in 1X 3',3-diaminobenzidine in stable peroxide buffer.

The quantification of DARPP32 and Iba1 positive cells in the left and right hemisphere of the brain was done by taking images (20X for DARPP32 and 40X for Iba1) with a Nikon Eclipse E600 microscope of each section. In order to consistently capture images between different sections, the first image was captured in the medial, dorsal edge of the striatum and the stage was moved 0.5cm toward the ventral edge. Once the ventral edge was reached, the stage was moved 0.5cm laterally and 0.5cm dorsally until ten images were captured. Random numbers were assigned to each image to eliminate bias when quantifying cells. The cells were counted using ImageJ software (NIH).

The quantification of NeuN positive cells was performed using the Nikon Eclipse E600 with a Chiu Technical Corporation Mercury 100-W lamp at 60X. The stereological method used for capturing DARPP32 and Iba1 images was also used to quantify the NeuN positive cells. The area of the striatum, caudate, and putamen for each section was measured by manually circling the DARPP32 stained regions using ImageJ software (NIH) on the injected and non-injected sides of every 20^{th} section through the striatum (29-35 sections per side per animal). The observer was blinded to the conditions. Total volume for each region was determined by multiplying the area by the section thickness (40 microns) by the number of sections between slides (20) and adding together for each animal. Statistical analysis was performed using Microsoft excel, paired and unpaired t-tests, N=3 or 4 animals per group.

Vector genome and miRNA distribution following injection

Silencing of an expanded mouse huntingtin in a knock-in model of HD and of the human *mHTT* transgene mRNA in a transgenic mouse model of HD have been observed. In this example, two cohorts of HD sheep (study 1 and study 2) were unilaterally injected the striatum. In study 1, the sheep were injected at 8-9 months of age with scAAV9-U6-miR^{HTT} (AAV9miRHTT) or scAAV9-CBA-empty (AAV9) where a non-coding stuffer sequence is inserted between the promoter and the poly-A signal. In study 2 the sheep were injected at 14 months of age with scAAV9-CBA-miR^{HTT} or scAAV9-CBA-empty (AAV9). The brains were harvested one and six months after AAV9-miR^{HTT} administration.

Genome copies were determined in a subset of regions (FIG. 26A) by droplet digital PCR (ddPCR, FIG. 26B). The genome copies were highest in the caudate and putamen on the injected compared to the non-injected side and were at the highest levels in the scAAV9-U6-miR^{HTT} treatment groups at 1 and 6 months post-injection. Small amounts of vector genome were present in the cortex and liver, but were undetectable in the adrenals (FIG. 26B).

RNA quality was measured using the fragment analyzer, which generates a score, called the RNA Quality Number (RQN). The RQN is generated by analyzing the electro-pherogram and integrates a number of different measures of RNA integrity, such as ratio between the 28S and 18S ribosomal peak sharpness and baseline. Scores generally range from 0 (completely degraded) to 10. Samples with scores greater than 5 were used to analyze the levels of artificial miR guide strand. Two animals from study 1 and two from study 2 were excluded from the analysis due to low RQN scores. The levels of the artificial miRNA guide strands were measured using ddPCR and normalized to the endogenous let7e* (FIG. 27). The relative quantity of artificial miR antisense strand was 3.5-1000 fold higher on the injected side than the non-injected side and higher at one month compared to six months post-injection. miRNA guide strands were detected at low levels on the side contralateral to injection with AAV9-miR^{HTT}.

A single administration of scAAV9-miR^{HTT} long-term reduces the human mutant huntingtin mRNA in caudate and putamen

HTT mRNA in the anterior and medial striatum was measured using a branched DNA (bDNA) assay that specifically recognizes human and not sheep *HTT* mRNA. This assay does not require RNA isolation and all samples were included in the analysis. At one-month post-injection, closest to the injection in the medial block, scAAV9-U6-miR^{HTT} (study 1) reduced human *HTT* mRNA by more than 50% in both the caudate and putamen (FIG. 28). No

significant silencing was detected in the anterior striatum, which was farther from the injection site (FIG. 28). At six-months post-injection, mRNA silencing was pronounced in the caudate (FIG. 28). In the scAAV9-CβA-miR^{HTT} cohort (study 2), marked silencing of *HTT* mRNA occurred in the medial putamen, medial caudate and anterior striatum (FIG. 28) at one month and in the medial caudate and part of the medial putamen (FIG. 28) at six-months post-injection. The anterior striatum did not show significant lowering at six months. There was no significant silencing of the endogenous sheep *HTT* mRNA (FIG. 29A).

Western blot assay and electrochemiluminescence (MSD assay) show that scAAV9-miR^{HTT} reduces human mutant huntingtin protein in the caudate and putamen

HTT protein was detected by Western blot (FIG. 30) and electrochemiluminesence (Meso Scale Discovery (MSD, FIG. 30) in the same sample preparations. In study 1, the 3BH510 antibody which preferentially detects mHTT (mutant HTT) compared to wild-type HTT, was used to detect mHTT protein by Western blot (FIG. 30). One month after treatment with scAAV9-U6-miR^{HTT}, there was a significant reduction in mHTT protein in the caudate, putamen and anterior striatum, and in putamen at six-months post-treatment, compared to treatment with AAV9 lacking miR^{HTT}. In study 2 (FIG. 30, bottom), scAAV9-CβA-miR^{HTT} treatment significantly silenced mHTT at one month post-injection in the putamen and at six months post-injection in caudate, putamen and anterior striatum.

Results with the MSD assay using MW1 for detection showed that scAAV9-U6-miR^{HTT} treatment (study 1) significantly lowered mHTT protein levels in the caudate, putamen, and anterior striatum at one and six-months post-treatment. scAAV9-CBA- miR^{HTT} markedly silenced mHTT protein in caudate at one month post-injection and in caudate, putamen and anterior striatum 6 months after treatment (FIG. 31). These results indicated that there was good agreement between results with the MSD assay (FIG. 31) and those obtained by Western blot assay (FIG. 30).

Table 2: Mean percent of mutant huntingtin protein lowering by Western blot and MSD assays in Studies 1 and 2. The human mutant huntingtin protein was measured by Western blot with anti-htt polyQ antibody 3B5H10 in study 1 and antibodies 3B5H10, MAB2166 (anti-HTT443-456), which does not recognize sheep HTT (Reid et al., 2013), and anti-polyQ monoclonal antibody MW1 in study 2. In the MSD assays MW1 was used as the detection antibody. This table

reports the mean percent mHTT lowering for the caudate, putamen, and anterior striatum. Percent lowering was calculated by dividing the average signal for the injected side in the AAV9miRHTT treated sheep by the average signal for the injected side in the AAV9 alone treated animals.

Table 1. Mean percent of mutant huntingtin protein lowering by Western blot and MSD assays in Studies 1 and 2.

Study# (promoter)	Study 1 (U6)				Study 2 (CBA)							
Assay mutant htt antibody	Western blot (3B5H10)		MSD MW1		Western blot (3B5H10)		Western blot (MAB2166)		Western blot (MW1)		MSD MW1	
Post-injection Interval (months)	1	6	1	6	1	6	1	6	1	6	1	6
Caudate	78**	30	71**	74**	16	30, 43	61	46, 58*	50*	65*, 70	43*	40*, 47*
Putamen	61*	47*	73**	50*	40*	55*, 44	68*	65*, 67*	54	51*, 56*	42	63**, 56**
Anterior Striatum	63**	22	49*	33	46	60**, 48	46	81*, 62*	-8	74*, 53*	22	62**, 67**

Since antibodies that detect mHTT may have different sensitivities, two other antibodies to detect human mHTT protein by Western blot, MAB2166 and MW1, were included in study 2 (Table 2). In the HD transgenic sheep MAB2166 recognizes only human huntingtin and not sheep HTT. MW1 preferentially recognizes the expanded polyglutamine region in HTT and was also used for detection of mHTT in the MSD assay. Table 2 compares the mean percent lowering of mHTT detected by Western blot with three anti-mHTT antibodies (3B5H10, 2166, and MW1) and by MSD assay with MW1 in studies 1 and 2. All three antibodies in Western blot analysis detected significant mHTT lowering in multiple neostriatal regions in study 2 (49% to 81%). Results of mHTT lowering by MSD assay were consistent when two samples from the same striatal region were analyzed in study 2. A comparison of the results by Western blots and by MSD assays with MW1 in study 2 are also noteworthy. There was good agreement between these two different methods of mHTT detection in the magnitude of mHTT lowering.

By Western blot analysis, the cortex overlying the AAV9-miR^{HTT} injected striatum did not show a decline in mHTT protein levels compared to the AAV9 injected cortex (FIG. 32A). A low level of mRNA guide strand was detected in the caudate and putamen on the side contralateral to the AAV9-miR^{HTT} injected striatum. These regions did not show reduced levels of mHTT protein by Western blot analysis (FIG. 32B).

To investigate whether treatment with AAV9-miR^{HTT} against the human HD gene affected the levels of endogenous sheep HTT, the levels of the human transgene mHTT with levels of endogenous sheep HTT were directly compared using Western blot analysis by taking advantage of differences in migration of the two proteins on SDS PAGE (FIG. 29B). Western

blot analysis with Ab1 antibody, which recognizes htt1-17, showed that unlike human mHTT, the endogenous sheep HTT was not lowered by treatment with miR^{HTT} (FIG. 29B).

DARPP32 labeled neurons and striatal volume are unaffected by miRNA treatment

To examine the safety of injection of the AAV vectors, immunohistochemistry for DARPP32, a marker of medium spiny neurons, was performed and the number of DARPP32 positive cells was counted. There was no significant difference between the number of cells in the AAV9-miR^{HTT} treated and AAV9 treated groups (Table 3) and no significant difference between treatment groups in the number of cells stained for NeuN, a marker of neuronal cells. Striatal volumes were determined using cross-sectional area measurements of striatum in DARPP32 labeled sections and were found to be unchanged compared to controls after miRNA treatment (Table 4).

Table 3: Number of DARPP32 and Neu N positive cells. Data were analyzed by paired t-test (injected to non-injected side). A significant difference was found between injected and non-injected sides only in WT sheep injected with AAV9-CBA-miRHTT at six months post injection, [1]p=0.002 by paired t-test.

Study# (promoter)	Group		Post-injection interval (months)	Side	# of DARPP32 positive cells	# of NeuN positive cells
Study 1 (U6)	HD	AAV9	1	inj	4201±389	
				non-inj	4056±488	
			6	inj	2819±614	1185±70
				non-inj	3314±364	1368±180
		AAV9 miRHTT	1	inj	4327±1444	
				non-inj	4587±838	
			6	inj	3884±1222	1547±315
				non-inj	4149±924	1633±262
Study 2 (CBA)	HD	AAV9	6	inj	2459±85	1111±314
				non-inj	2324±347	1184±330
		AAV9 miRHTT	6	inj	2061±321	1404±61
				non-inj	2084±460	1499±46
		No Injection	6	left	1852±232	1440±76
				right	2047±306	1183±220
Study 2 (CBA)	WT	AAV9	6	inj	2121±96	1157±180
				non-inj	2148±146	1106±86
		AAV9	6	inj	1799±223	1285±151[1]

		miRHTT No Injection	6	non-inj	1895±327	1434±142
				left	1963±181	1188±328
				right	2101±219	1056±258

Table 4: Striatal volume. Volume was determined from cross-sectional areas of 29-35 40 µm sections per side per animal, N=3 sheep per group. [1]p=0.01, [2]p=0.03, [3]p=0.02, using a paired t test.

				Volume (mm3), Mean±SD		
				Study 1 (U6)		Study 2 (CBA)
				1 month post-injection	6 months post-injection	6 months post-injection
Caudate	HD	AAV9	inj	293±54	241±13	
			non-inj	300±48	248±17	
			% inj/non-inj	97.3±4.2	97.2±3.2	
		AAV9 miRHTT	inj	257±48	302±15	280±79
			non-inj	290±48	325±33	304±49
			% inj/non-inj	88.6±9.3	93.8±15	91.1±12
	WT	AAV9 miRHTT	inj			283±67[1]
			non-inj			309±65
			% inj/non-inj			91.4±2.7
Putamen	HD	AAV9	inj	298±46	247±33	
			non-inj	307±52	274±11	
			% inj/non-inj	97.0±1.4	90.3±13	
		AAV9 miRHTT	inj	278±57	318±39[2]	281±10
			non-inj	285±50	340±45	314±21
			% inj/non-inj	97.5±8.9	93.6±1.2	89.9±6.9
	WT	AAV9 miRHTT	inj			292±45
			non-inj			329±63
			% inj/non-inj			89.2±3.7
Striatum (Rostral pole + Caudate + Putamen)	HD	AAV9	inj	1041±172	947±28	
			non-inj	1034±150	1006±49	
			% inj/non-inj	101±2.3	94.3±5.6	
		AAV9 miRHTT	inj	1023±85	1030±31[3]	1165±145
			non-inj	1037±96	1110±31	1222±116
			% inj/non-inj	98.8±4.7	92.8±1.7	95.1±4.0
	WT	AAV9 miRHTT	inj			1159±76
			non-inj			1210±32
			% inj/non-inj			95.7±3.8

A transient increase in activated microglia occurs after direct injection with scAAV9

Immuno-histochemical localization of Iba1, a protein which is localized to microglia and upregulated upon their activation, was investigated. Labeled cells were identified based on morphology as resting or activated microglia (Table 5). Injection of scAAV9-U6-miR^{HTT} or the

corresponding control vector increased the number of activated microglia on the injected side at one-month post-injection, but six months after injection the injected and non-injected sides were indistinguishable. In the second study, the microglial response was examined only at the study end point (6 months) at which time, there was no significant difference between groups. The findings suggest that the transient increase in activated microglia is independent of AAV cargo and can occur with any vector or with surgery alone.

Table 5: Number and classification based on morphology of IBA1 positive cells. Statistical analysis was done by paired t-test (injected side vs. non-injected side). A significant increase in activated microglia on the injected side compared to non-injected side was found in HD sheep injected with AAV9-U6-miR^{HTT} ([1] p=0.01) and in WT sheep injected with AAV9 ([2] p=0.006) at six months. A significant decrease in resting microglia was found at one month in HD sheep injected with both AAV9 ([3] p=0.05) and AAV9-U6-miR^{HTT} ([4] p=0.04) and a significant increase in total microglia was found in HD sheep injected with AAV9 at six months in study 2 ([4] p=0.04). All analyses were done by paired t-test comparing injected to non-injected side.

Study# (promoter)	Group		Post-injection interval (months)	Side	# of Iba1 activated microglia	# of Iba1 resting microglia	Total # Iba1 positive cells
Study 1 (U6)	HD	AAV9	1	inj	253±178	180±102[3]	433±168
				non-inj	2.0±2	305±70	307±68
			6	inj	29±19	351±104	380±123
				non-inj	12±6	377±214	388±219
		AAV9 miRHTT	1	inj	195±2	201±77[4]	396±135
				non-inj	2.8±3	347±116	350±113
			6	inj	29±8[1]	279±202	308±194
				non-inj	6.7±4	311±148	318±144
Study 2 (CBA)	HD	AAV9	6	inj	38±10	263±31	301±23[4]
				non-inj	23±3	248±29	271±32
		AAV9 miRHTT	6	inj	10±4	240±38	251±37
				non-inj	6.0±4	260±32	266±36
		No Injection	6	left	8.3±5	256±90	265±85
				right	13±17	192±112	204±100
Study 2 (CBA)	WT	AAV9	6	inj	16±4[2]	288±39	303±35
				non-inj	7.0±4	256±50	263±46
		AAV9 miRHTT	6	inj	22±18	261±29	283±26
				non-inj	8.7±7	303±59	312±55
		No	6	left	10±12	246±108	256±96

		Injection		right	9.3±8	298±63	307±69

scAAV9-miRH^{TT} treatment does not affect blood counts, electrolytes, or liver and kidney function

Blood samples were taken at four times: baseline (pretreatment), 28 (or 30) days, 90 days, and 180 days post treatment. A complete blood count, electrolytes were measured, and liver and kidney function tests were performed (Table 6). No changes in any of these measurements were found between AAV9-miR^{HTT} injected sheep and controls. In addition, there were no changes in weight at these times.

Table 6: Clinical pathology and complete blood counts for all sheep.

	Baseline				Day 28				Day 90				Day 180			
	Control		Test		Control		Test		Control		Test		Control		Test	
	Mean	n	Mean	n	Mean	n	Mean	n	Mean	n	Mean	n	Mean	n	Mean	n
sodium mmol/L	146	19	147	22	148	18	148	20	146	10	145	10	146	10	145	10
potassium mmol/L	4.14	18	4.15	22	5.26	18	5.17	20	5.21	10	5.38	9	5.21	10	5.38	9
chloride mmol/L	105	19	106	22	109	18	109	20	109	10	109	10	109	10	109	10
bicarbonate mmol/L	27	19	26	22	26	18	26	20	26	10	26	10	26	10	26	10
Anion mmol/L	18	18	19	22	18	18	18	20	17	10	16	10	17	10	16	10
glucose mmol/L	4.57	18	4.15	21	2.73	18	3.48	20	2.89	10	2.70	10	2.89	10	2.70	10
urea mmol/L	7.56	19	7.44	22	5.46	18	5.24	20	6.08	10	5.75	10	6.08	10	5.75	10
creatinine µmol/L	53	19	51	22	62	18	60	20	57	10	55	10	57	10	55	10
cholesterol mmol/L	1	19	1	22	2	18	1	20	1	10	2	10	1	10	2	10
osmo mmol/L	291	18	292	22	293	18	294	20	291	10	288	9	291	10	288	9
urate mmol/L	0.00	19	0.09	22	0.00	18	0.00	20	0.00	10	0.00	10	0.00	10	0.00	10
phosphate mmol/L	2.31	19	2.31	22	2.09	18	1.97	20	1.95	10	2.14	10	1.95	10	2.14	10
T Cal mmol/L	2.48	19	2.40	22	2.44	18	2.42	20	2.54	10	2.46	10	2.54	10	2.46	10
Ion Cal mmol/L	1.28	18	1.25	22	1.28	13	1.26	12	1.33	10	1.29	10	1.33	10	1.29	10
albumin g/L	36	19	35	22	35	18	35	20	35	10	35	10	35	10	35	10
globulin g/L	29	19	29	22	28	18	28	20	28	10	28	10	28	10	28	10
Total Protein g/L	65	19	64	22	63	18	63	20	63	10	64	10	63	10	64	10
Total Bilirubin	2	19	1	22	1	18	1	20	0	10	1	10	0	10	1	10

µmol/L																
GGT U/L	55	19	58	22	58	18	63	20	47	10	51	10	47	10	51	10
ALP U/L	153	19	140	22	157	18	151	20	202	10	211	10	202	10	211	10
ALT U/L	16	19	16	22	15	18	17	20	21	10	21	10	21	10	21	10
AST U/L	84	19	78	22	82	18	91	20	111	10	107	9	111	10	107	9
LDH U/L	498	18	496	22	532	18	536	20	624	10	577	9	624	10	577	9

	Baseline				Day 28				Day 90				Day 180			
	Control		Test		Control		Test		Control		Test		Control		Test	
	Mean	n	Mean	n	Mean	n	Mean	n	Mean	n	Mean	n	Mean	n	Mean	n
Haemoglobin g/L	102	19	100	22	115	18	113	21	115	10	120	10	115	10	120	10
Red Blood Cells $x10^{12}$/L	9.02	19	9.07	22	10.17	18	10.47	21	10.07	10	10.41	10	10.07	10	10.41	10
Packed Cell Volume L/L	0.34	19	0.34	22	0.38	18	0.40	21	0.39	10	0.40	10	0.39	10	0.40	10
Mean Cell Volume fl	37.71	19	37.97	22	37.79	18	37.94	21	38.39	10	37.92	10	38.39	10	37.92	10
Mean Cell Haem pg	11.28	19	11.08	22	11.36	18	11.30	21	11.44	10	11.52	10	11.44	10	11.52	10
Mean Cell Haem Conc g/L	299	19	292	22	301	18	298	21	299	10	305	10	299	10	305	10
Red cell Dist Width %	20	19	20	22	20	18	20	21	19	10	19	10	19	10	19	10
Platelets $x10^9$/L	343	16	334	22	397	17	347	20	305	9	241	9	305	9	241	9
White Cell Count 10^9/L	4.87	19	5.35	22	5.82	18	5.89	21	5.89	10	6.16	10	5.89	10	6.16	10
Neutrophils %	40	19	41	22	46	18	64	21	40	10	41	10	40	10	41	10
Lymphocytes %	58	19	56	22	51	18	51	21	53	10	58	10	53	10	58	10
Monocytes %	1	19	1	22	2	18	2	21	4	10	5	10	4	10	5	10
Eosinophils %	1	19	1	22	1	18	0	21	4	10	2	10	4	10	2	10
Basophils %	0	19	0	22	0	18	0	21	0	10	0	10	0	10	0	10

SEQUENCES

>SEQ ID NO: 1 Huntingtin mRNA; NCBI Ref. Seq NM_002111.8)

GCTGCCGGGACGGGTCCAAGATGGACGGCCGCTCAGGTTCTGCTTTTACCTGCGGC
CCAGAGCCCCATTCATTGCCCCGGTGCTGAGCGGCGCCGCGAGTCGGCCCGAGGCC
TCCGGGGACTGCCGTGCCGGGCGGGAGACCGCCATGGCGACCCTGGAAAAGCTGAT
GAAGGCCTTCGAGTCCCTCAAGTCCTTCCAGCAGCAGCAGCAGCAGCAGCAGCAGC
AGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAACAGCCGCCACCGCCGCC
GCCGCCGCCGCCGCCTCCTCAGCTTCCTCAGCCGCCGCCGCAGGCACAGCCGCTGCT
GCCTCAGCCGCAGCCGCCCCCGCCGCCGCCCCCGCCGCCACCCGGCCCGGCTGTGG

CTGAGGAGCCGCTGCACCGACCAAAGAAAGAACTTTCAGCTACCAAGAAAGACCGT
GTGAATCATTGTCTGACAATATGTGAAAACATAGTGGCACAGTCTGTCAGAAATTCT
CCAGAATTTCAGAAACTTCTGGGCATCGCTATGGAACTTTTTCTGCTGTGCAGTGAT
GACGCAGAGTCAGATGTCAGGATGGTGGCTGACGAATGCCTCAACAAAGTTATCAA
AGCTTTGATGGATTCTAATCTTCCAAGGTTACAGCTCGAGCTCTATAAGGAAATTAA
AAAGAATGGTGCCCCTCGGAGTTTGCGTGCTGCCCTGTGGAGGTTTGCTGAGCTGGC
TCACCTGGTTCGGCCTCAGAAATGCAGGCCTTACCTGGTGAACCTTCTGCCGTGCCT
GACTCGAACAAGCAAGAGACCCGAAGAATCAGTCCAGGAGACCTTGGCTGCAGCT
GTTCCCAAAATTATGGCTTCTTTTGGCAATTTTGCAAATGACAATGAAATTAAGGTT
TTGTTAAAGGCCTTCATAGCGAACCTGAAGTCAAGCTCCCCCACCATTCGGCGGAC
AGCGGCTGGATCAGCAGTGAGCATCTGCCAGCACTCAAGAAGGACACAATATTTCT
ATAGTTGGCTACTAAATGTGCTCTTAGGCTTACTCGTTCCTGTCGAGGATGAACACT
CCACTCTGCTGATTCTTGGCGTGCTGCTCACCCTGAGGTATTTGGTGCCCTTGCTGCA
GCAGCAGGTCAAGGACACAAGCCTGAAAGGCAGCTTCGGAGTGACAAGGAAAGAA
ATGGAAGTCTCTCCTTCTGCAGAGCAGCTTGTCCAGGTTTATGAACTGACGTTACAT
CATACACAGCACCAAGACCACAATGTTGTGACCGGAGCCCTGGAGCTGTTGCAGCA
GCTCTTCAGAACGCCTCCACCCGAGCTTCTGCAAACCCTGACCGCAGTCGGGGGCA
TTGGGCAGCTCACCGCTGCTAAGGAGGAGTCTGGTGGCCGAAGCCGTAGTGGGAGT
ATTGTGGAACTTATAGCTGGAGGGGGTTCCTCATGCAGCCCTGTCCTTTCAAGAAAA
CAAAAAGGCAAAGTGCTCTTAGGAGAAGAAGAAGCCTTGGAGGATGACTCTGAAT
CGAGATCGGATGTCAGCAGCTCTGCCTTAACAGCCTCAGTGAAGGATGAGATCAGT
GGAGAGCTGGCTGCTTCTTCAGGGGTTTCCACTCCAGGGTCAGCAGGTCATGACATC
ATCACAGAACAGCCACGGTCACAGCACACACTGCAGGCGGACTCAGTGGATCTGGC
CAGCTGTGACTTGACAAGCTCTGCCACTGATGGGGATGAGGAGGATATCTTGAGCC
ACAGCTCCAGCCAGGTCAGCGCCGTCCCATCTGACCCTGCCATGGACCTGAATGAT
GGGACCCAGGCCTCGTCGCCCATCAGCGACAGCTCCCAGACCACCACCGAAGGGCC
TGATTCAGCTGTTACCCCTTCAGACAGTTCTGAAATTGTGTTAGACGGTACCGACAA
CCAGTATTTGGGCCTGCAGATTGGACAGCCCCAGGATGAAGATGAGGAAGCCACAG
GTATTCTTCCTGATGAAGCCTCGGAGGCCTTCAGGAACTCTTCCATGGCCCTTCAAC
AGGCACATTTATTGAAAAACATGAGTCACTGCAGGCAGCCTTCTGACAGCAGTGTT
GATAAATTTGTGTTGAGAGATGAAGCTACTGAACCGGGTGATCAAGAAAACAAGCC
TTGCCGCATCAAAGGTGACATTGGACAGTCCACTGATGATGACTCTGCACCTCTTGT
CCATTGTGTCCGCCTTTTATCTGCTTCGTTTTTGCTAACAGGGGGAAAAAATGTGCT
GGTTCCGGACAGGGATGTGAGGGTCAGCGTGAAGGCCCTGGCCCTCAGCTGTGTGG
GAGCAGCTGTGGCCCTCCACCCGGAATCTTTCTTCAGCAAACTCTATAAAGTTCCTC
TTGACACCACGGAATACCCTGAGGAACAGTATGTCTCAGACATCTTGAACTACATC
GATCATGGAGACCCACAGGTTCGAGGAGCCACTGCCATTCTCTGTGGGACCCTCAT
CTGCTCCATCCTCAGCAGGTCCCGCTTCCACGTGGGAGATTGGATGGGCACCATTAG
AACCCTCACAGGAAATACATTTTCTTTGGCGGATTGCATTCCTTTGCTGCGGAAAAC
ACTGAAGGATGAGTCTTCTGTTACTTGCAAGTTAGCTTGTACAGCTGTGAGGAACTG
TGTCATGAGTCTCTGCAGCAGCAGCTACAGTGAGTTAGGACTGCAGCTGATCATCG
ATGTGCTGACTCTGAGGAACAGTTCCTATTGGCTGGTGAGGACAGAGCTTCTGGAA
ACCCTTGCAGAGATTGACTTCAGGCTGGTGAGCTTTTTGGAGGCAAAAGCAGAAAA
CTTACACAGAGGGGCTCATCATTATACAGGGCTTTTAAAACTGCAAGAACGAGTGC
TCAATAATGTTGTCATCCATTTGCTTGGAGATGAAGACCCCAGGGTGCGACATGTTG
CCGCAGCATCACTAATTAGGCTTGTCCCAAAGCTGTTTTATAAATGTGACCAAGGAC
AAGCTGATCCAGTAGTGGCCGTGGCAAGAGATCAAAGCAGTGTTTACCTGAAACTT
CTCATGCATGAGACGCAGCCTCCATCTCATTTCTCCGTCAGCACAATAACCAGAATA

TATAGAGGCTATAACCTACTACCAAGCATAACAGACGTCACTATGGAAAATAACCT
TTCAAGAGTTATTGCAGCAGTTTCTCATGAACTAATCACATCAACCACCAGAGCACT
CACATTTGGATGCTGTGAAGCTTTGTGTCTTCTTTCCACTGCCTTCCCAGTTTGCATT
TGGAGTTTAGGTTGGCACTGTGGAGTGCCTCCACTGAGTGCCTCAGATGAGTCTAGG
AAGAGCTGTACCGTTGGGATGGCCACAATGATTCTGACCCTGCTCTCGTCAGCTTGG
TTCCCATTGGATCTCTCAGCCCATCAAGATGCTTTGATTTTGGCCGGAAACTTGCTT
GCAGCCAGTGCTCCCAAATCTCTGAGAAGTTCATGGGCCTCTGAAGAAGAAGCCAA
CCCAGCAGCCACCAAGCAAGAGGAGGTCTGGCCAGCCCTGGGGGACCGGGCCCTG
GTGCCCATGGTGGAGCAGCTCTTCTCTCACCTGCTGAAGGTGATTAACATTTGTGCC
CACGTCCTGGATGACGTGGCTCCTGGACCCGCAATAAAGGCAGCCTTGCCTTCTCTA
ACAAACCCCCCTTCTCTAAGTCCCATCCGACGAAAGGGGAAGGAGAAAGAACCAG
GAGAACAAGCATCTGTACCGTTGAGTCCCAAGAAAGGCAGTGAGGCCAGTGCAGCT
TCTAGACAATCTGATACCTCAGGTCCTGTTACAACAAGTAAATCCTCATCACTGGGG
AGTTTCTATCATCTTCCTTCATACCTCAAACTGCATGATGTCCTGAAAGCTACACAC
GCTAACTACAAGGTCACGCTGGATCTTCAGAACAGCACGGAAAAGTTTGGAGGGTT
TCTCCGCTCAGCCTTGGATGTTCTTTCTCAGATACTAGAGCTGGCCACACTGCAGGA
CATTGGGAAGTGTGTTGAAGAGATCCTAGGATACCTGAAATCCTGCTTTAGTCGAG
AACCAATGATGGCAACTGTTTGTGTTCAACAATTGTTGAAGACTCTCTTTGGCACAA
ACTTGGCCTCCCAGTTTGATGGCTTATCTTCCAACCCCAGCAAGTCACAAGGCCGAG
CACAGCGCCTTGGCTCCTCCAGTGTGAGGCCAGGCTTGTACCACTACTGCTTCATGG
CCCCGTACACCCACTTCACCCAGGCCCTCGCTGACGCCAGCCTGAGGAACATGGTG
CAGGCGGAGCAGGAGAACGACACCTCGGGATGGTTTGATGTCCTCCAGAAAGTGTC
TACCCAGTTGAAGACAAACCTCACGAGTGTCACAAAGAACCGTGCAGATAAGAATG
CTATTCATAATCACATTCGTTTGTTTGAACCTCTTGTTATAAAAGCTTTAAAACAGTA
CACGACTACAACATGTGTGCAGTTACAGAAGCAGGTTTTAGATTTGCTGGCGCAGC
TGGTTCAGTTACGGGTTAATTACTGTCTTCTGGATTCAGATCAGGTGTTTATTGGCTT
TGTATTGAAACAGTTTGAATACATTGAAGTGGGCCAGTTCAGGGAATCAGAGGCAA
TCATTCCAAACATCTTTTTCTTCTTGGTATTACTATCTTATGAACGCTATCATTCAAA
ACAGATCATTGGAATTCCTAAAATCATTCAGCTCTGTGATGGCATCATGGCCAGTGG
AAGGAAGGCTGTGACACATGCCATACCGGCTCTGCAGCCCATAGTCCACGACCTCT
TTGTATTAAGAGGAACAAATAAAGCTGATGCAGGAAAAGAGCTTGAAACCCAAAA
AGAGGTGGTGGTGTCAATGTTACTGAGACTCATCCAGTACCATCAGGTGTTGGAGA
TGTTCATTCTTGTCCTGCAGCAGTGCCACAAGGAGAATGAAGACAAGTGGAAGCGA
CTGTCTCGACAGATAGCTGACATCATCCTCCCAATGTTAGCCAAACAGCAGATGCA
CATTGACTCTCATGAAGCCCTTGGAGTGTTAAATACATTATTTGAGATTTTGGCCCC
TTCCTCCCTCCGTCCGGTAGACATGCTTTTACGGAGTATGTTCGTCACTCCAAACAC
AATGGCGTCCGTGAGCACTGTTCAACTGTGGATATCGGGAATTCTGGCCATTTTGAG
GGTTCTGATTTCCCAGTCAACTGAAGATATTGTTCTTTCTCGTATTCAGGAGCTCTCC
TTCTCTCCGTATTTAATCTCCTGTACAGTAATTAATAGGTTAAGAGATGGGGACAGT
ACTTCAACGCTAGAAGAACACAGTGAAGGGAAACAAATAAAGAATTTGCCAGAAG
AAACATTTTCAAGGTTTCTATTACAACTGGTTGGTATTCTTTTAGAAGACATTGTTAC
AAAACAGCTGAAGGTGGAAATGAGTGAGCAGCAACATACTTTCTATTGCCAGGAAC
TAGGCACACTGCTAATGTGTCTGATCCACATCTTCAAGTCTGGAATGTTCCGGAGAA
TCACAGCAGCTGCCACTAGGCTGTTCCGCAGTGATGGCTGTGGCGGCAGTTTCTACA
CCCTGGACAGCTTGAACTTGCGGGCTCGTTCCATGATCACCACCCACCCGGCCCTGG
TGCTGCTCTGGTGTCAGATACTGCTGCTTGTCAACCACACCGACTACCGCTGGTGGG
CAGAAGTGCAGCAGACCCCGAAAAGACACAGTCTGTCCAGCACAAAGTTACTTAGT
CCCCAGATGTCTGGAGAAGAGGAGGATTCTGACTTGGCAGCCAAACTTGGAATGTG

CAATAGAGAAATAGTACGAAGAGGGGCTCTCATTCTCTTCTGTGATTATGTCTGTCA
GAACCTCCATGACTCCGAGCACTTAACGTGGCTCATTGTAAATCACATTCAAGATCT
GATCAGCCTTTCCCACGAGCCTCCAGTACAGGACTTCATCAGTGCCGTTCATCGGAA
CTCTGCTGCCAGCGGCCTGTTCATCCAGGCAATTCAGTCTCGTTGTGAAAACCTTTC
AACTCCAACCATGCTGAAGAAAACTCTTCAGTGCTTGGAGGGGATCCATCTCAGCC
AGTCGGGAGCTGTGCTCACGCTGTATGTGGACAGGCTTCTGTGCACCCCTTTCCGTG
TGCTGGCTCGCATGGTCGACATCCTTGCTTGTCGCCGGGTAGAAATGCTTCTGGCTG
CAAATTTACAGAGCAGCATGGCCCAGTTGCCAATGGAAGAACTCAACAGAATCCAG
GAATACCTTCAGAGCAGCGGGCTCGCTCAGAGACACCAAAGGCTCTATTCCCTGCT
GGACAGGTTTCGTCTCTCCACCATGCAAGACTCACTTAGTCCCTCTCCTCCAGTCTCT
TCCCACCCGCTGGACGGGGATGGGCACGTGTCACTGGAAACAGTGAGTCCGGACAA
AGACTGGTACGTTCATCTTGTCAAATCCCAGTGTTGGACCAGGTCAGATTCTGCACT
GCTGGAAGGTGCAGAGCTGGTGAATCGGATTCCTGCTGAAGATATGAATGCCTTCA
TGATGAACTCGGAGTTCAACCTAAGCCTGCTAGCTCCATGCTTAAGCCTAGGGATG
AGTGAAATTTCTGGTGGCCAGAAGAGTGCCCTTTTTGAAGCAGCCCGTGAGGTGAC
TCTGGCCCGTGTGAGCGGCACCGTGCAGCAGCTCCCTGCTGTCCATCATGTCTTCCA
GCCCGAGCTGCCTGCAGAGCCGGCGGCCTACTGGAGCAAGTTGAATGATCTGTTTG
GGGATGCTGCACTGTATCAGTCCCTGCCCACTCTGGCCCGGGCCCTGGCACAGTACC
TGGTGGTGGTCTCCAAACTGCCCAGTCATTTGCACCTTCCTCCTGAGAAAGAGAAGG
ACATTGTGAAATTCGTGGTGGCAACCCTTGAGGCCCTGTCCTGGCATTTGATCCATG
AGCAGATCCCGCTGAGTCTGGATCTCCAGGCAGGGCTGGACTGCTGCTGCCTGGCC
CTGCAGCTGCCTGGCCTCTGGAGCGTGGTCTCCTCCACAGAGTTTGTGACCCACGCC
TGCTCCCTCATCTACTGTGTGCACTTCATCCTGGAGGCCGTTGCAGTGCAGCCTGGA
GAGCAGCTTCTTAGTCCAGAAAGAAGGACAAATACCCCAAAAGCCATCAGCGAGG
AGGAGGAGGAAGTAGATCCAAACACACAGAATCCTAAGTATATCACTGCAGCCTGT
GAGATGGTGGCAGAAATGGTGGAGTCTCTGCAGTCGGTGTTGGCCTTGGGTCATAA
AAGGAATAGCGGCGTGCCGGCGTTTCTCACGCCATTGCTAAGGAACATCATCATCA
GCCTGGCCCGCCTGCCCCTTGTCAACAGCTACACACGTGTGCCCCCACTGGTGTGGA
AGCTTGGATGGTCACCCAAACCGGGAGGGGATTTTGGCACAGCATTCCCTGAGATC
CCCGTGGAGTTCCTCCAGGAAAAGGAAGTCTTTAAGGAGTTCATCTACCGCATCAA
CACACTAGGCTGGACCAGTCGTACTCAGTTTGAAGAAACTTGGGCCACCCTCCTTGG
TGTCCTGGTGACGCAGCCCCTCGTGATGGAGCAGGAGGAGAGCCCACCAGAAGAA
GACACAGAGAGGACCCAGATCAACGTCCTGGCCGTGCAGGCCATCACCTCACTGGT
GCTCAGTGCAATGACTGTGCCTGTGGCCGGCAACCCAGCTGTAAGCTGCTTGGAGC
AGCAGCCCCGGAACAAGCCTCTGAAAGCTCTCGACACCAGGTTTGGGAGGAAGCTG
AGCATTATCAGAGGGATTGTGGAGCAAGAGATTCAAGCAATGGTTTCAAAGAGAGA
GAATATTGCCACCCATCATTTATATCAGGCATGGGATCCTGTCCCTTCTCTGTCTCCG
GCTACTACAGGTGCCCTCATCAGCCACGAGAAGCTGCTGCTACAGATCAACCCCGA
GCGGGAGCTGGGGAGCATGAGCTACAAACTCGGCCAGGTGTCCATACACTCCGTGT
GGCTGGGGAACAGCATCACACCCCTGAGGGAGGAGGAATGGGACGAGGAAGAGGA
GGAGGAGGCCGACGCCCCTGCACCTTCGTCACCACCCACGTCTCCAGTCAACTCCA
GGAAACACCGGGCTGGAGTTGACATCCACTCCTGTTCGCAGTTTTTGCTTGAGTTGT
ACAGCCGCTGGATCCTGCCGTCCAGCTCAGCCAGGAGGACCCCGGCCATCCTGATC
AGTGAGGTGGTCAGATCCCTTCTAGTGGTCTCAGACTTGTTCACCGAGCGCAACCAG
TTTGAGCTGATGTATGTGACGCTGACAGAACTGCGAAGGGTGCACCCTTCAGAAGA
CGAGATCCTCGCTCAGTACCTGGTGCCTGCCACCTGCAAGGCAGCTGCCGTCCTTGG
GATGGACAAGGCCGTGGCGGAGCCTGTCAGCCGCCTGCTGGAGAGCACGCTCAGGA
GCAGCCACCTGCCCAGCAGGGTTGGAGCCCTGCACGGCGTCCTCTATGTGCTGGAG

TGCGACCTGCTGGACGACACTGCCAAGCAGCTCATCCCGGTCATCAGCGACTATCTC
CTCTCCAACCTGAAAGGGATCGCCCACTGCGTGAACATTCACAGCCAGCAGCACGT
ACTGGTCATGTGTGCCACTGCGTTTTACCTCATTGAGAACTATCCTCTGGACGTAGG
GCCGGAATTTTCAGCATCAATAATACAGATGTGTGGGGTGATGCTGTCTGGAAGTG
AGGAGTCCACCCCCTCCATCATTTACCACTGTGCCCTCAGAGGCCTGGAGCGCCTCC
TGCTCTCTGAGCAGCTCTCCCGCCTGGATGCAGAATCGCTGGTCAAGCTGAGTGTGG
ACAGAGTGAACGTGCACAGCCCGCACCGGGCCATGGCGGCTCTGGGCCTGATGCTC
ACCTGCATGTACACAGGAAAGGAGAAAGTCAGTCCGGGTAGAACTTCAGACCCTAA
TCCTGCAGCCCCCGACAGCGAGTCAGTGATTGTTGCTATGGAGCGGGTATCTGTTCT
TTTTGATAGGATCAGGAAAGGCTTTCCTTGTGAAGCCAGAGTGGTGGCCAGGATCC
TGCCCCAGTTTCTAGACGACTTCTTCCCACCCCAGGACATCATGAACAAAGTCATCG
GAGAGTTTCTGTCCAACCAGCAGCCATACCCCCAGTTCATGGCCACCGTGGTGTATA
AGGTGTTTCAGACTCTGCACAGCACCGGGCAGTCGTCCATGGTCCGGGACTGGGTC
ATGCTGTCCCTCTCCAACTTCACGCAGAGGGCCCCGGTCGCCATGGCCACGTGGAG
CCTCTCCTGCTTCTTTGTCAGCGCGTCCACCAGCCCGTGGGTCGCGGCGATCCTCCC
ACATGTCATCAGCAGGATGGGCAAGCTGGAGCAGGTGGACGTGAACCTTTTCTGCC
TGGTCGCCACAGACTTCTACAGACACCAGATAGAGGAGGAGCTCGACCGCAGGGCC
TTCCAGTCTGTGCTTGAGGTGGTTGCAGCCCCAGGAAGCCCATATCACCGGCTGCTG
ACTTGTTTACGAAATGTCCACAAGGTCACCACCTGCTGAGCGCCATGGTGGGAGAG
ACTGTGAGGCGGCAGCTGGGGCCGGAGCCTTTGGAAGTCTGCGCCCTTGTGCCCTG
CCTCCACCGAGCCAGCTTGGTCCCTATGGGCTTCCGCACATGCCGCGGGCGGCCAG
GCAACGTGCGTGTCTCTGCCATGTGGCAGAAGTGCTCTTTGTGGCAGTGGCCAGGC
AGGGAGTGTCTGCAGTCCTGGTGGGGCTGAGCCTGAGGCCTTCCAGAAAGCAGGAG
CAGCTGTGCTGCACCCCATGTGGGTGACCAGGTCCTTTCTCCTGATAGTCACCTGCT
GGTTGTTGCCAGGTTGCAGCTGCTCTTGCATCTGGGCCAGAAGTCCTCCCTCCTGCA
GGCTGGCTGTTGGCCCCTCTGCTGTCCTGCAGTAGAAGGTGCCGTGAGCAGGCTTTG
GGAACACTGGCCTGGGTCTCCCTGGTGGGGTGTGCATGCCACGCCCCGTGTCTGGAT
GCACAGATGCCATGGCCTGTGCTGGGCCAGTGGCTGGGGGTGCTAGACACCCGGCA
CCATTCTCCCTTCTCTCTTTTCTTCTCAGGATTTAAAATTTAATTATATCAGTAAAGA
GATTAATTTTAACGTAACTCTTTCTATGCCCGTGTAAAGTATGTGAATCGCAAGGCC
TGTGCTGCATGCGACAGCGTCCGGGGTGGTGGACAGGGCCCCCGGCCACGCTCCCT
CTCCTGTAGCCACTGGCATAGCCCTCCTGAGCACCCGCTGACATTTCCGTTGTACAT
GTTCCTGTTTATGCATTCACAAGGTGACTGGGATGTAGAGAGGCGTTAGTGGGCAG
GTGGCCACAGCAGGACTGAGGACAGGCCCCCATTATCCTAGGGGTGCGCTCACCTG
CAGCCCCTCCTCCTCGGGCACAGACGACTGTCGTTCTCCACCCACCAGTCAGGGACA
GCAGCCTCCCTGTCACTCAGCTGAGAAGGCCAGCCCTCCCTGGCTGTGAGCAGCCTC
CACTGTGTCCAGAGACATGGGCCTCCCACTCCTGTTCCTTGCTAGCCCTGGGGTGGC
GTCTGCCTAGGAGCTGGCTGGCAGGTGTTGGGACCTGCTGCTCCATGGATGCATGCC
CTAAGAGTGTCACTGAGCTGTGTTTTGTCTGAGCCTCTCTCGGTCAACAGCAAAGCT
TGGTGTCTTGGCACTGTTAGTGACAGAGCCCAGCATCCCTTCTGCCCCCGTTCCAGC
TGACATCTTGCACGGTGACCCCTTTTAGTCAGGAGAGTGCAGATCTGTGCTCATCGG
AGACTGCCCCACGGCCCTGTCAGAGCCGCCACTCCTATCCCCAGGCCAGGTCCCTG
GACCAGCCTCCTGTTTGCAGGCCCAGAGGAGCCAAGTCATTAAAATGGAAGTGGAT
TCTGGATGGCCGGGCTGCTGCTGATGTAGGAGCTGGATTTGGGAGCTCTGCTTGCCG
ACTGGCTGTGAGACGAGGCAGGGGCTCTGCTTCCTCAGCCCTAGAGGCGAGCCAGG
CAAGGTTGGCGACTGTCATGTGGCTTGGTTTGGTCATGCCCGTCGATGTTTTGGGTA
TTGAATGTGGTAAGTGGAGGAAATGTTGGAACTCTGTGCAGGTGCTGCCTTGAGAC
CCCCAAGCTTCCACCTGTCCCTCTCCTATGTGGCAGCTGGGGAGCAGCTGAGATGTG

GACTTGTATGCTGCCCACATACGTGAGGGGGAGCTGAAAGGGAGCCCCTCCTCTGA
GCAGCCTCTGCCAGGCCTGTATGAGGCTTTTCCCACCAGCTCCCAACAGAGGCCTCC
CCCAGCCAGGACCACCTCGTCCTCGTGGCGGGGCAGCAGGAGCGGTAGAAAGGGG
TCCGATGTTTGAGGAGGCCCTTAAGGGAAGCTACTGAATTATAACACGTAAGAAAA
TCACCATTCCGTATTGGTTGGGGGCTCCTGTTTCTCATCCTAGCTTTTTCCTGGAAAG
CCCGCTAGAAGGTTTGGGAACGAGGGGAAAGTTCTCAGAACTGTTGGCTGCTCCCC
ACCCGCCTCCCGCCTCCCCCGCAGGTTATGTCAGCAGCTCTGAGACAGCAGTATCAC
AGGCCAGATGTTGTTCCTGGCTAGATGTTTACATTTGTAAGAAATAACACTGTGAAT
GTAAAACAGAGCCATTCCCTTGGAATGCATATCGCTGGGCTCAACATAGAGTTTGTC
TTCCTCTTGTTTACGACGTGATCTAAACCAGTCCTTAGCAAGGGGCTCAGAACACCC
CGCTCTGGCAGTAGGTGTCCCCCACCCCCAAAGACCTGCCTGTGTGCTCCGGAGATG
AATATGAGCTCATTAGTAAAAATGACTTCACCCACGCATATACATAAAGTATCCAT
GCATGTGCATATAGACACATCTATAATTTTACACACACACCTCTCAAGACGGAGAT
GCATGGCCTCTAAGAGTGCCCGTGTCGGTTCTTCCTGGAAGTTGACTTTCCTTAGAC
CCGCCAGGTCAAGTTAGCCGCGTGACGGACATCCAGGCGTGGGACGTGGTCAGGGC
AGGGCTCATTCATTGCCCACTAGGATCCCACTGGCGAAGATGGTCTCCATATCAGCT
CTCTGCAGAAGGGAGGAAGACTTTATCATGTTCCTAAAAATCTGTGGCAAGCACCC
ATCGTATTATCCAAATTTTGTTGCAAATGTGATTAATTTGGTTGTCAAGTTTTGGGG
GTGGGCTGTGGGGAGATTGCTTTTGTTTTCCTGCTGGTAATATCGGGAAAGATTTTA
ATGAAACCAGGGTAGAATTGTTTGGCAATGCACTGAAGCGTGTTTCTTTCCCAAAAT
GTGCCTCCCTTCCGCTGCGGGCCCAGCTGAGTCTATGTAGGTGATGTTTCCAGCTGC
CAAGTGCTCTTTGTTACTGTCCACCCTCATTTCTGCCAGCGCATGTGTCCTTTCAAGG
GGAAAATGTGAAGCTGAACCCCCTCCAGACACCCAGAATGTAGCATCTGAGAAGGC
CCTGTGCCCTAAAGGACACCCCTCGCCCCCATCTTCATGGAGGGGGTCATTTCAGAG
CCCTCGGAGCCAATGAACAGCTCCTCCTCTTGGAGCTGAGATGAGCCCCACGTGGA
GCTCGGGACGGATAGTAGACAGCAATAACTCGGTGTGTGGCCGCCTGGCAGGTGGA
ACTTCCTCCCGTTGCGGGGTGGAGTGAGGTTAGTTCTGTGTGTCTGGTGGGTGGAGT
CAGGCTTCTCTTGCTACCTGTGAGCATCCTTCCCAGCAGACATCCTCATCGGGCTTT
GTCCCTCCCCCGCTTCCTCCCTCTGCGGGGAGGACCCGGGACCACAGCTGCTGGCCA
GGGTAGACTTGGAGCTGTCCTCCAGAGGGGTCACGTGTAGGAGTGAGAAGAAGGA
AGATCTTGAGAGCTGCTGAGGGACCTTGGAGAGCTCAGGATGGCTCAGACGAGGAC
ACTCGCTTGCCGGGCCTGGGCCTCCTGGGAAGGAGGGAGCTGCTCAGAATGCCGCA
TGACAACTGAAGGCAACCTGGAAGGTTCAGGGGCCGCTCTTCCCCCATGTGCCTGT
CACGCTCTGGTGCAGTCAAAGGAACGCCTTCCCCTCAGTTGTTTCTAAGAGCAGAGT
CTCCCGCTGCAATCTGGGTGGTAACTGCCAGCCTTGGAGGATCGTGGCCAACGTGG
ACCTGCCTACGGAGGGTGGGCTCTGACCCAAGTGGGGCCTCCTTGTCCAGGTCTCAC
TGCTTTGCACCGTGGTCAGAGGGACTGTCAGCTGAGCTTGAGCTCCCCTGGAGCCA
GCAGGGCTGTGATGGGCGAGTCCCGGAGCCCCACCCAGACCTGAATGCTTCTGAGA
GCAAAGGGAAGGACTGACGAGAGATGTATATTTAATTTTTTAACTGCTGCAAACAT
TGTACATCCAAATTAAAGGAAAAAAATGGAAACCATCAAAAAAAAAAAAAAAAAA

>SEQ ID NO: 2
TAAATGTGCCTGTTGAAGGGC

>SEQ ID NO: 3
AAGAGGTGCAGAGTCATCATC

>SEQ ID NO: 4

TTCTGGAGGACATCAAACCAT

>SEQ ID NO: 5
TGAACTGGCCCACTTCAATGT

>SEQ ID NO: 6
TTCCATTGGCAACTGGGCCAT

>SEQ ID NO: 7
TAAGCATGGAGCTAGCAGGCT

>SEQ ID NO: 8
TAGCGTTGAAGTACTGTCCCC

SEQ ID NO: 9
TTGAGGCAGCAGCGGCTGTGC

>SEQ ID NO: 10
TTCATCAGCTTTTCCAGGGTC

>SEQ ID NO: 11
TGGAATTCTCGGGTGCCAAGG

>SEQ ID NO: 12
CCTTGGCACCCGAGAATTCCA

>SEQ ID NO: 13
GUUCAGAGUUCUACAGUCCGACGAUC

>SEQ ID NO: 14
AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA

>SEQ ID NO: 15
CAAGCAGAAGACGGCATACGAGATNNNNNNGTGACTGGAGTTCCTTGGCACCCGA
GAATTCCA

>SEQ ID NO: 16
LOCUS dsCB-GFP-mir155- 5483 bp DNA circular SYN 11-OCT-2012
DEFINITION Ligation of 6433 into dsCB-GFP-mirFlank-ployA*
ACCESSION dsCB-GFP-mir155-
KEYWORDS .
SOURCE Unknown.
ORGANISM Unknown
Unclassified.
REFERENCE 1 (bases 1 to 5483)
AUTHORS Self
JOURNAL Unpublished.
COMMENT SECID/File created by SciEd Central, Scientific & Educational Software

```
COMMENT     SECNOTES|Vector molecule:  dsCB-GFP-mirFlank-ployA*
            Fragment ends: BsmBI
            Fragment size: 5419
          Insert molecule:  6433
            Fragment ends:
            Fragment size: 64
FEATURES             Location/Qualifiers
     misc_feature    662..767
                     /gene="mutated ITR"
                     /SECDrawAs="Region"
     misc_feature    814..1093
                     /gene="CMV enhancer"
                     /SECDrawAs="Region"
     misc_feature    870..899
                     /gene="tentative for"
                     /SECDrawAs="Region"
     misc_feature    1100..1126
                     /gene="Probe"
                     /SECDrawAs="Region"
     misc_feature    1100..1369
                     /gene="B-Actin promoter"
                     /product="Chicken"
                     /SECDrawAs="Region"
     misc_feature    complement (1168..1190)
                     /gene="rev"
                     /SECDrawAs="Region"
     misc_feature    1435..1465
                     /gene="SV40_late_19s_int"
                     /SECDrawAs="Region"
     misc_feature    1435..1531
                     /gene="modSV40_late_16s_int"
                     /SECDrawAs="Region"
     CDS             1605..2314
                     /gene="GFP'"
                     /SECDrawAs="Gene"
     misc_feature    2341..2357
                     /gene="'MCS"
                     /SECDrawAs="Region"
     misc_feature    2372..2395
                     /gene="5'miR Flank'"
                     /SECDrawAs="Region"
     misc_feature    2460..2504
                     /gene="3'miR Flank"
                     /SECDrawAs="Region"
     misc_feature    2573..2699
                     /gene="Poly A signal"
                     /product="Rabbit globin poly A"
                     /SECDrawAs="Region"
```

misc_feature complement (2788..2917)
/gene="3' ITR"
/SECDrawAs="Region"
CDS 3680..4537
/gene="Amp(R)"
/SECDrawAs="Gene"
ORIGIN
1 gcccaatacg caaaccgcct ctccccgcgc gttggccgat tcattaatgc agctgattct
61 aacgaggaaa gcacgttata cgtgctcgtc aaagcaacca tagtacgcgc cctgtagcgg
121 cgcattaagc gcggcgggtg tggtggttac gcgcagcgtg accgctacac ttgccagcgc
181 cctagcgccc gctcctttcg ctttcttccc ttcctttctc gccacgttcg ccggctttcc
241 ccgtcaagct ctaaatcggg ggctcccttt agggttccga tttagtgctt tacggcacct
301 cgaccccaaa aaacttgatt agggtgatgg ttcacgtagt gggccatcgc cctgatagac
361 ggtttttcgc cctttgacgt tggagtccac gttctttaat agtggactct tgttccaaac
421 tggaacaaca ctcaacccta tctcggtcta ttcttttgat ttataaggga ttttgccgat
481 ttcggcctat tggttaaaaa atgagctgat ttaacaaaaa tttaacgcga attttaacaa
541 aatattaacg cttacaattt aaatatttgc ttatacaatc ttcctgtttt tggggctttt
601 ctgattatca accggggtac atatgattga catgctagtt ttacgattac cgttcatcgc
661 cctgcgcgct cgctcgctca ctgaggccgc ccgggcaaag cccgggcgtc gggcgacctt
721 tggtcgcccg gcctcagtga gcgagcgagc gcgcagagag ggagtggaat tcacgcgtgg
781 atctgaattc aattcacgcg tggtacctct ggtcgttaca taacttacgg taaatggccc
841 gcctggctga ccgcccaacg acccccgccc attgacgtca ataatgacgt atgttcccat
901 agtaacgcca atagggactt tccattgacg tcaatgggtg gagtatttac ggtaaactgc
961 ccacttggca gtacatcaag tgtatcatat gccaagtacg ccccctattg acgtcaatga
1021 cggtaaatgg cccgcctggc attatgccca gtacatgacc ttatgggact ttcctacttg
1081 gcagtacatc tactcgaggc cacgttctgc ttcactctcc ccatctcccc cccctcccca
1141 cccccaattt tgtatttatt tatttttttaa ttattttgtg cagcgatggg ggcgggggggg
1201 ggggggggggc gcgcgccagg cggggcgggg cggggcgagg ggcggggcgg ggcgaggcgg
1261 agaggtgcgg cggcagccaa tcagagcggc gcgctccgaa agtttccttt tatggcgagg
1321 cggcggcggc ggcggcccta taaaaagcga agcgcgcggc gggcgggagc gggatcagcc
1381 accgcggtgg cggcctagag tcgacgagga actgaaaaac cagaaagtta actggtaagt
1441 ttagtctttt tgtcttttat ttcaggtccc ggatccggtg gtggtgcaaa tcaaagaact
1501 gctcctcagt ggatgttgcc tttacttcta ggcctgtacg gaagtgttac ttctgctcta
1561 aaagctgcgg aattgtaccc gcggccgatc caccggtcgc caccatggtg agcaagggcg
1621 aggagctgtt caccggggtg gtgcccatcc tggtcgagct ggacggcgac gtaaacggcc
1681 acaagttcag cgtgtccggc gagggcgagg gcgatgccac ctacggcaag ctgaccctga
1741 agttcatctg caccaccggc aagctgcccg tgccctggcc caccctcgtg accaccctga
1801 cctacggcgt gcagtgcttc agccgctacc ccgaccacat gaagcagcac gacttcttca
1861 agtccgccat gcccgaaggc tacgtccagg agcgcaccat cttcttcaag gacgacggca
1921 actacaagac ccgcgccgag gtgaagttcg agggcgacac cctggtgaac cgcatcgagc
1981 tgaagggcat cgacttcaag gaggacggca acatcctggg gcacaagctg gagtacaact
2041 acaacagcca caacgtctat atcatggccg acaagcagaa gaacggcatc aaggtgaact
2101 tcaagatccg ccacaacatc gaggacggca gcgtgcagct cgccgaccac taccagcaga
2161 acaccccat cggcgacggc cccgtgctgc tgcccgacaa ccactacctg agcacccagt
2221 ccgccctgag caaagacccc aacgagaagc gcgatcacat ggtcctgctg gagttcgtga
2281 ccgccgccgg gatcactctc ggcatggacg agctgtacaa gtaaagcggc cctagcgttt
2341 ccggcgacgg tgctagcgtc gaccagtgga tcctggaggc ttgctgaagg ctgtatgctg
2401 taagcatgga gctagcaggc tgttttggcc actgactgac agcctgctct ccatgcttac

2461 aggacacaag gcctgttact agcactcaca tggaacaaat ggcccagatc tggccgcact
2521 cgaaaacggg ccctctagac tcgaggacgg ggtgaactac gcctgaggat ccgatctttt
2581 tccctctgcc aaaaattatg gggacatcat gaagcccctt gagcatctga cttctggcta
2641 ataaaggaaa tttattttca ttgcaatagt gtgttggaat tttttgtgtc tctcactcgg
2701 aagcaattcg ttgatctgaa tttcgaccac ccataatacc cattaccctg gtagataagt
2761 agcatggcgg gttaatcatt aactacaagg aacccctagt gatggagttg gccactccct
2821 ctctgcgcgc tcgctcgctc actgaggccg ggcgaccaaa ggtcgcccga cgcccgggct
2881 ttgcccgggc ggcctcagtg agcgagcgag cgcgcagcct taattaacct aattcactgg
2941 ccgtcgtttt acaacgtcgt gactgggaaa accctggcgt tacccaactt aatcgccttg
3001 cagcacatcc cctttcgcc agctggcgta atagcgaaga ggcccgcacc gatcgccctt
3061 cccaacagtt gcgcagcctg aatggcgaat gggacgcgcc ctgtagcggc gcattaagcg
3121 cggcgggtgt ggtggttacg cgcagcgtga ccgctacact tgccagcgcc ctagcgcccg
3181 ctcctttcgc tttcttccct tcctttctcg ccacgttcgc cggctttccc cgtcaagctc
3241 taaatcgggg gctcccttta gggttccgat ttagtgcttt acggcacctc gaccccaaaa
3301 aacttgatta gggtgatggt tcacgtagtg ggccatcgcc ctgatagacg gtttttcgcc
3361 ctttgacgtt ggagtccacg ttctttaata gtggactctt gttccaaact ggaacaacac
3421 tcaaccctat ctcggtctat tcttttgatt tataagggat tttgccgatt tcggcctatt
3481 ggttaaaaaa tgagctgatt taacaaaaat ttaacgcgaa ttttaacaaa atattaacgc
3541 ttacaattta ggtggcactt ttcggggaaa tgtgcgcgga acccctattt gtttatttttt
3601 ctaaatacat tcaaatatgt atccgctcat gagacaataa ccctgataaa tgcttcaata
3661 atattgaaaa aggaagagta tgagtattca acatttccgt gtcgcccttа ttccctttttt
3721 tgcggcattt tgccttcctg tttttgctca cccagaaacg ctggtgaaag taaaagatgc
3781 tgaagatcag ttgggtgcac gagtgggtta catcgaactg gatctcaaca gcggtaagat
3841 ccttgagagt tttcgccccg aagaacgttt tccaatgatg agcactttta aagttctgct
3901 atgtggcgcg gtattatccc gtattgacgc cgggcaagag caactcggtc gccgcataca
3961 ctattctcag aatgacttgg ttgagtactc accagtcaca gaaaagcatc ttacggatgg
4021 catgacagta agagaattat gcagtgctgc cataaccatg agtgataaca ctgcggccaa
4081 cttacttctg acaacgatcg gaggaccgaa ggagctaacc gcttttttgc acaacatggg
4141 ggatcatgta actcgccttg atcgttggga accggagctg aatgaagcca taccaaacga
4201 cgagcgtgac accacgatgc ctgtagcaat ggcaacaacg ttgcgcaaac tattaactgg
4261 cgaactactt actctagctt cccggcaaca attaatagac tggatggagg cggataaagt
4321 tgcaggacca cttctgcgct cggcccttcc ggctggctgg tttattgctg ataaatctgg
4381 agccggtgag cgtgggtctc gcggtatcat tgcagcactg gggccagatg gtaagccctc
4441 ccgtatcgta gttatctaca cgacggggag tcaggcaact atggatgaac gaaatagaca
4501 gatcgctgag ataggtgcct cactgattaa gcattggtaa ctgtcagacc aagtttactc
4561 atatatactt tagattgatt taaaacttca tttttaattt aaaaggatct aggtgaagat
4621 cctttttgat aatctcatga ccaaaatccc ttaacgtgag ttttcgttcc actgagcgtc
4681 agaccccgta gaaaagatca aaggatcttc ttgagatcct ttttttctgc gcgtaatctg
4741 ctgcttgcaa acaaaaaaac caccgctacc agcggtggtt tgtttgccgg atcaagagct
4801 accaactctt tttccgaagg taactggctt cagcagagcg cagataccaa atactgttct
4861 tctagtgtag ccgtagttag gccaccactt caagaactct gtagcaccgc ctacatacct
4921 cgctctgcta atcctgttac cagtggctgc tgccagtggc gataagtcgt gtcttaccgg
4981 gttggactca agacgatagt taccggataa ggcgcagcgg tcgggctgaa cgggggttc
5041 gtgcacacag cccagcttgg agcgaacgac ctacaccgaa ctgagatacc tacagcgtga
5101 gctatgagaa agcgccacgc ttcccgaagg gagaaaggcg gacaggtatc cggtaagcgg
5161 cagggtcgga acaggagagc gcacgaggga gcttccaggg ggaaacgcct ggtatcttta
5221 tagtcctgtc gggtttcgcc acctctgact tgagcgtcga tttttgtgat gctcgtcagg
5281 ggggcggagc ctatggaaaa acgccagcaa cgcggccttt ttacggttcc tggccttttg

5341 ctggcctttt gctcacatgt tctttcctgc gttatcccct gattctgtgg ataaccgtat
5401 taccgccttt gagtgagctg ataccgctcg ccgcagccga acgaccgagc gcagcgagtc
5461 agtgagcgag gaagcggaag agc

>SEQ ID NO: 17
//LOCUS pdsU6-Mir-htt-64 5686 bp DNA circular SYN 17-SEP-2013
DEFINITION Ligation of 6433 into pU6-miRNAFlank-GFP*
ACCESSION pdsU6-Mir-htt-64
KEYWORDS .
SOURCE Unknown.
ORGANISM Unknown
Unclassified.
REFERENCE 1 (bases 1 to 5686)
AUTHORS Self
JOURNAL Unpublished.
COMMENT SECID/File created by SciEd Central, Scientific & Educational Software
COMMENT SECNOTES|Vector molecule: pU6-miRNAFlank-GFP*
Fragment ends: BsmBI
Fragment size: 5622
Insert molecule: 6433
Fragment ends:
Fragment size: 64
FEATURES Location/Qualifiers
misc_feature 662..767
/gene="mutated ITR"
/SECDrawAs="Region"
misc_feature 777..1041
/gene="U6 promoter"
/SECDrawAs="Region"
misc_signal 1041..1041
/gene="Pol III Start"
/product="Transcriptional Start "
/SECDrawAs="Label"
CDS 1042..1065
/gene="5' miR Flank'"
/SECDrawAs="Gene"
CDS 1130..1175
/gene="miR 3' Flank"
/SECDrawAs="Gene"
misc_signal 1176..1181
/gene="Pol III term"
/product="pol III terminator"
/SECDrawAs="Label"
misc_feature 1199..1478
/gene="CMV enhancer"
/SECDrawAs="Region"
misc_feature 1255..1284
/gene="tentative for"

/SECDrawAs="Region"
misc_feature 1485..1754
/gene="B-Actin promoter"
/product="Chicken"
/SECDrawAs="Region"
misc_feature 1485..1511
/gene="Probe"
/SECDrawAs="Region"
misc_feature complement (1553..1575)
/gene="rev"
/SECDrawAs="Region"
misc_feature 1820..1916
/gene="modSV40_late_16s_int"
/SECDrawAs="Region"
misc_feature 1820..1850
/gene="SV40_late_19s_int"
/SECDrawAs="Region"
CDS 1990..2699
/gene="GFP'"
/SECDrawAs="Gene"
misc_feature 2726..2737
/gene="'MCS'"
/SECDrawAs="Region"
misc_feature 2776..2902
/gene="Poly A signal"
/product="Rabbit globin poly A"
/SECDrawAs="Region"
misc_feature complement (2991..3120)
/gene="3' ITR"
/SECDrawAs="Region"
CDS 3883..4740
/gene="Amp(R)"
/SECDrawAs="Gene"
ORIGIN
1 gcccaatacg caaaccgcct ctccccgcgc gttggccgat tcattaatgc agctgattct
61 aacgaggaaa gcacgttata cgtgctcgtc aaagcaacca tagtacgcgc cctgtagcgg
121 cgcattaagc gcggcgggtg tggtggttac gcgcagcgtg accgctacac ttgccagcgc
181 cctagcgccc gctcctttcg ctttcttccc ttcctttctc gccacgttcg ccggctttcc
241 ccgtcaagct ctaaatcggg ggctcccttt agggttccga tttagtgctt tacggcacct
301 cgaccccaaa aaacttgatt agggtgatgg ttcacgtagt gggccatcgc cctgatagac
361 ggtttttcgc cctttgacgt tggagtccac gttctttaat agtggactct tgttccaaac
421 tggaacaaca ctcaacccta tctcggtcta ttcttttgat ttataaggga ttttgccgat
481 ttcggcctat tggttaaaaa atgagctgat ttaacaaaaa tttaacgcga attttaacaa
541 aatattaacg cttacaattt aaatatttgc ttatacaatc ttcctgtttt tggggctttt
601 ctgattatca accggggtac atatgattga catgctagtt ttacgattac cgttcatcgc
661 cctgcgcgct cgctcgctca ctgaggccgc ccgggcaaag cccgggcgtc gggcgacctt
721 tggtcgcccg gcctcagtga gcgagcgagc gcgcagagag ggagtggaat tctataaagg
781 tcgggcagga agagggccta tttcccatga ttccttcata tttgcatata cgatacaagg

841 ctgttagaga gataattaga attaatttga ctgtaaacac aaagatatta gtacaaaata
901 cgtgacgtag aaagtaataa tttcttgggt agtttgcagt tttaaaatta tgttttaaaa
961 tggactatca tatgcttacc gtaacttgaa agtatttcga tttcttggct ttatatatct
1021 tgtggaaagg acgaaacacc gcctggaggc ttgctgaagg ctgtatgctg taagcatgga
1081 gctagcaggc tgttttggcc actgactgac agcctgctct ccatgcttac aggacacaag
1141 gcctgttact agcactcaca tggaacaaat ggccctttt tctagtggta cctctggtcg
1201 ttacataact tacggtaaat ggcccgcctg gctgaccgcc caacgacccc cgcccattga
1261 cgtcaataat gacgtatgtt cccatagtaa cgccaatagg gactttccat tgacgtcaat
1321 gggtggagta tttacggtaa actgcccact tggcagtaca tcaagtgtat catatgccaa
1381 gtacgccccc tattgacgtc aatgacggta aatggcccgc ctggcattat gcccagtaca
1441 tgaccttatg ggactttcct acttggcagt acatctactc gaggccacgt tctgcttcac
1501 tctccccatc tccccccccc ccccacccc aattttgtat ttatttattt tttaattatt
1561 ttgtgcagcg atgggggcgg gggggggggg ggggcgcgcg ccaggcgggg cggggcgggg
1621 cgaggggcgg ggcggggcga ggcggagagg tgcggcggca gccaatcaga gcggcgcgct
1681 ccgaaagttt ccttttatgg cgaggcggcg gcggcggcgg ccctataaaa agcgaagcgc
1741 gcggcgggcg ggagcgggat cagccaccgc ggtggcggcc tagagtcgac gaggaactga
1801 aaaaccagaa agttaactgg taagtttagt ctttttgtct tttatttcag gtcccggatc
1861 cggtggtggt gcaaatcaaa gaactgctcc tcagtggatg ttgcctttac ttctaggcct
1921 gtacggaagt gttacttctg ctctaaaagc tgcggaattg tacccgcggc cgatccaccg
1981 gtcgccacca tggtgagcaa gggcgaggag ctgttcaccg gggtggtgcc catcctggtc
2041 gagctggacg gcgacgtaaa cggccacaag ttcagcgtgt ccggcgaggg cgagggcgat
2101 gccacctacg gcaagctgac cctgaagttc atctgcacca ccggcaagct gcccgtgccc
2161 tggcccaccc tcgtgaccac cctgacctac ggcgtgcagt gcttcagccg ctaccccgac
2221 cacatgaagc agcacgactt cttcaagtcc gccatgcccg aaggctacgt ccaggagcgc
2281 accatcttct tcaaggacga cggcaactac aagacccgcg ccgaggtgaa gttcgagggc
2341 gacaccctgg tgaaccgcat cgagctgaag ggcatcgact tcaaggagga cggcaacatc
2401 ctggggcaca agctggagta caactacaac agccacaacg tctatatcat ggccgacaag
2461 cagaagaacg gcatcaaggt gaacttcaag atccgccaca acatcgagga cggcagcgtg
2521 cagctcgccg accactacca gcagaacacc cccatcggcg acggccccgt gctgctgccc
2581 gacaaccact acctgagcac ccagtccgcc ctgagcaaag accccaacga gaagcgcgat
2641 cacatggtcc tgctggagtt cgtgaccgcc gccgggatca ctctcggcat ggacgagctg
2701 tacaagtaaa gcggccctag cgtttccggc gacggtgcta gactcgagga cggggtgaac
2761 tacgcctgag gatccgatct ttttccctct gccaaaaatt atggggacat catgaagccc
2821 cttgagcatc tgacttctgg ctaataaagg aaatttattt tcattgcaat agtgtgttgg
2881 aatttttgt gtctctcact cggaagcaat tcgttgatct gaatttcgac cacccataat
2941 acccattacc ctggtagata agtagcatgg cgggttaatc attaactaca aggaacccct
3001 agtgatggag ttggccactc cctctctgcg cgctcgctcg ctcactgagg ccgggcgacc
3061 aaaggtcgcc cgacgcccgg gctttgcccg ggcggcctca gtgagcgagc gagcgcgcag
3121 ccttaattaa cctaattcac tggccgtcgt tttacaacgt cgtgactggg aaaaccctgg
3181 cgttacccaa cttaatcgcc ttgcagcaca tccccctttc gccagctggc gtaatagcga
3241 agaggcccgc accgatcgcc cttcccaaca gttgcgcagc ctgaatggcg aatgggacgc
3301 gccctgtagc ggcgcattaa gcgcggcggg tgtggtggtt acgcgcagcg tgaccgctac
3361 acttgccagc gccctagcgc ccgctccttt cgctttcttc ccttcctttc tcgccacgtt
3421 cgccggcttt ccccgtcaag ctctaaatcg ggggctccct ttagggttcc gatttagtgc
3481 tttacggcac ctcgacccca aaaaacttga ttagggtgat ggttcacgta gtgggccatc
3541 gccctgatag acggtttttc gccctttgac gttggagtcc acgttcttta atagtggact
3601 cttgttccaa actggaacaa cactcaaccc tatctcggtc tattcttttg atttataagg
3661 gattttgccg atttcggcct attggttaaa aaatgagctg atttaacaaa aatttaacgc

3721 gaattttaac aaaatattaa cgcttacaat ttaggtggca cttttcgggg aaatgtgcgc
3781 ggaaccccta tttgtttatt tttctaaata cattcaaata tgtatccgct catgagacaa
3841 taaccctgat aaatgcttca ataatattga aaaaggaaga gtatgagtat tcaacatttc
3901 cgtgtcgccc ttattcccct ttttgcggca ttttgccttc ctgtttttgc tcacccagaa
3961 acgctggtga aagtaaaaga tgctgaagat cagttgggtg cacgagtggg ttacatcgaa
4021 ctggatctca acagcggtaa gatccttgag agttttcgcc ccgaagaacg ttttccaatg
4081 atgagcactt ttaaagttct gctatgtggc gcggtattat cccgtattga cgccgggcaa
4141 gagcaactcg gtcgccgcat acactattct cagaatgact tggttgagta ctcaccagtc
4201 acagaaaagc atcttacgga tggcatgaca gtaagagaat tatgcagtgc tgccataacc
4261 atgagtgata acactgcggc caacttactt ctgacaacga tcggaggacc gaaggagcta
4321 accgcttttt tgcacaacat gggggatcat gtaactcgcc ttgatcgttg ggaaccggag
4381 ctgaatgaag ccataccaaa cgacgagcgt gacaccacga tgcctgtagc aatggcaaca
4441 acgttgcgca aactattaac tggcgaacta cttactctag cttcccggca acaattaata
4501 gactggatgg aggcggataa agttgcagga ccacttctgc gctcggccct tccggctggc
4561 tggtttattg ctgataaatc tggagccggt gagcgtgggt ctcgcggtat cattgcagca
4621 ctggggccag atggtaagcc ctcccgtatc gtagttatct acacgacggg gagtcaggca
4681 actatggatg aacgaaatag acagatcgct gagataggtg cctcactgat taagcattgg
4741 taactgtcag accaagttta ctcatatata ctttagattg atttaaaact tcatttttaa
4801 tttaaaagga tctaggtgaa gatccttttt gataatctca tgaccaaaat cccttaacgt
4861 gagttttcgt tccactgagc gtcagacccc gtagaaaaga tcaaaggatc ttcttgagat
4921 cctttttttc tgcgcgtaat ctgctgcttg caaacaaaaa aaccaccgct accagcggtg
4981 gtttgtttgc cggatcaaga gctaccaact ctttttccga aggtaactgg cttcagcaga
5041 gcgcagatac caaatactgt tcttctagtg tagccgtagt taggccacca cttcaagaac
5101 tctgtagcac cgcctacata cctcgctctg ctaatcctgt taccagtggc tgctgccagt
5161 ggcgataagt cgtgtcttac cgggttggac tcaagacgat agttaccgga taaggcgcag
5221 cggtcgggct gaacgggggg ttcgtgcaca gcccagct tggagcgaac gacctacacc
5281 gaactgagat acctacagcg tgagctatga gaaagcgcca cgcttcccga agggagaaag
5341 gcggacaggt atccggtaag cggcagggtc ggaacaggag agcgcacgag ggagcttcca
5401 gggggaaacg cctggtatct ttatagtcct gtcgggtttc gccacctctg acttgagcgt
5461 cgatttttgt gatgctcgtc agggggggcgg agcctatgga aaaacgccag caacgcggcc
5521 tttttacggt tcctggcctt ttgctggcct tttgctcaca tgttctttcc tgcgttatcc
5581 cctgattctg tggataaccg tattaccgcc tttgagtgag ctgataccgc tcgccgcagc
5641 cgaacgaccg agcgcagcga gtcagtgagc gaggaagcgg aagagc

>SEQ ID NO: 18
LOCUS pCVscAsaq+-mir64 5155 bp DNA circular SYN 17-SEP-2013
DEFINITION Ligation of 6433 into pCVscAsaq+-mirFlank*
ACCESSION pCVscAsaq+-mir64
KEYWORDS .
SOURCE Unknown.
ORGANISM Unknown
Unclassified.
REFERENCE 1 (bases 1 to 5155)
AUTHORS Self
JOURNAL Unpublished.
COMMENT SECID/File created by SciEd Central, Scientific & Educational Software
COMMENT SECNOTES|Vector molecule: pCVscAsaq+-mirFlank*
Fragment ends: BsmBI

```
            Fragment size: 5090
          Insert molecule:  6433
            Fragment ends:
            Fragment size: 64
FEATURES          Location/Qualifiers
   misc_feature   1..105
                  /gene="ITR"
                  /SECDrawAs="Region"
   misc_feature   182..449
                  /gene="CMV"
                  /product="CMV Enhancer"
                  /SECDrawAs="Region"
   CDS            448..753
                  /gene="CB promoter"
                  /product="Promoter Eukaryotic"
                  /SECDrawAs="Gene"
   CDS            754..1819
                  /gene="Intron"
                  /product="Intron"
                  /SECDrawAs="Gene"
   CDS            1820..1839
                  /gene="MCS"
                  /SECDrawAs="Gene"
   misc_feature   1843..1847
                  /gene=""MCS"
                  /SECDrawAs="Region"
   misc_feature   1862..1885
                  /gene="5'miR Flank'"
                  /SECDrawAs="Region"
   misc_feature   1950..1994
                  /gene="3'miR Flank"
                  /SECDrawAs="Region"
   CDS            2002..2128
                  /gene="RBG pA"
                  /product="PolyA Signal"
                  /SECDrawAs="Gene"
   misc_feature   complement (2002..2128)
                  /gene="RBG\pA"
                  /SECDrawAs="Info only"
   misc_feature   2139..2281
                  /gene="3'ITR"
                  /SECDrawAs="Region"
   CDS            2317..2509
                  /gene="lacZ"
                  /SECDrawAs="Gene"
   CDS            2510..2965
                  /gene="f1 ori"
                  /SECDrawAs="Gene"
```

misc_feature 3097..3957
/gene="bla-AmpR"
/SECDrawAs="Region"
misc_feature 4117..4731
/gene="rep-pMB1"
/SECDrawAs="Region"
ORIGIN
1 ctgcgcgctc gctcgctcac tgaggccgcc cgggcaaagc ccgggcgtcg ggcgaccttt
61 ggtcgcccgg cctcagtgag cgagcgagcg cgcagagagg gagtgtagcc atgctctagg
121 aagatcaatt caattcacgc gtcgacattg attattgact agctctggtc gttacataac
181 ttacggtaaa tggcccgcct ggctgaccgc ccaacgaccc ccgcccattg acgtcaataa
241 tgacgtatgt tcccatagta acgccaatag ggactttcca ttgacgtcaa tgggtggagt
301 atttacggta aactgcccac ttggcagtac atcaagtgta tcatatgcca agtccgcccc
361 ctattgacgt caatgacggt aaatggcccg cctggcatta tgcccagtac atgaccttac
421 gggactttcc tacttggcag tacatctacg tattagtcat cgctattacc atggtcgagg
481 tgagccccac gttctgcttc actctcccca tctccccccc ctccccaccc ccaattttgt
541 atttatttat tttttaatta ttttgtgcag cgatgggggc ggggggggggg gggggcgcgcg
601 cgccaggcgg ggcggggcgg ggcgaggggc ggggcggggc gaggcggaga ggtgcggcgg
661 cagccaatca gagcggcgcg ctccgaaagt ttcctttttat ggcgaggcgg cggcggcggc
721 ggccctataa aaagcgaagc gcgcggcggg cgggagtcgc tgcgcgctgc cttcgccccg
781 tgccccgctc cgccgccgcc tcgcgccgcc cgccccggct ctgactgacc gcgttactcc
841 cacaggtgag cgggcgggac ggcccttctc ctccgggctg taattagcgc ttggtttaat
901 gacggcttgt ttcttttctg tggctgcgtg aaagccttga ggggctccgg gagggccctt
961 tgtgcggggg ggagcggctc ggggggtgcg tgcgtgtgtg tgtgcgtggg gagcgccgcg
1021 tgcggctccg cgctgcccgg cggctgtgag cgctgcgggc gcggcgcggg gctttgtgcg
1081 ctccgcagtg tgcgcgaggg gagcgcggcc gggggcggtg ccccgcggtg cgggggggggc
1141 tgcgagggga acaaaggctg cgtgcggggt gtgtgcgtgg gggggtgagc agggggtgtg
1201 ggcgcgtcgg tcgggctgca acccccccctg cacccccctc cccgagttgc tgagcacggc
1261 ccggcttcgg gtgcggggct ccgtacgggg cgtggcgcgg ggctcgccgt gccgggcggg
1321 gggtggcggc aggtgggggt gccgggcggg gcggggccgc ctcgggccgg ggagggctcg
1381 ggggaggggc gcggcggccc ccggagcgcc ggcggctgtc gaggcgcggc gagccgcagc
1441 cattgccttt tatggtaatc gtgcgagagg gcgcagggac ttcctttgtc ccaaatctgt
1501 gcggagccga aatctgggag gcgccgccgc accccctcta gcgggcgcgg ggcgaagcgg
1561 tgcggcgccg gcaggaagga atgggcgggg gagggccttc gtgcgtcgcc gcgccgccgt
1621 ccccttctcc ctctccagcc tcggggctgt ccgcgggggg acggctgcct tcgggggggga
1681 cggggcaggg cggggttcgg cttctggcgt gtgaccggcg gctctagagc ctctgctaac
1741 catgttcatg ccttcttctt tttcctacag ctcctgggca acgtgctggt tattgtgctg
1801 tctcatcatt ttggcaaaga attcatcgat accgtcgacg atctagcgtc gaccagtgga
1861 tcctggaggc ttgctgaagg ctgtatgctg taagcatgga gctagcaggc tgttttggcc
1921 actgactgac agcctgctct ccatgcttac aggacacaag gcctgttact agcactcaca
1981 tggaacaaat ggcccagatc cgatcttttt ccctctgcca aaaattatgg ggacatcatg
2041 aagccccttg agcatctgac ttctggctaa taaaggaaat ttattttcat tgcaatagtg
2101 tgttggaatt ttttgtgtct ctcactcgat cagatctgag gaacccctag tgatggagtt
2161 ggccactccc tctctgcgcg ctcgctcgct cactgaggcc gcccgggcaa agcccgggcg
2221 tcgggcgacc tttggtcgcc cggcctcagt gagcgagcga gcgcgcagag agggagtggc
2281 ccccccccccc ccccccccct gcattctaga gagctccaat tcgccctata gtgagtcgta
2341 ttacgcgcgc tcactggccg tcgttttaca acgtcgtgac tgggaaaacc ctggcgttac
2401 ccaacttaat cgccttgcag cacatccccc tttcgccagc tggcgtaata gcgaagaggc

2461 ccgcaccgat cgcccttccc aacagttgcg cagcctgaat ggcgaatgga aattgtaagc
2521 gttaatattt tgttaaaatt cgcgttaaat ttttgttaaa tcagctcatt ttttaaccaa
2581 taggccgaaa tcggcaaaat cccttataaa tcaaaagaat agaccgagat agggttgagt
2641 gttgttccag tttggaacaa gagtccacta ttaaagaacg tggactccaa cgtcaaaggg
2701 cgaaaaaccg tctatcaggg cgatggccca ctacgtgaac catcacccta atcaagtttt
2761 ttggggtcga ggtgccgtaa agcactaaat cggaacccta aagggagccc ccgatttaga
2821 gcttgacggg gaaagccggc gaacgtggcg agaaaggaag ggaagaaagc gaaaggagcg
2881 ggcgctaggg cgctggcaag tgtagcggtc acgctgcgcg taaccaccac acccgccgcg
2941 cttaatgcgc cgctacaggg cgcgtcaggt ggcacttttc ggggaaatgt gcgcggaacc
3001 cctatttgtt tatttttcta atacattca aatatgtatc cgctcatgag acaataaccc
3061 tgataaatgc ttcaataata ttgaaaaagg aagagtatga gtattcaaca tttccgtgtc
3121 gcccttattc cctttttgc ggcattttgc cttcctgttt ttgctcaccc agaaacgctg
3181 gtgaaagtaa aagatgctga agatcagttg ggtgcacgag tgggttacat cgaactggat
3241 ctcaacagcg gtaagatcct tgagagtttt cgccccgaag aacgttttcc aatgatgagc
3301 acttttaaag ttctgctatg tggcgcggta ttatcccgta ttgacgccgg gcaagagcaa
3361 ctcggtcgcc gcatacacta ttctcagaat gacttggttg agtactcacc agtcacagaa
3421 aagcatctta cggatggcat gacagtaaga gaattatgca gtgctgccat aaccatgagt
3481 gataacactg cggccaactt acttctgaca acgatcggag gaccgaagga gctaaccgct
3541 tttttgcaca acatggggga tcatgtaact cgccttgatc gttgggaacc ggagctgaat
3601 gaagccatac caaacgacga gcgtgacacc acgatgcctg tagcaatggc aacaacgttg
3661 cgcaaactat taactggcga actacttact ctagcttccc ggcaacaatt aatagactgg
3721 atggaggcgg ataaagttgc aggaccactt ctgcgctcgg cccttccggc tggctggttt
3781 attgctgata aatctggagc cggtgagcgt gggtctcgcg gtatcattgc agcactgggg
3841 ccagatggta agccctcccg tatcgtagtt atctacacga cggggagtca ggcaactatg
3901 gatgaacgaa atagacagat cgctgagata ggtgcctcac tgattaagca ttggtaactg
3961 tcagaccaag tttactcata tatactttag attgatttaa aacttcattt ttaatttaaa
4021 aggatctagg tgaagatcct ttttgataat ctcatgacca aaatccctta acgtgagttt
4081 tcgttccact gagcgtcaga ccccgtagaa aagatcaaag gatcttcttg agatcctttt
4141 tttctgcgcg taatctgctg cttgcaaaca aaaaaaccac cgctaccagc ggtggtttgt
4201 ttgccggatc aagagctacc aactcttttt ccgaaggtaa ctggcttcag cagagcgcag
4261 ataccaaata ctgtccttct agtgtagccg tagttaggcc accacttcaa gaactctgta
4321 gcaccgccta catacctcgc tctgctaatc ctgttaccag tggctgctgc cagtggcgat
4381 aagtcgtgtc ttaccgggtt ggactcaaga cgatagttac cggataaggc gcagcggtcg
4441 ggctgaacgg ggggttcgtg cacacagccc agcttggagc gaacgaccta caccgaactg
4501 agatacctac agcgtgagct atgagaaagc gccacgcttc ccgaagggag aaaggcggac
4561 aggtatccgg taagcggcag ggtcggaaca ggagagcgca cgagggagct tccagggga
4621 aacgcctggt atctttatag tcctgtcggg tttcgccacc tctgacttga gcgtcgattt
4681 ttgtgatgct cgtcaggggg gcggagccta tggaaaaacg ccagcaacgc ggcctttta
4741 cggttcctgg ccttttgctg gccttttgct cacatgttct ttcctgcgtt atcccctgat
4801 tctgtggata accgtattac cgcctttgag tgagctgata ccgctcgccg cagccgaacg
4861 accgagcgca gcgagtcagt gagcgaggaa gcggaagagc gcccaatacg caaaccgcct
4921 ctccccgcgc gttggccgat tcattaatgc agctggcacg acaggtttcc cgactggaaa
4981 gcgggcagtg agcgcaacgc aattaatgtg agttagctca ctcattaggc accccaggct
5041 ttacacttta tgcttccggc tcgtatgttg tgtggaattg tgagcggata acaatttcac
5101 acaggaaaca gctatgacca tgattacgcc agatttaatt aaggccttaa ttagg

>SEQ ID NO: 19
LOCUS U6-mir6433-BGHpA 5223 bp DNA circular SYN 12-SEP-2013

```
DEFINITION  Ligation of dsAAV CB MCS** into U6-MiRBA-6433-GFP**
ACCESSION   U6-mir6433-BGHpA
KEYWORDS    .
SOURCE      Unknown.
  ORGANISM  Unknown
            Unclassified.
REFERENCE   1  (bases 1 to 5223)
  AUTHORS   Self
  JOURNAL   Unpublished.
COMMENT     SECID/File created by SciEd Central, Scientific & Educational Software
COMMENT     SECNOTES|Vector molecule:  U6-MiRBA-6433-GFP**
              Fragment ends: blunt and EagI
              Fragment size: 4954
            Insert molecule:  dsAAV CB MCS**
              Fragment ends: EagI and blunt
              Fragment size: 269
FEATURES             Location/Qualifiers
     misc_feature    662..767
                     /gene="mutated ITR"
                     /SECDrawAs="Region"
     misc_feature    777..1041
                     /gene="U6 promoter"
                     /SECDrawAs="Region"
     misc_signal     1041..1041
                     /gene="Pol III Start"
                     /product="Transcriptional Start "
                     /SECDrawAs="Label"
     CDS             1042..1065
                     /gene="5' miR Flank'"
                     /SECDrawAs="Gene"
     CDS             1130..1175
                     /gene="miR 3' Flank"
                     /SECDrawAs="Gene"
     misc_signal     1176..1181
                     /gene="Pol III term"
                     /product="pol III terminator"
                     /SECDrawAs="Label"
     misc_feature    1199..1478
                     /gene="CMV enhancer"
                     /SECDrawAs="Region"
     misc_feature    1255..1284
                     /gene="tentative for"
                     /SECDrawAs="Region"
     misc_feature    1485..1511
                     /gene="Probe"
                     /SECDrawAs="Region"
     misc_feature    1485..1754
                     /gene="B-Actin promoter"
```

```
                /product="Chicken"
                /SECDrawAs="Region"
     misc_feature    complement (1553..1575)
                /gene="rev"
                /SECDrawAs="Region"
     misc_feature    1820..1850
                /gene="SV40_late_19s_int"
                /SECDrawAs="Region"
     misc_feature    1820..1916
                /gene="modSV40_late_16s_int"
                /SECDrawAs="Region"
     misc_feature    2034..2230
                /gene="BGHpA"
                /SECDrawAs="Region"
     misc_feature    2263..2274
                /gene="'MCS'"
                /SECDrawAs="Region"
     misc_feature    2313..2439
                /gene="Poly A signal"
                /product="Rabbit globin poly A"
                /SECDrawAs="Region"
     misc_feature    complement (2528..2657)
                /gene="3' ITR"
                /SECDrawAs="Region"
     CDS             3420..4277
                /gene="Amp(R)"
                /SECDrawAs="Gene"
ORIGIN
        1 gcccaatacg caaaccgcct ctccccgcgc gttggccgat tcattaatgc agctgattct
       61 aacgaggaaa gcacgttata cgtgctcgtc aaagcaacca tagtacgcgc cctgtagcgg
      121 cgcattaagc gcggcgggtg tggtggttac gcgcagcgtg accgctacac ttgccagcgc
      181 cctagcgccc gctcctttcg ctttcttccc ttcctttctc gccacgttcg ccggctttcc
      241 ccgtcaagct ctaaatcggg ggctcccttt agggttccga tttagtgctt tacggcacct
      301 cgaccccaaa aaacttgatt agggtgatgg ttcacgtagt gggccatcgc cctgatagac
      361 ggtttttcgc cctttgacgt tggagtccac gttctttaat agtggactct tgttccaaac
      421 tggaacaaca ctcaacccta tctcggtcta ttcttttgat ttataaggga ttttgccgat
      481 ttcggcctat tggttaaaaa atgagctgat ttaacaaaaa tttaacgcga attttaacaa
      541 aatattaacg cttacaattt aaatatttgc ttatacaatc ttcctgtttt tggggctttt
      601 ctgattatca accggggtac atatgattga catgctagtt ttacgattac cgttcatcgc
      661 cctgcgcgct cgctcgctca ctgaggccgc ccgggcaaag cccgggcgtc gggcgacctt
      721 tggtcgcccg gcctcagtga gcgagcgagc gcgcagagag ggagtggaat tctataaagg
      781 tcgggcagga agagggccta tttcccatga ttccttcata tttgcatata cgatacaagg
      841 ctgttagaga gataattaga attaatttga ctgtaaacac aaagatatta gtacaaaata
      901 cgtgacgtag aaagtaataa tttcttgggt agtttgcagt tttaaaatta tgtttaaaa
      961 tggactatca tatgcttacc gtaacttgaa agtatttcga tttcttggct ttatatatct
     1021 tgtggaaagg acgaaacacc gcctggaggc ttgctgaagg ctgtatgctg taagcatgga
     1081 gctagcaggc tgttttggcc actgactgac agcctgctct ccatgcttac aggacacaag
     1141 gcctgttact agcactcaca tggaacaaat ggccctttt tctagtggta cctctggtcg
```

1201 ttacataact tacggtaaat ggcccgcctg gctgaccgcc caacgacccc cgcccattga
1261 cgtcaataat gacgtatgtt cccatagtaa cgccaatagg gactttccat tgacgtcaat
1321 gggtggagta tttacggtaa actgcccact tggcagtaca tcaagtgtat catatgccaa
1381 gtacgccccc tattgacgtc aatgacggta aatggcccgc ctggcattat gcccagtaca
1441 tgaccttatg ggactttcct acttggcagt acatctactc gaggccacgt tctgcttcac
1501 tctccccatc tccccccccc ccccacccc aattttgtat ttatttattt tttaattatt
1561 ttgtgcagcg atgggggcgg gggggggggg ggggcgcgcg ccaggcgggg cggggcgggg
1621 cgaggggcgg ggcggggcga ggcggagagg tgcggcggca gccaatcaga gcggcgcgct
1681 ccgaaagttt ccttttatgg cgaggcggcg gcggcggcgg ccctataaaa agcgaagcgc
1741 gcggcgggcg ggagcgggat cagccaccgc ggtggcggcc tagagtcgac gaggaactga
1801 aaaaccagaa agttaactgg taagtttagt ctttttgtct tttatttcag gtcccggatc
1861 cggtggtggt gcaaatcaaa gaactgctcc tcagtggatg ttgcctttac ttctaggcct
1921 gtacggaagt gttacttctg ctctaaaagc tgcggaattg tacccgcggc cgcgtttaaa
1981 ccctgcaggt ctagaaagct tatcgatacc gtcgactaga gctcgctgat cagcctcgac
2041 tgtgccttct agttgccagc catctgttgt ttgcccctcc cccgtgcctt ccttgaccct
2101 ggaaggtgcc actcccactg tcctttccta ataaaatgag gaaattgcat cgcattgtct
2161 gagtaggtgt cattctattc tgggggggtgg ggtggggcag gacagcaagg gggaggattg
2221 ggaagacaat agcagggtac aagtaaagcg gccctagcgt ttccggcgac ggtgctagac
2281 tcgaggacgg ggtgaactac gcctgaggat ccgatctttt tccctctgcc aaaaattatg
2341 gggacatcat gaagcccctt gagcatctga cttctggcta ataaaggaaa tttatttca
2401 ttgcaatagt gtgttggaat tttttgtgtc tctcactcgg aagcaattcg ttgatctgaa
2461 tttcgaccac ccataatacc cattaccctg gtagataagt agcatggcgg gttaatcatt
2521 aactacaagg aacccctagt gatggagttg gccactccct ctctgcgcgc tcgctcgctc
2581 actgaggccg ggcgaccaaa ggtcgcccga cgcccgggct ttgcccgggc ggcctcagtg
2641 agcgagcgag cgcgcagcct taattaacct aattcactgg ccgtcgtttt acaacgtcgt
2701 gactgggaaa accctggcgt tacccaactt aatcgccttg cagcacatcc cccttcgcc
2761 agctggcgta atagcgaaga ggcccgcacc gatcgccctt cccaacagtt gcgcagcctg
2821 aatggcgaat gggacgcgcc ctgtagcggc gcattaagcg cggcgggtgt ggtggttacg
2881 cgcagcgtga ccgctacact tgccagcgcc ctagcgcccg ctcctttcgc tttcttccct
2941 tcctttctcg ccacgttcgc cggctttccc cgtcaagctc taaatcgggg gctcccttta
3001 gggttccgat ttagtgcttt acggcacctc gaccccaaaa aacttgatta gggtgatggt
3061 tcacgtagtg ggccatcgcc ctgatagacg gtttttcgcc ctttgacgtt ggagtccacg
3121 ttctttaata gtggactctt gttccaaact ggaacaacac tcaaccctat ctcggtctat
3181 tcttttgatt tataagggat tttgccgatt tcggcctatt ggttaaaaaa tgagctgatt
3241 taacaaaaat ttaacgcgaa ttttaacaaa atattaacgc ttacaattta ggtggcactt
3301 ttcggggaaa tgtgcgcgga acccctattt gtttatttttt ctaaatacat tcaaatatgt
3361 atccgctcat gagacaataa ccctgataaa tgcttcaata atattgaaaa aggaagagta
3421 tgagtattca acatttccgt gtcgcccttа ttcccttttt tgcggcattt tgccttcctg
3481 tttttgctca cccagaaacg ctggtgaaag taaaagatgc tgaagatcag ttgggtgcac
3541 gagtgggtta catcgaactg gatctcaaca gcggtaagat ccttgagagt tttcgccccg
3601 aagaacgttt tccaatgatg agcacttttа aagttctgct atgtggcgcg gtattatccc
3661 gtattgacgc cgggcaagag caactcggtc gccgcataca ctattctcag aatgacttgg
3721 ttgagtactc accagtcaca gaaaagcatc ttacggatgg catgacagta agagaattat
3781 gcagtgctgc cataaccatg agtgataaca ctgcggccaa cttacttctg acaacgatcg
3841 gaggaccgaa ggagctaacc gcttttttgc acaacatggg ggatcatgta actcgccttg
3901 atcgttggga accggagctg aatgaagcca taccaaacga cgagcgtgac accacgatgc
3961 ctgtagcaat ggcaacaacg ttgcgcaaac tattaactgg cgaactactt actctagctt
4021 cccggcaaca attaatagac tggatggagg cggataaagt tgcaggacca cttctgcgct

4081 cggcccttcc ggctggctgg tttattgctg ataaatctgg agccggtgag cgtgggtctc
4141 gcggtatcat tgcagcactg gggccagatg gtaagccctc ccgtatcgta gttatctaca
4201 cgacggggag tcaggcaact atggatgaac gaaatagaca gatcgctgag ataggtgcct
4261 cactgattaa gcattggtaa ctgtcagacc aagtttactc atatatactt tagattgatt
4321 taaaacttca tttttaattt aaaaggatct aggtgaagat ccttttttgat aatctcatga
4381 ccaaaatccc ttaacgtgag ttttcgttcc actgagcgtc agaccccgta gaaaagatca
4441 aaggatcttc ttgagatcct ttttttctgc gcgtaatctg ctgcttgcaa acaaaaaaac
4501 caccgctacc agcggtggtt tgtttgccgg atcaagagct accaactctt tttccgaagg
4561 taactggctt cagcagagcg cagataccaa atactgttct tctagtgtag ccgtagttag
4621 gccaccactt caagaactct gtagcaccgc ctacatacct cgctctgcta atcctgttac
4681 cagtggctgc tgccagtggc gataagtcgt gtcttaccgg gttggactca agacgatagt
4741 taccggataa ggcgcagcgg tcgggctgaa cgggggggttc gtgcacacag cccagcttgg
4801 agcgaacgac ctacaccgaa ctgagatacc tacagcgtga gctatgagaa agcgccacgc
4861 ttcccgaagg gagaaaggcg gacaggtatc cggtaagcgg cagggtcgga acaggagagc
4921 gcacgaggga gcttccaggg ggaaacgcct ggtatcttta tagtcctgtc gggtttcgcc
4981 acctctgact tgagcgtcga tttttgtgat gctcgtcagg ggggcggagc ctatggaaaa
5041 acgccagcaa cgcggccttt ttacggttcc tggccttttg ctggcctttt gctcacatgt
5101 tctttcctgc gttatcccct gattctgtgg ataaccgtat taccgccttt gagtgagctg
5161 ataccgctcg ccgcagccga acgaccgagc gcagcgagtc agtgagcgag gaagcggaag
5221 agc

>SEQ ID NO: 20 AAV9 Capsid Protein
MAADGYLPDWLEDNLSEGIREWWALKPGAPQPKANQQHQDNARGLVLPGYKYLGPG
NGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLKYNHADAEFQERLKEDTSFGG
NLGRAVFQAKKRLLEPLGLVEEAAKTAPGKKRPVEQSPQEPDSSAGIGKSGAQPAKKR
LNFGQTGDTESVPDPQPIGEPPAAPSGVGSLTMASGGGAPVADNNEGADGVGSSSGNW
HCDSQWLGDRVITTSTRTWALPTYNNHLYKQISNSTSGGSSNDNAYFGYSTPWGYFDF
NRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTDNNGVKTIANNLTSTVQV
FTDSDYQLPYVLGSAHEGCLPPFPADVFMIPQYGYLTLNDGSQAVGRSSFYCLEYFPSQ
MLRTGNNFQFSYEFENVPFHSSYAHSQSLDRLMNPLIDQYLYYLSKTINGSGQNQQTLK
FSVAGPSNMAVQGRNYIPGPSYRQQRVSTTVTQNNNSEFAWPGASSWALNGRNSLMN
PGPAMASHKEGEDRFFPLSGSLIFGKQGTGRDNVDADKVMITNEEEIKTTNPVATESYG
QVATNHQSAQAQAQTGWVQNQGILPGMVWQDRDVYLQGPIWAKIPHTDGNFHPSPL
MGGFGMKHPPPQILIKNTPVPADPPTAFNKDKLNSFITQYSTGQVSVEIEWELQKENSKR
WNPEIQYTSNYYKSNNVEFAVNTEGVYSEPRPIGTRYLTRNL

>SEQ ID NO: 21
GCCTGGAGGCTTGCTGAAGGCTGTATGCTGTAAGCATGGAGCTAGCAGGCTGTTTT
GGCCACTGACTGACAGCCTGCTCTCCTAGCTTACAGGACACAAGGCCTGTTACTAGC
ACTCACATAACAAATGGCCCTTTT

>SEQ ID NO: 22
GCTCGAGTGAGCGCAGCCTGCTAGCTCCATGCTTACTGTAAAGCCACCAGATGGGT
AAGCATGGAGCTAGCAGGCTTCGCCTACTAGTTTT

CLAIMS

What is claimed is:

1. An isolated nucleic acid comprising a transgene encoding one or more mature, single-stranded miRNAs, wherein the nucleic acid sequence of the transgene encoding each mature, single-stranded miRNA comprises the sequence set forth in SEQ ID NO: 7, and is flanked by a heterologous miRNA backbone sequence.

2. The isolated nucleic acid of claim 1, wherein each heterologous miRNA backbone sequence is a mir-155 backbone sequence, a mir-30 backbone sequence, or a mir-64 backbone sequence.

3. The isolated nucleic acid of claim 1, wherein the transgene comprises a promoter.

4. The isolated nucleic acid of claim 3, wherein the promoter is a chicken beta-actin (CBA) promoter or a U6 promoter.

5. The isolated nucleic acid of claim 1, wherein the transgene comprises the sequence set forth in SEQ ID NO: 21 or 22.

6. The isolated nucleic acid of claim 1, wherein the transgene is flanked by adeno-associated virus (AAV) inverted terminal repeats (ITRs), or variants thereof.

7. The isolated nucleic acid of claim 6, wherein the ITR variant lacks a functional terminal resolution site (TRS).

8. The isolated nucleic acid of claim 7, wherein the ITR variant lacking a TRS is a ΔTRS ITR.

9. A vector comprising an isolated nucleic acid comprising a transgene encoding one or more mature, single-stranded miRNAs, wherein the nucleic acid sequence of the transgene encoding each mature, single-stranded miRNA comprises the sequence set forth in SEQ ID NO: 7, and is flanked by a heterologous miRNA backbone sequence.

10. The vector of claim 9, wherein the vector is a plasmid.

11. The vector of claim 10, wherein each heterologous miRNA backbone sequence is a mir-155 backbone sequence, a mir-30 backbone sequence, or a mir-64 backbone sequence.

12. The vector of claim 10, wherein the transgene comprises a promoter.

13. The vector of claim 12, wherein the promoter is a chicken beta-actin (CBA) promoter or a U6 promoter.

14. The vector of claim 10, wherein the transgene comprises the sequence set forth in SEQ ID NO: 21 or 22.

15. The vector of claim 10, wherein the transgene is flanked by adeno-associated virus (AAV) inverted terminal repeats (ITRs), or variants thereof.

16. The vector of claim 15, wherein the ITR variant lacks a functional terminal resolution site (TRS).

17. The vector of claim 16, wherein the ITR variant lacking a TRS is a ΔTRS ITR.

18. A recombinant AAV (rAAV) comprising:

(i) a capsid protein; and,

(ii) an isolated nucleic acid comprising a transgene encoding one or more mature, single-stranded miRNAs, wherein the nucleic acid sequence of the transgene encoding each mature, single-stranded miRNA comprises the sequence set forth in SEQ ID NO: 7, and is flanked by a heterologous miRNA backbone sequence.

19. The rAAV of claim 18, wherein the transgene is flanked by full-length AAV ITR sequences.

20. The rAAV of claim 18, wherein the transgene is flanked by a full-length AAV ITR and a ΔTRS ITR.

21. The rAAV of claim 18, wherein the capsid protein is an AAV9 capsid protein.

22. The rAAV of claim 21, wherein the capsid protein comprises the sequence set forth in SEQ ID NO: 20.