AAV EXPRESSION OF MIRNA TO SUPPRESS HUMAN APOE MRNA

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of the filing date of U.S. application No. 63/575,493, filed on April 5, 2024, and U.S. application No. 63/690,122, filed on September 3, 2024, the disclosures of which are incorporated by reference herein.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING

This application contains a Sequence Listing which has been submitted electronically in ST26 format and hereby incorporated by reference in its entirety. Said ST26 file, created on April 4, 2025, is named 1676211WO1.xml and is 117,277 bytes in size.

BACKGROUND

Apolipoprotein E (APOE) is an important central nervous system (CNS) apolipoprotein intimately involved in the pathogenesis of the most common late-onset familial and sporadic forms of Alzheimer's disease (AD; Yu et al., 2014). In the general population, there are 3 common APOE alleles (ε4, ε3, and ε2) that encode the 3 APOE isoforms expressed primarily in the liver and brain. *APOE4* carriers have a markedly increased risk of developing AD (3-15 fold for heterozygotes and homozygotes, respectively, compared with *APOE3* homozygotes) and an earlier age-of-onset for developing the disease (approximately 5 years for each ε4 allele; Corder et al.,1993; Farrer et al., 1997; Lambert et al., 2013; Saunders et al., 1993; Strittmatter et al., 1993). The fact that 45% of AD patients carry at least 1 ε4 allele (compared with only 15% of age-matched healthy controls) makes *APOE4* by far the most common genetic risk factor for late-onset AD, the most common form of AD. By contrast, *APOE2* is a protective allele reducing AD risk by approximately 50% and markedly delaying the age-of-onset (Corder et al.,1994; Farrer et al., 1997; Suri et al., 2013; Talbot et al., 1994; Yu et al., 2014).

The major physiological differences between APOE3, the most common isoform, and APOE2 and APOE4, are due to differences in amino acids at 1 of 2 positions, residues 112 (APOE4) and 158 (APOE2), which are cysteine-arginine interchanges (Hatters et al., 2006). Differences in these 2 amino acids result in differences in protein structure, and the corresponding binding affinities of these APOE isoforms to lipoproteins, lipoprotein receptors, and in regulating Aβ aggregation, degradation, efflux, and phagocytosis (Castellano et al., 2011; Deane et al., 2008; Hashimoto et al., 2012; Hatters et al., 2006; Holtzman et al., 2012; Li et al., 2012; Manelli et al., 2004; Walker et al., 2000; Yu et al., 2014; Zhao et al., 2009).

SUMMARY

In one embodiment, the present disclosure provides a gene therapy vector for Alzheimer's disease. In one embodiment, a gene therapy vector comprises an AAV expression vector encoding the human APOE2 gene and either in *cis* or in *trans* artificial microRNA(s) that target endogenous APOE4. This vector system silences the expression of detrimental endogenous APOE4 in combination with supplementation of the beneficial APOE2 gene from a gene therapy vector, e.g., an AAV vector. As disclosed herein, exemplary artificial microRNA sequences were designed that target the endogenous APOE4 mRNA for suppression. The microRNAs (miRNAs) may be incorporated in sequences that are 5' to the APOE2 coding sequence, e.g., in a n intron such as the CAG promoter intron, or sequences that are 3' to the APOE2 coding sequence, e.g., sequences that are 5' to the polyA tail of the vector transgene plasmid coding for the human APOE2 coding sequence. Alternatively, the microRNA(s) may be inserted between a PolIII promoter, e.g., a U6 promoter, and a terminator following the polyA site of the APOE2 expression cassette. The vector-derived human APOE2 DNA sequence optionally includes silent nucleotide changes to decrease or inhibit suppression by the microRNAs and in one embodiment may include a tag such as a HA tag for detection, e.g., for pre-clinical detection studies. In one embodiment, the expression construct is packaged into an AAV capsid of a serotype that targets astrocytes and glial cells (for example AAV9) the prominent sites of endogenous APOE expression in the CNS, but can be provided in other vectors, e.g., other viral vectors, plasmids, nanoparticle or liposomes.

The homozygous APOE4 genotype is the major risk factor for the development of early Alzheimer's disease. Genome engineering studies in mouse models of human APOE4-dependent pathology have established that reduction of APOE4 expression can rescue the phenotype. It was hypothesized that APOE4 could be suppressed in the CNS of APOE4 homozygotes using adeno-associated virus (AAV) expression of microRNAs (miRNA) designed to hybridize to APOE mRNA. 9 different miRNAs targeting APOE were screened following transfection in HEK293T and HuH7 cells. APOE suppression was greatest with mir2A (targeting coding region nt330-351) and mirN4 (3' untranslated region nt1142-1162). miRNA expression cassettes were designed with two copies of each of these two miRNAs co-expressed with a mCherry transgene. To enhance delivery of these miRNAs, an engineered AAVrh.10 variant was identified from a screen of multiple peptide insertions into capsid loop IV and substitutions in loop VIII. This led to identifying the AAV.S2 capsid with enhanced transduction of both neurons and glia and enhanced distribution in the brain. The engineered capsid was used to deliver the APOE miRNA suppression cassette to the hippocampus of TRE4 mice (human APOE4 knock-in replacement of the murine apoE locus). Two weeks after intra-hippocampus administration, regional expression of miRNA at the injection site was quantified at the mRNA level relative to an endogenous

reference. The AAV.S2 capsid provided 2.31 ± 0.37-fold higher expression of miRNA over that provided by AAVrh.10 ($p<0.05$). In the targeted region, a single intra-hippocampus AAV.S2 administration suppressed hippocampal APOE4 mRNA levels by 76.5 ± 3.9% compared to 41.3 ± 3.3% with the same cassette delivered by the wildtype AAVrh.10 capsid ($p<0.0001$). Thus, an expression cassette with two different miRNAs targeting APOE4 delivered by the AAV.S2 capsid generated highly significant suppression of APOE4 in the CNS.

In one embodiment, a gene therapy vector is provided comprising a first promoter operably linked to a nucleic acid sequence comprising an open reading frame encoding APOE2 and a 3' untranslated region, and an isolated nucleotide sequence is provided comprising one or more RNAi nucleic acid sequences for inhibition of APOE4 mRNA. In one embodiment, the vector comprises the nucleotide sequence. In one embodiment, the nucleotide sequence is inserted 5' or 3' to the open reading frame. In one embodiment, the nucleotide sequence is inserted 5' and 3' to the open reading frame. In one embodiment, the nucleotide sequence is on a different vector. In one embodiment, the isolated nucleotide sequence comprises a second promoter operably linked to the one or more RNAi nucleic acid sequences. In one embodiment, the gene therapy vector is a viral vector. In one embodiment, the different vector is a viral vector. In one embodiment, the viral vector is an AAV, adenovirus, lentivirus, herpesvirus or retrovirus vector. In one embodiment, the AAV is AAV5, AAV9 or AAVrh10. In one embodiment, the APOE4 is human APOE4. In one embodiment, the APOE2 is human APOE2. In one embodiment, the first promoter is a PolI promoter, e.g., a constitutive promoter or a regulatable promoter, for example, an inducible promoter. In one embodiment, the second promoter is a PolIII promoter. In one embodiment, the isolated nucleotide sequence comprises nucleic acid for one or more miRNA comprising two or more of the RNAi nucleic acid sequences, e.g., one or more RNAi sequences are embedded in a miRNA sequence. In one embodiment, the RNAi comprises siRNA including a plurality of siRNA sequences. In one embodiment, the RNAi comprises shRNA sequences of about 15 to 25 nucleotides in length. In one embodiment, the open reading frame for APOE2 comprises a plurality of silent nucleotide substitutions relative to SEQ ID NO:6, e.g., the open reading frame comprises SEQ ID NO:7 or nucleotide sequence with at least 70%, 75% 80%, 85%, 90%, 95%, 97% or 98% nucleic acid sequence identity to SEQ ID NO:7 and encodes APOE2, or the open reading frame encodes APOE2 and comprises a nucleotide sequence with at least 70%, 75% 80%, 85%, 90%, 95%, 97% or 98% nucleic acid sequence identity to GAAAGAACTCAAAGCTTATAAGAGCGAGCTGGAGG (SEQ ID NO:13) but which sequence is not SEQ ID NO:7. In one embodiment, the plurality of the silent nucleotide substitutions in the APOE2 open reading frame are not in the RNAi nucleic acid sequence in the isolated nucleotide sequence, that is the sequence with the nucleotide

substitutions differs from the RNAi nucleotide sequence so that the mRNA having the nucleotide substitutions does not bind to, e.g., for a duplex with, the RNAi sequences, e.g., isolated RNAi or RNAi sequences expressed from a vector. In one embodiment, at least 50%, 60%, 70%, 80% or 90% of codons in the open reading frame for APOE2 have a silent nucleotide substitution. In one embodiment, at least 5%, 10%, 20%, 30%, or 40%, of codons in the open reading frame for APOE2 have a silent nucleotide substitution, e.g., in a portion of APOE2 sequences that correspond to the RNAi sequences. That is, the silent nucleotide substitutions in a human APOE2 coding sequence result in a sequence that differs from endogenous human APOE4 sequences and differs from the APOE4 RNAi sequences. In one embodiment, the APOE4 that is inhibited has a sequence having at least 80%, 85%, 90%, 95% or more amino acid sequence identity to a polypeptide encoded by SEQ ID NO:22. In one embodiment, the APOE2 has a sequence having at least 80%, 85%, 90%, 95% or more amino acid sequence identity to a polypeptide encoded by SEQ ID NO:9. In one embodiment, the one or more RNAi nucleic acid sequences have at least 60%, 70%, 80%, 90% or more nucleotide sequence identity to one of SEQ ID Nos. 1-4, 20-22, 33-41 or 80 or the complement thereof. In one embodiment, one or more RNAi nucleic acid sequences, e.g., miRNA, that target APOE mRNA have at least 70%, 75%, 80%, 85%, 90%, 95% or more nucleic acid complementary sequence identity to the APOE mRNA. In one embodiment, a APOE gene, e.g., transgene, that resists targeting has less than 70%, 65%, 60%, 55% or 50% or less identity to the miRNA. In one embodiment, a APOE gene, e.g., transgene, that resists targeting has at least 80%, 82%, 84%, 85%, 87%, 89%, 90%, 92%, 94%, 95%, 96%, 97%, 98%, 99% or more nucleotide sequence identity to one of SEQ ID Nos. 67-70 and in one embodiment, the APOE gene that resists targeting has SEQ ID NO:82, SEQ ID NO:85, GTCGCTTTTGGGATTACCTGCGCTGGGTGCAGACGTTAATTCCTTCGG (SEQ ID NO:91), ATTACCTGCGCTGGGTGCAGACGTTA (SEQ ID NO:92) ATAAcCAtTAGAc (SEQ ID NO:93), aagcgcaacaaatcagactc (SEQ ID NO:94), atAAGAGTGAGTTAGAAGAGCA (SEQ ID NO:95), a portion thereof, or a sequence with 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 nucleotide substitutions. In one embodiment, the vector has a first promoter, e.g., a PolI or PolII promoter, operably linked to a nucleic acid sequence comprising an open reading frame encoding human APOE2 and an isolated nucleotide sequence having one or more RNAi nucleic acid sequences for inhibition of human APOE4 mRNA. In one embodiment, the nucleotide sequence is inserted 5' to the open reading frame. In one embodiment, the nucleotide sequence is inserted 3' to the open reading frame. In one embodiment, the nucleotide sequence is inserted 5' and 3' to the open reading frame. In one embodiment, the isolated nucleotide sequence comprises a second promoter operably linked to the one or more RNAi nucleic acid sequences. In one embodiment, the RNAi nucleic acid sequence is about 125 to 500, e.g., about 150 to 175, nucleotides in length.

In one embodiment the gene therapy vector may have 2, 3, 4 or more copies of the RNAi nucleic acid sequence which may include miRNA sequences, e.g., miRNA sequences which flank the APOE4 inhibitory sequences.

In one embodiment, a method to prevent, inhibit or treat Alzheimer's disease in a mammal is provided comprising: administering to the mammal an effective amount of a composition comprising the gene therapy vector. In one embodiment, the composition comprises nanoparticles comprising the gene therapy vector or the different vector, or both. In one embodiment, the gene therapy vector or the different vector, or both, comprise a viral vector. In one embodiment, the mammal is a E2/E4 heterozygote. In one embodiment, the mammal is a E4/E4 homozygote. In one embodiment, the composition is systemically administered. In one embodiment, the composition is orally administered. In one embodiment, the composition is intravenously administered. In one embodiment, the composition is locally administered. In one embodiment, the composition is injected. In one embodiment, the composition is administered to the central nervous system. In one embodiment, the composition is administered to the brain. In one embodiment, the composition is a sustained release composition. In one embodiment, the mammal is a human. In one embodiment, the RNAi nucleic acid sequences comprise a plurality of miRNA sequences.

In one embodiment, a method to prevent, inhibit or treat a disease associated with APOE4 expression in a mammal is provided comprising: administering to the mammal an effective amount of a composition comprising the gene therapy vector. In one embodiment, the composition comprises liposomes comprising the gene therapy vector or the different vector, or both. In one embodiment, the composition comprises nanoparticles comprising the gene therapy vector or the different vector, or both. In one embodiment, the gene therapy vector or the different vector, or both, comprise a viral vector. In one embodiment, the mammal is a E2/E4 heterozygote. In one embodiment, the mammal is a E4/E4 homozygote. In one embodiment, the composition is systemically administered. In one embodiment, the composition is orally administered. In one embodiment, the composition is intravenously administered. In one embodiment, the composition is locally administered. In one embodiment, the composition is injected. In one embodiment, the composition is administered to the central nervous system. In one embodiment, the composition is administered to the brain. In one embodiment, the composition is a sustained release composition. In one embodiment, the mammal is a human. In one embodiment, the RNAi sequences comprise a plurality of miRNA sequences.

In one embodiment, a gene therapy vector is provided comprising one or more RNAi nucleic acid sequences for inhibition of APOE4 expression. In one embodiment, the one or more RNAi nucleic acid sequences correspond to sequences in an APOE coding region. In one

embodiment, the one or more RNAi nucleic acid sequences are inserted 5' or 3' to an open reading frame. In one embodiment, the one or more RNAi nucleic acid sequences are inserted 5' and 3' to an open reading frame. In one embodiment, different RNAi sequences are in the vector. In one embodiment, there are from 1 to 5 copies of the RNAi sequences. In one embodiment, the one or more RNAi nucleic acid sequences correspond to sequences in an APOE non-coding region. In one embodiment, the gene therapy vector is a viral vector. In one embodiment, the viral vector is an AAV, adenovirus, lentivirus, herpesvirus or retrovirus vector. In one embodiment, the AAV is AAV5, AAV9 or AAVrh10. In one embodiment, the APOE4 is human APOE4. In one embodiment, the vector further comprises a coding region for APOE2 that is resistant to the one or more RNAi nucleic acid sequences. In one embodiment, a sperate vector comprises a coding region for APOE2 that is resistant to the one or more RNAi nucleic acid sequences. In one embodiment, the APOE2 is human APOE2. In one embodiment, the vector comprises at least two different RNAi sequences. In one embodiment, one of the RNAi sequences comprises one of SEQ ID Nos. 33-41, the complement thereof, or a sequence with at least 80%, 82%, 85%, 87%, 90%, 92%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity thereto. In one embodiment, one or more RNAi nucleic acid sequences, e.g., miRNA, that target APOE mRNA have at least 70%, 75%, 80%, 85%, 90%, 95% or more nucleic acid complementary sequence identity to the APOE mRNA. In one embodiment, a APOE gene, e.g., transgene, that resists targeting has less than 70%, 65%, 60%, 55% or 50% or less identity to the miRNA. In one embodiment, the RNAi comprises miRNA including a plurality of miRNA sequences. In one embodiment, the open reading frame for APOE2 comprises a plurality of silent nucleotide substitutions relative to SEQ ID NO:6. In one embodiment, the plurality of the silent nucleotide substitutions in the APOE2 open reading frame are not in the RNAi nucleic acid sequence in the isolated nucleotide sequence.

Also provided is a composition comprising the gene therapy vector and optionally a pharmaceutically acceptable carrier. Further provided is a method to prevent, inhibit or treat a neurological disease in a mammal, comprising: administering to the mammal an effective amount of a composition comprising the gene therapy vector. In addition, a method to prevent, inhibit or treat a disease associated with APOE4 expression in a mammal is provided, comprising: administering to the mammal an effective amount of a composition comprising the gene therapy vector. In one embodiment, the mammal is a E2/E4 heterozygote. In one embodiment, the mammal is a E4/E4 homozygote. In one embodiment, the composition is systemically administered. In one embodiment, the composition is orally administered. In one embodiment, the composition is intravenously administered. In one embodiment, the composition is locally administered. In one embodiment, the composition is injected. In one embodiment, the

composition is administered to the central nervous system. In one embodiment, the composition is administered to the brain. In one embodiment, the composition is a sustained release composition. In one embodiment, the mammal is a human. In one embodiment, the RNAi sequences comprise a plurality of miRNA sequences each comprising the one or more RNAi nucleic acid sequences for inhibition of APOE4 mRNA. In one embodiment, one of the miRNA sequences in the vector is inserted 5' to the open reading frame and another is inserted 3' to the open reading frame. In one embodiment, the RNAi sequences comprise a miRNA sequence comprising one or more of SEQ ID Nos. 33-41 or the complement thereof. In one embodiment, the miRNA sequence in the vector is inserted 5' to the open reading frame. In one embodiment, the miRNA sequence in the vector is inserted 3' to the open reading frame. In one embodiment, vectors of the disclosure can encode one or more miRNA sequences that target an APOE mRNA sequence set forth in any one of SEQ ID NOs: 33-41.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Production of inhibitory RNAs from an exemplary target transcript template (Boudreau and Davidson. 2012. Methods in Enzymology, Volume 507). (SEQ ID NOS: 14-18)

Figure 2. Pathways to inhibit mRNA (Borel et al., 2014. *Mol Ther* 22:692-701).

Figures 3A-3B. Exemplary construct for miRNA insertion(s). A) Insertion into the promoter intron. B) Insertion between transgene and polyA. Multiple miRNAs can be placed in tandem for enhanced silencing of APOE4. Level of APOE2-HA and miRNA are similar. A lower level of miRNA expression compared to U6 promoter for fewer off target effects and lower potential for toxicity.

Figures 4A-4B. Exemplary construct for miRNA insertion(s). Multiple miRNAs can be placed in tandem for enhanced silencing of APOE4. PolIII promoters can be used for transcription of rRNA, tRNA and miRNA. Defined terminator, no polyA. U6 promoter drives high expression of miRNA for fewer off target effects and lower potential for toxicity. Reporter gene is useful to maintain length and track expression.

Figures 5A-5B. Exemplary vector to silence APOE4 and express APOE2. miRNA knocks down APOE4 and vector encoded APOE2 is resistant to miRNA. CAG promoter results in a similar level of APOE2-HA and miRNA expression. miRNA can be inserted into, for example, the CAG intron or 3' untranslated region.

Figure 6. APOE knock down of expression by four different siRNAs *in vitro*. 4 different siRNAs targeting the coding sequence of APOE were generated and siRNAs were transfected into U87 cells and APOE mRNA copies quantified by RT-PCR. (SEQ ID NOS: 1-5)

Figure 7. Exemplary strategy.

Figure 8. Exemplary miRNA targets.

Figure 9. Exemplary miRNA structure. (SEQ ID NO: 23)

Figure 10. *In Vitro* assessment of miRNAs in 2 cell types.

Figure 11. *In Vitro* Suppression of APOE mRNA by Single miRNAs (SEQ ID NOS: 33-41).

Figure 12. Combination of 2 miRNAs Provides Additional Silencing

Figure 13. miRNA Combinations in a Single Vector.

Figure 14. Exemplary miRNA Targets.

Figures 15A-15D. A) APOE2 Gene to Circumvent Silencing by mirK8 and mir2A (SEQ ID NOS:67 and 96-98). B) APOE gene resistant to K3 (SEQ ID NOS:68 and 99-102). C) APOE gene resistant to K13 (SEQ ID NOS:69 and 103-106). D) APOE gene resistant to K7 (SEQ ID NOS:70 and 107-110).

Figure 16. mRNA Expression from Engineered APOE cDNA.

Figure 17. *In Vivo* Silencing of Human APOE in TRE4 Mouse Brain.

Figures 18A-18D. miRNA combinations for suppression of APOE. A) Schematic of miRNA targets in the APOE cDNA showing targeting of non-coding region by miRNA candidates. B, C). miRNA-mediated suppression of APOE *in vitro*. B) HEK293T and C) HuH7 cells were transfected with plasmids expressing miRNAs as part of a CAG-mCherry expression cassette. After 48 hours, APOE mRNA levels were assessed by TaqMan using relative quantitation with GAPDH reference. Results are average and standard error of 3 biological replicates and normalized to the control transfected with the pCAG-mCherry plasmid with no miRNA. See Table 4 for details regarding the sequence of the miRNA targets. Control $p<0.0001$ compared to all other groups. *$p<0.05$; **$p<0.005$. D) AAVR dependence of transduction of AAAVrh.10 variants. Equal doses of 39 different variant capsids with luciferase expression cassette were used to transduce HeLa cells with or without AAVR. Data is plotted as transduction of AAVR+ versus transduction of AAVR – cell line. Original rh.10 (orange) and AAVR independent (red) capsids are indicated.

Figures 19A-19E. *In vitro* assessment of AAVrh.10 capsid modified variants. rh.10 and rh.10 variant capsids were used to package an AAV2 genome expressing mCherry driven by the CAG promoter. Four different CNS-related cell types: U87 (human glioblastoma, ATCC:HTB-14), SVGp12 (human fetal glia, ATCC:CRL-8621), SH-SY5Y (human neuroblastoma, ATCC:CRL-2266) and HMC3 (human microglia, ATCC:CRL-3304) were infected at a ratio of 10^4 genome copies of AAV per cell and cultured for 72 hours. The percentage of mCherry positive cells was counted by fluorescence microscopy and values presented in the cells of the heat map. A-D. Quantitative data for the 4 best capsids compared to AAVrh.10. A) U87; B)

SVG-p12; C) SH-SY5Y; and D) HMC3. p values are derived from unpaired t-test, n=3 data points per capsid per cell line. For data for all lines tested, see Table 6. E. Heat map for all assessed capsids. See Table 5 for sequences of the AAVrh.10 variants.

Figures 20A-20D. Quantitative assessment of vector spread following direct administration to the hippocampus. Cohorts of 4 seven-week-old male C57Bl/6 mice received a unilateral hippocampal administration of $2.5x10^{10}$ genome copies of AAV.S2 (referred to as "S2") or AAVrh.10 control ("rh.10"). After 4 weeks, the whole brain was sliced into 3 mm coronal sections. A) Sectioning scheme. B) Vector genome level/µg total DNA; C) mCherry mRNA level (copies/µg input RNA). D) mCherry levels by ELISA normalized to mg total protein. All graphs show mean ± SEM; p values are derived from unpaired t- test and compare S2 to rh.10 for each brain section. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$, # $p>0.05$.

Figures 21A-21D. Immunohistochemical assessment of transduction efficiency by AAV.S2 compared to AAVrh.10. Mice received bilateral hippocampus administration of $2.5x10^{10}$ genome copies of AAV.S2 (referred to as "S2") or AAVrh.10 ("rh.10") control. Sections of fixed brains were stained by DAPI and then imaged in blue and red channels to visualize nuclei and mCherry expression respectively. The merged images were used to quantify the percentage of cells that were mCherry positive. A) Images of hippocampus. B) Images of cortex. C) Quantitation (percentage) of mCherry positive cells. D) Amount (integrated density) of mCherry per cell. Quantification is the mean ± SEM for n=7-9 random fields in n=3 brains/cohort. Bar 50 µm.

Figures 22A-22D. Colocalization of AAV.S2 vs AAVrh.10 transduced cells with a neuronal and glial marker following CNS administration. Sections of fixed brains from mice administered with either AAVrh.10mCherry or AAVrh.10 S2-mCherry as described in Figure 21 were processed for immunofluorescence imaging using anti-NeuN neuronal marker or anti-GFAP astroglial marker and stained with DAPI. A) Images of hippocampus were merged to show colocalization of NeuN (green) and mCherry (red) which when colocalized show as yellow. B) Individual images of hippocampus were merged to show colocalization of GFAP (green) and mCherry (red) which when colocalized show as yellow. C) High magnification view of representative fields in the hippocampus showing yellow colocalization indicated by white arrows. Left, AAVrh.10; right, AAV.S2. Upper panels are NeuN colocalization and lower panels GFAP colocalization. D) Quantification of colocalization of mCherry with NeuN and GFAP (mean ± SEM for n=5 random fields in n=3 brains/cohort). The number of astroglia was determined the in the same way using GFAP and mCherry colocalization. Bar 400 µm.

Figures 23A-23E. *In vivo* suppression of APOE4 following hippocampus administration of the AAV.S2 vector expressing APOE suppressing miRNAs. TRE4 mice received unilateral

AAV administration into the right hippocampus (dose $2.5x10^{10}$ gc in 2 µl). After 2 weeks, the brains were removed and sliced into 3 mm coronal sections. Sections C and D represent the targeted injection area and A is distant (see Figure 20A). A) Schematic of the constructs with miRNAs targeting APOE gene inserted into the intron or 3'untranslated regions of CAGmCherry cassette. B) miRNA expression. Regional expression of miRNA level was assessed using qPCR for mCherry mRNA across brain sections with absolute quantitation using a plasmid containing mCherry to create a standard curve (mCherry is in the same primary transcript and is a proxy for mir2A and mirN4). miRNA2A level was assessed directly by qRT-PCR with mir2A-specfic probe and an endogenous mouse standard of miRNA361. Data is mean plus standard error for n=5 mice per cohort for all sections, C-D) corresponding to the region of vector administration. C) miRNA-mediated APOE4 suppression assessed by APOE mRNA level (qPCR) with a standard of a plasmid containing APOE cDNA. D) Determination of APOE4 protein level in sections from mice receiving AAVrh.10.S2 compared to cohorts. Mean and standard error from all mice (n=5). E) Correlation of APOE and mir2A expression across all samples of brains injected with mirAPOE expression cassette with either AAVrh.10 or AAV.S2 capsid.

Figure 24. In vivo comparison of APOE mRNA level from silencing resistant APOE-SR gene with and without mir2A. The indicated expression cassettes were inserted into AAVrh.10 variant S2 vector and injected into the hippocampus of TRE4 mice expressing the humanAPOE4 gene at a dose of 2.5×10^{10} genome copies bilaterally. After 4 weeks, the brains were removed and cut into 2mm coronal slices. Slice D corresponds to the injection site while A,B,C are more frontal. Total APOE mRNA level was determined qPCR using a primer and probe set designed to region common to all APOE variants. The suppression of endogenous APOE4 gene in TRE4 mice by mir2A (red) is evident in the lower expression compared to the PBS control (blue). Introduction of either native APOE2 gene (green) or APOE2-SR (purple) results in APOE levels approximately 3 fold over endogenous close to the injection site. The addition of mir2A to the vector with the APOE2-SR (orange) gene does not suppress total APOE at the injection site demonstrating that it is silencing resistant.

Figure 25. In vivo comparison of APOE protein level from silencing resistant APOE-SR gene with and without mir2A. The indicated expression cassettes were inserted into AAVrh.10 variant S2 vector and injected into the hippocampus of TRE4 mice expressing the humanAPOE4 gene at a dose of 2.5×10^{10} genome copies bilaterally. After 4 weeks, the brains were removed and cut into 2mm coronal slices. Total APOE protein level was determined by ELISA. Slice D corresponds to the injection site while A, B, and C are more frontal. Total APOE protein level was determined by ELISA. The suppression of endogenous APOE4 gene in TRE4 mice by mir2A (red) is evident in the lower expression compared to the PBS control (blue). Introduction

of either native APOE2 gene (green) or APOE2-SR (purple) results in APOE levels approximately 2 fold over endogenous close to the injection site. The addition of mir2A to the vector with the APOE2-SR (orange) gene does not suppress total APOE demonstrating that it is silencing resistant.

DETAILED DESCRIPTION

Definitions

A "vector" refers to a macromolecule or association of macromolecules that comprises or associates with a polynucleotide, and which can be used to mediate delivery of the polynucleotide to a cell, either *in vitro* or *in vivo*. Illustrative vectors include, for example, plasmids, viral vectors, liposomes and other gene delivery vehicles. The polynucleotide to be delivered, sometimes referred to as a "target polynucleotide" or "transgene," may comprise a coding sequence of interest in gene therapy (such as a gene encoding a protein of therapeutic interest), a coding sequence of interest in vaccine development (such as a polynucleotide expressing a protein, polypeptide or peptide suitable for eliciting an immune response in a mammal), and/or a selectable or detectable marker.

"Transduction," "transfection," "transformation" or "transducing" as used herein, are terms referring to a process for the introduction of an exogenous polynucleotide into a host cell leading to expression of the polynucleotide, e.g., the transgene in the cell, and includes the use of recombinant virus to introduce the exogenous polynucleotide to the host cell. Transduction, transfection or transformation of a polynucleotide in a cell may be determined by methods well known to the art including, but not limited to, protein expression (including steady state levels), e.g., by ELISA, flow cytometry and Western blot, measurement of DNA and RNA by hybridization assays, e.g., Northern blots, Southern blots and gel shift mobility assays. Methods used for the introduction of the exogenous polynucleotide include well-known techniques such as viral infection or transfection, lipofection, transformation and electroporation, as well as other non-viral gene delivery techniques. The introduced polynucleotide may be stably or transiently maintained in the host cell.

"Gene delivery" refers to the introduction of an exogenous polynucleotide into a cell for gene transfer, and may encompass targeting, binding, uptake, transport, localization, replicon integration and expression.

"Gene transfer" refers to the introduction of an exogenous polynucleotide into a cell which may encompass targeting, binding, uptake, transport, localization and replicon integration, but is distinct from and does not imply subsequent expression of the gene.

"Gene expression" or "expression" refers to the process of gene transcription, translation, and post-translational modification.

An "infectious" virus or viral particle is one that comprises a polynucleotide component which it is capable of delivering into a cell for which the viral species is trophic. The term does not necessarily imply any replication capacity of the virus.

The term "polynucleotide" refers to a polymeric form of nucleotides of any length, including deoxyribonucleotides or ribonucleotides, or analogs thereof. A polynucleotide may comprise modified nucleotides, such as methylated or capped nucleotides and nucleotide analogs, and may be interrupted by non-nucleotide components. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The term polynucleotide, as used herein, refers interchangeably to double- and single-stranded molecules. Unless otherwise specified or required, any embodiment described herein that is a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

An "isolated" polynucleotide, e.g., plasmid, virus, polypeptide or other substance refers to a preparation of the substance devoid of at least some of the other components that may also be present where the substance or a similar substance naturally occurs or is initially prepared from. Thus, for example, an isolated substance may be prepared by using a purification technique to enrich it from a source mixture. Isolated nucleic acid, peptide or polypeptide is present in a form or setting that is different from that in which it is found in nature. For example, a given DNA sequence (e.g., a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences, such as a specific mRNA sequence encoding a specific protein, are found in the cell as a mixture with numerous other mRNAs that encode a multitude of proteins. The isolated nucleic acid molecule may be present in single-stranded or double-stranded form. When an isolated nucleic acid molecule is to be utilized to express a protein, the molecule will contain at a minimum the sense or coding strand (i.e., the molecule may single-stranded), but may contain both the sense and anti-sense strands (i.e., the molecule may be double-stranded). Enrichment can be measured on an absolute basis, such as weight per volume of solution, or it can be measured in relation to a second, potentially interfering substance present in the source mixture. Increasing enrichments of the embodiments of this invention are envisioned. Thus, for example, a 2-fold enrichment, 10-fold enrichment, 100-fold enrichment, or a 1000-fold enrichment.

A "transcriptional regulatory sequence" refers to a genomic region that controls the transcription of a gene or coding sequence to which it is operably linked. Transcriptional regulatory sequences of use in the present invention generally include at least one transcriptional promoter and may also include one or more enhancers and/or terminators of transcription.

"Operably linked" refers to an arrangement of two or more components, wherein the components so described are in a relationship permitting them to function in a coordinated manner. By way of illustration, a transcriptional regulatory sequence or a promoter is operably linked to a coding sequence if the TRS or promoter promotes transcription of the coding sequence. An operably linked TRS is generally joined in *cis* with the coding sequence, but it is not necessarily directly adjacent to it.

"Heterologous" means derived from a genotypically distinct entity from the entity to which it is compared. For example, a polynucleotide introduced by genetic engineering techniques into a different cell type is a heterologous polynucleotide (and, when expressed, can encode a heterologous polypeptide). Similarly, a transcriptional regulatory element such as a promoter that is removed from its native coding sequence and operably linked to a different coding sequence is a heterologous transcriptional regulatory element.

A "terminator" refers to a polynucleotide sequence that tends to diminish or prevent read-through transcription (i.e., it diminishes or prevent transcription originating on one side of the terminator from continuing through to the other side of the terminator). The degree to which transcription is disrupted is typically a function of the base sequence and/or the length of the terminator sequence. In particular, as is well known in numerous molecular biological systems, particular DNA sequences, generally referred to as "transcriptional termination sequences" are specific sequences that tend to disrupt read-through transcription by RNA polymerase, presumably by causing the RNA polymerase molecule to stop and/or disengage from the DNA being transcribed. Typical example of such sequence-specific terminators include polyadenylation ("polyA") sequences, e.g., SV40 polyA. In addition to or in place of such sequence-specific terminators, insertions of relatively long DNA sequences between a promoter and a coding region also tend to disrupt transcription of the coding region, generally in proportion to the length of the intervening sequence. This effect presumably arises because there is always some tendency for an RNA polymerase molecule to become disengaged from the DNA being transcribed, and increasing the length of the sequence to be traversed before reaching the coding region would generally increase the likelihood that disengagement would occur before transcription of the coding region was completed or possibly even initiated. Terminators may thus prevent transcription from only one direction ("uni-directional" terminators) or from both directions ("bi-directional" terminators), and may be comprised of sequence-specific termination sequences or sequence-non-specific terminators or both. A variety of such terminator sequences are known in the art; and illustrative uses of such sequences within the context of the present invention are provided below.

"Host cells," "cell lines," "cell cultures," "packaging cell line" and other such terms denote higher eukaryotic cells, such as mammalian cells including human cells, useful in the present invention, e.g., to produce recombinant virus or recombinant fusion polypeptide. These cells include the progeny of the original cell that was transduced. It is understood that the progeny of a single cell may not necessarily be completely identical (in morphology or in genomic complement) to the original parent cell.

"Recombinant," as applied to a polynucleotide means that the polynucleotide is the product of various combinations of cloning, restriction and/or ligation steps, and other procedures that result in a construct that is distinct from a polynucleotide found in nature. A recombinant virus is a viral particle comprising a recombinant polynucleotide. The terms respectively include replicates of the original polynucleotide construct and progeny of the original virus construct.

A "control element" or "control sequence" is a nucleotide sequence involved in an interaction of molecules that contributes to the functional regulation of a polynucleotide, including replication, duplication, transcription, splicing, translation, or degradation of the polynucleotide. The regulation may affect the frequency, speed, or specificity of the process, and may be enhancing or inhibitory in nature. Control elements known in the art include, for example, transcriptional regulatory sequences such as promoters and enhancers. A promoter is a DNA region capable under certain conditions of binding RNA polymerase and initiating transcription of a coding region usually located downstream (in the 3' direction) from the promoter. Promoters include AAV promoters, e.g., P5, P19, P40 and AAV ITR promoters, as well as heterologous promoters.

An "expression vector" is a vector comprising a region which encodes a gene product of interest, and is used for effecting the expression of the gene product in an intended target cell. An expression vector also comprises control elements operatively linked to the encoding region to facilitate expression of the protein in the target. The combination of control elements and a gene or genes to which they are operably linked for expression is sometimes referred to as an "expression cassette," a large number of which are known and available in the art or can be readily constructed from components that are available in the art.

The terms "polypeptide" and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, acetylation, phosphonylation, lipidation, or conjugation with a labeling component.

The term "exogenous," when used in relation to a protein, gene, nucleic acid, or polynucleotide in a cell or organism refers to a protein, gene, nucleic acid, or polynucleotide which has been introduced into the cell or organism by artificial or natural means. An exogenous

nucleic acid may be from a different organism or cell, or it may be one or more additional copies of a nucleic acid which occurs naturally within the organism or cell. By way of a non-limiting example, an exogenous nucleic acid is in a chromosomal location different from that of natural cells, or is otherwise flanked by a different nucleic acid sequence than that found in nature, e.g., an expression cassette which links a promoter from one gene to an open reading frame for a gene product from a different gene.

"Transformed" or "transgenic" is used herein to include any host cell or cell line, which has been altered or augmented by the presence of at least one recombinant DNA sequence. The host cells of the present invention are typically produced by transfection with a DNA sequence in a plasmid expression vector, as an isolated linear DNA sequence, or infection with a recombinant viral vector.

The term "sequence homology" means the proportion of base matches between two nucleic acid sequences or the proportion amino acid matches between two amino acid sequences. When sequence homology is expressed as a percentage, e.g., 50%, the percentage denotes the proportion of matches over the length of a selected sequence that is compared to some other sequence. Gaps (in either of the two sequences) are permitted to maximize matching; gap lengths of 15 bases or less are usually used, 6 bases or less e.g., with 2 bases or less. When using oligonucleotides as probes or treatments, the sequence homology between the target nucleic acid and the oligonucleotide sequence is generally not less than 17 target base matches out of 20 possible oligonucleotide base pair matches (85%); not less than 9 matches out of 10 possible base pair matches (90%), or not less than 19 matches out of 20 possible base pair matches (95%).

Two amino acid sequences are homologous if there is a partial or complete identity between their sequences. For example, 85% homology means that 85% of the amino acids are identical when the two sequences are aligned for maximum matching. Gaps (in either of the two sequences being matched) are allowed in maximizing matching; gap lengths of 5 or less or with 2 or less. Alternatively, two protein sequences (or polypeptide sequences derived from them of at least 30 amino acids in length) are homologous, as this term is used herein, if they have an alignment score of at more than 5 (in standard deviation units) using the program ALIGN with the mutation data matrix and a gap penalty of 6 or greater. The two sequences or parts thereof are more homologous if their amino acids are greater than or equal to 50% identical when optimally aligned using the ALIGN program.

The term "corresponds to" is used herein to mean that a polynucleotide sequence is structurally related to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is structurally related to all or a portion of a reference polypeptide sequence, e.g., they have at least 80%, 85%, 90%, 95% or more, e.g., 99% or 100%, sequence

identity. In contradistinction, the term "complementary to" is used herein to mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAC" and is complementary to a reference sequence "GTATA".

The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denote a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, e.g., at least 90 to 95 percent sequence identity, or at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 20-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison.

"Conservative" amino acid substitutions are, for example, aspartic-glutamic as polar acidic amino acids; lysine/arginine/histidine as polar basic amino acids; leucine/isoleucine/methionine/valine/alanine/glycine/proline as non-polar or hydrophobic amino acids; serine/ threonine as polar or uncharged hydrophilic amino acids. Conservative amino acid substitution also includes groupings based on side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. For example, it is reasonable to expect that replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid will not have a major effect on the properties of the resulting polypeptide. Whether an amino

acid change results in a functional polypeptide can readily be determined by assaying the specific activity of the polypeptide. Naturally occurring residues are divided into groups based on common side-chain properties: (1) hydrophobic: norleucine, met, ala, val, leu, ile; (2) neutral hydrophilic: cys, ser, thr; (3) acidic: asp, glu; (4) basic: asn, gln, his, lys, arg; (5) residues that influence chain orientation: gly, pro; and (6) aromatic; trp, tyr, phe.

The disclosure also envisions polypeptides with non-conservative substitutions. Non-conservative substitutions entail exchanging a member of one of the classes described above for another.

Exemplary human APOE sequences include but are not limited to:

mkvlwaallv tflagcqakv cqavctcpcp clrqqtcwqs gqrwclalgr fwdylrwvqt
lseqvqeell ssqvtqelra lmdetmkelk aykseleeql tpvaeetrar lskelqaaqa
rlgadmedv**c** grlvqyrgev qamlgqstee lrvrlashlr klrkrllrda ddlqk**r**lavy
qagaregaer glsairerlg plveqgrvra atvgslagqp lqeraqawge rlrarmeemg
srtrdrldev keqvaevrak leeqaqqirl qaeafqarlk swfeplvedm qrqwaglvek
vqaavgtsaa pvpsdnh (includes signal peptide, italicized above) (SEQ ID NO:8), as well as sequences with at least 80%, 85%, 90%, 95% or more, e.g., 99% or 100%, sequence identity thereto, including those with Cys at residue 112 (mature polypeptide numbering; c bolded above) and Cys at residue 158 (r bolded above) (APOE2) corresponding to SEQ ID NO:9, or with Arg at residue 112 (mature polypeptide numbering) and Arg at residue 158 (APOE4), corresponding to SEQ ID NO:10, where in one embodiment, APOE4 may have 31K, 46P, 79T, 130R, 163C, 292H and/or 314R, and APOE2 may have 43C, 152Q, 154C/S, 163C/P, 164Q, 172A, 176C, 242Q, 246C, 254E.

SEQ ID NO:9 includes

kv eqavetepep elrqqtewqs gqrwelalgr fwdylrwvqt
lseqvqeell ssqvtqelra lmdetmkelk aykseleeql tpvaeetrar lskelqaaqa
rlgadmedv**c** grlvqyrgev qamlgqstee lrvrlashlr klrkrllrda ddlqk**c**lavy
qagaregaer glsairerlg plveqgrvra atvgslagqp lqeraqawge rlrarmeemg
srtrdrldev keqvaevrak leeqaqqirl qaeafqarlk swfeplvedm qrqwaglvek
vqaavgtsaa pvpsdnh.

SEQ ID NO:10 includes

kv eqavetepep elrqqtewqs gqrwelalgr fwdylrwvqt
lseqvqeell ssqvtqelra lmdetmkelk aykseleeql tpvaeetrar lskelqaaqa
rlgadmedv**r** grlvqyrgev qamlgqstee lrvrlashlr klrkrllrda ddlqk**r**lavy
qagaregaer glsairerlg plveqgrvra atvgslagqp lqeraqawge rlrarmeemg
srtrdrldev keqvaevrak leeqaqqirl qaeafqarlk swfeplvedm qrqwaglvek
vqaavgtsaa pvpsdnh.

Exemplary human APOE nucleic acid sequences, e.g., those for silent nucleotide substitutions if they encode APOE2, include but are not limited to:

ggaacttgat gctcagagag gacaagtcat ttgcccaagg tcacacagct ggcaactggc
agagccagga ttcacgccct ggcaatttga ctccagaatc ctaaccttaa cccagaagca
cggcttcaag cccctggaaa ccacaatacc tgtggcagcc agggggaggt gctggaatct
catttcacat gtggggaggg ggctcccctg tgctcaaggt cacaaccaaa gaggaagctg
tgattaaaac ccaggtccca tttgcaaagc ctcgactttt agcaggtgca tcatactgtt
cccacccctc ccatcccact tctgtccagc cgcctagccc cacttccttt tttttctttt
tttgagacag tctccctctt gctgaggctg gagtgcagtg gcgagatctc ggctcactgt
aacctccgcc tcccgggttc aagcgattct cctgcctcag cctcccaagt agctaggatt
acaggcgccc gccaccacgc ctggctaact tttgtatttt tagtagagat ggggtttcac
catgttggcc aggctggtct caaactcctg accttaagtg attcgcccac tgtggcctcc
caaagtgctg ggattacagg cgtgagctac cgcccccagc ccctcccatc ccacttctgt
ccagcccccc agccctactt tctttctggg atccaggagt ccagatcccc agcccccctc
ccagattaca ttcatccagg cacaggaaag gacagggtca ggaaaggagg actctgggcg
gcagcctcca cattcccctt ccacgcttgg cccccagaat ggaggagggt gtctggatta
ctgggcgagg tgtcctccct tcctggggac tgtgggggct ggtcaaaaga cctctatgcc
ccacctcctt cctccctctg ccctgctgtg cctggggcag ggggagaaca gcccacctcg
tgactggggg ctggcccagc ccgccctatc cctgggggag ggggcgggac aggggagccc
ctataattgg acaagtctgg gatccttgag tcctactcag ccccagcgga ggtgaaggac
gtccttcccc aggagccg (SEQ ID NO:11)

or

ccccagcgga ggtgaaggac gtccttcccc aggagccgac tggccaatca caggcaggaa
gatgaaggtt ctgtgggctg cgttgctggt cacattcctg gcaggatgcc aggccaaggt
ggagcaagcg gtggagacag agccggagcc cgagctgcgc cagcagaccg agtggcagag
cggccagcgc tgggaactgg cactgggtcg cttttgggat tacctgcgct gggtgcagac
actgtctgag caggtgcagg aggagctgct cagctcccaa gtcacccaag aactgagggc
gctgatggac gagaccatga aggagttgaa ggcctacaaa tcggaactgg aggaacaact
gaccccggta gcggaggaga cgcgggcacg gctgtccaag gagctgcaga cggcgcaggc
ccggctgggc gcggacatgg aggacgtgtg cggccgcctg gtgcagtacc gcggcgaggt
gcaggccatg ctcggccaga gcaccgagga gctgcgggtg cgcctcgcct cccacctgcg
caagctgcgt aagcggctcc tccgcgatcc cgatgacctg cagaagcgcc tggcagtgta
ccaggccggg gcccgcgagg gcgccgagcg cggcctcagc gccatccgcg agcgcctggg
gcccctggtg gaacagggcc gcgtgcgggc cgccactgtg ggctccctgg ccggccagcc
gctacaggag cgggcccagg cctggggcga gcggctgcgc gcgcggatgg aggagatggg
cagtcggacc cgcgaccgcc tggacgaggt gaaggagcag gtggcggagg tgcgcgccaa
gctggaggag caggcccagc agatacgcct gcaggccgag gccttccagg cccgcctcaa
gagctggttc gagccccgtg tggaagacat gcagcgccag tgggccgggc tggtggagaa
ggtgcaggct gccgtgggca ccagcgccgc ccctgtgccc agcgacaatc actgaacgcc
gaagcctgca gccatgcgac cccacgccac cccgtgcctc ctgccgccgc gcagcctgca
gcgggagacc ctgtccccgc cccagccgtc ctcctggggt ggaccctagt ttaataaaga
ttcaccaagt ttcacgc (SEQ ID NO:12),

as well as sequences with at least 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95% or more, e.g., 99% or 100%, sequence identity thereto that encode an APOE.

Compositions and Methods

Alzheimer's disease (AD) directly affects 5 million Americans and is rapidly increasing in prevalence and economic impact. Existing drugs have little impact on the underlying disease process and no preventive therapies are currently available. Inheritance of the variant APOE4 gene conveys a high risk for the development of AD, while inheritance of the APOE2 gene is protective, reducing the risk of developing AD by about 50% and delaying the age of onset. APOE4 is associated with increased brain amyloid load and greater memory impairment in AD. Conversely, APOE2 attenuates these effects. In humans, the odds ratio of developing AD with E4/E4 homozygous genotype is 14.9 and is reduced to 2.6 in E2/E4 heterozygotes. APOE4 may be associated with abnormal brain function apart from its role in promoting amyloid production.

Apolipoprotein E (APOE) is a 299 amino acid protein that functions as a carrier of lipids (Phillips, 2014). In the brain, APOE is expressed by astrocytes and glial cells and acts as the major transporter of cholesterol and other lipids to neurons (Rebeck, 2014). Common variants in the APOE gene (APOE3 APOE4 and APOE2) influence the risk the development of Alzheimer's disease (AD) (Wolters et al., 2019; Chen et al., 2021). Compared to homozygotes for the common APOE3 allele, APOE4 homozygotes have a markedly higher risk of developing AD while APOE2 homozygotes have a lower risk (Wolters et al., 2019; Chen et al., 2021). Based on the observations that almost all APOE4 homozygotes exhibit AD pathology, higher AD biomarkers, earlier symptoms and markedly increased lifetime risk for AD dementia compared to APOE3 homozygotes, it has been proposed that APOE4 homozygosity represents a distinct genetic form of AD (Fortea et al., 2024). There is considerable evidence that APOE4 is a toxic gain-of-function associated with a variety of abnormal biological processes related to the pathogenesis of AD, including dysfunction of CNS cholesterol metabolism (Jeong et al., 2019), synaptic defects (Lane-Donovan & Herz, 2017), mitochondrial dysfunction (Pires & Rego, 2023), leaky blood-brain barrier (Jackson et al., 2022), neuroinflammation (Arnaud et al., 2022), increased amyloid β deposition and decreased amyloid β clearance11 and increased tau pathology including increased neurofilament accumulation (Farfel et al., 2016).

Based on this biological and epidemiologic evidence that APOE4 is toxic to the CNS, it has been proposed that lowering CNS APOE4 levels of APOE4 homozygotes could be therapeutic strategy to prevent/treat APOE4-related AD (Li et al., 2022). Consistent with this concept, studies in humanized APOE4 mice have shown that genetic removal of APOE4 reduces neurodegeneration and tau pathology (Rao et al., 2023) and epidemiologic studies demonstrate that APOE4/null individuals have a reduced risk of AD compared to APOE4 homozygotes (Chemparathy et al., 2023).

The present disclosure provides for a gene therapy vector for expression of APOE2,

sequences to inhibit APOE4 expression, and methods of using the APOE2 and APOE4 inhibitory sequences.

The present disclosure provides for a gene therapy vector for expression of APOE2, sequences to inhibit APOE4 expression, and methods of using the APOE2 and APOE4 inhibitory sequences. The disclosure provides a gene therapy vector comprising an RNAi sequence that can comprise, consist essentially of, or consist of a nucleic acid sequence at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% (or any percentage in between) identical to SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, or SEQ ID NO: 41, or the complement thereof.

Exemplary APOE4 target sequences:

Name	APOE target sequence
2A	Ggagttgaaggcctacaaatc (SEQ ID NO: 33)
N1	Aatcacaggcaggaagatgaa (SEQ ID NO: 34)
N2	Ctggccaatcacaggcaggaa (SEQ ID NO: 35)
N3	Agtttaataaagattcaccaa (SEQ ID NO: 36)
N4	Ataaagattcaccaagtttca (SEQ ID NO: 37)
K3	Gcccagcgacaatcactgaacg (SEQ ID NO: 38)
K7	Acaaatcggaactggaggaaca (SEQ ID NO: 39)
K8	gcagacactgtctgagcaggtg (SEQ ID NO: 40)
K13	Aggcccagcagatacgcctgca (SEQ ID NO: 41)

In some embodiments, the RNAi sequence comprises SEQ ID NO: 33, or the complement thereof. In some embodiments, the RNAi sequence comprises SEQ ID NO: 34, or the complement thereof. In some embodiments, the RNAi sequence comprises SEQ ID NO: 35, or the complement thereof. In some embodiments, the RNAi sequence comprises SEQ ID NO: 36, or the complement thereof. In some embodiments, the RNA sequence comprises SEQ ID NO: 37, or the complement thereof. In some embodiments, the RNAi sequence comprises SEQ ID NO: 38, or the complement thereof. In some embodiments, the RNAi sequence comprises SEQ ID NO: 39, or the complement thereof. In some embodiments, the RNAi sequence comprises SEQ ID NO: 40, or the complement thereof. In some embodiments, the RNAi sequence comprises SEQ ID NO: 41, or the complement thereof. In some embodiments, the RNAi sequence comprises SEQ ID NO: 33, or the complement thereof.

The disclosure provides a gene therapy vector comprising one or more RNAi sequences

that bind a target APOE4 sequence that can comprise, consist essentially of, or consist of a nucleic acid sequence at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% (or any percentage in between) identical to SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, or SEQ ID NO: 41, or the complement thereof.

The disclosure provides a gene therapy vector comprising one or more miRNA sequences that bind a target APOE4 sequence that can comprise, consist essentially of, or consist of a nucleic acid sequence at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% (or any percentage in between) identical to SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, or SEQ ID NO: 41, or the complement thereof. In some embodiments, a gene therapy vector comprises two miRNA sequences that can comprise, consist essentially of, or consist of a nucleic acid sequence at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% (or any percentage in between) identical to SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, or SEQ ID NO: 41, or the complement thereof. In some embodiments, a gene therapy vector comprises three miRNA sequences that can comprise, consist essentially of, or consist of a nucleic acid sequence at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% (or any percentage in between) identical to SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, or SEQ ID NO: 41, or the complement thereof. In some embodiments, a gene therapy vector comprises four miRNA sequences that can comprise, consist essentially of, or consist of a nucleic acid sequence at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% (or any percentage in between) identical to SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, or SEQ ID NO: 41, or the complement thereof.

Exemplary microRNAs are SEQ ID NO:23, SEQ ID NO:24, or SEQ ID NO:25.

Exemplary shRNAs are SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:20, SEQ ID NO:21 and SEQ ID NO:22.

Exemplary vector, e.g., AAV-CAG-APOE2-SR-2XMIR2A(I)-2XMIRN4(PA) (4087 BP), has the following sequence:

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CTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGCCCGGCCTCAGTGAG
CGAGCGAGCGCGCAGAGAGGGAGTGGCCAACTCCATCACTAGGGGTTCCTGCGGCCGCTCTAGAAACTAGTTATTAATAG
TAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGG
CTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATT
GACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCT
ATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTA
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CATCTACGTATTAGTCATCGCTATTACCATGGTCGAGGTGAGCCCCACGTTCTGCTCACTCTCCCCATCTCCCCCCCCT
CCCCACCCCCAATTTTGTATTTATTTATTTTTTAATTATTTTGTGCAGCGATGGGGGCGGGGGGGGGGGGGGGCGCGCG
CCAGGCGGGGCGGGGCGGGGCGAGGGGCGGGGCGGGGCGAGGCGGAGAGGTGCGGCGGCAGCCAATCAGAGCGGCGCT
CCGAAAGTTTCCTTTTATGGCGAGGCGGCGGCGGCGGCGCCCTATAAAAAGCGAAGCGCGCGGCGGGCGGGAGTCGCTG
CGCGCTGCCTTCGCCCCGTGCCCCGCTCCGCCGCCGCCTCGCGCCGCCCGCCCCGGCTCTGACTGACCGCGTTACTCCCA
CAGGTGAGCGGGCGGGACGGCCCTTCTCCTCCGGGCTGTAATTAGCGCTTGGTTTAATGACGGCTTGTTTCTTTTCTGTG
GCTGCGCGAAAGCCTTGAGGGGCTCCGGGAGGGCCCTTTGTGCGGGGGGAGCGGCTCGGGGGGTGCGTGCGTGTGTGTGT
GCGTGGGGAGCGCCGCGTGCGGCTCCGCGCTGCCCGGCGGCTGTGAGCGCTGCGGGCGCGGCGCGGGGCTTTGTGCGCTC
CGCAGTGTGCGCGAGGGGAGCGCGGCCGGGGGCGGTGCCCCGCGGTGCGGGGGGGGCTGCGAGGGGAACAAAGGCTGCGT
GCGGGGCGTGTGCGTGGGGGGGTGAGCAGGGGGTGTGGGCGCGTCGGTCGGGCTGCAACCCCCCCTGCACCCCCCTCCCC
GAGTTGCTGAGCACGGCCCGGCTTCGGGTGCGGGGCTCCGTACGGGGCGTGGCGCGGGGCTCGCCGTGCCGGGCGGGGGG
TGGCGGCAGGTGGGGGTGCCGGGCGGGGCGGGGCCGCCTCGGGCCGGGGAGGGCTCGGGGGAGGGGCGCGGCGGCCCCCG
GAGCGCCGGCGGCTCACCTAGGCCTGGAGGCTTGCTTTGGGCTGTATGCTGATTTGTAGGCCTTCAACTCCTGTTTTGGC
CACTGACTGACAGGAGTTGAAGTCACAAATCAGGACACAAGGCCCTTTATCAGCACTCACATGGAACAAATGGCCACCGT
GGGAGGATGACAAACGCGTCCTGGAGGCTTGCTTTGGGCTGTATGCTGATTTGTAGGCCTTCAACTCCTGTTTTGGCCAC
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CCTGTTACTAGCACTCACATGGAACAAATGGCCACCGTGGGAGGATGACCTCGAGAATTAATTAAGAATTCACTCCTCAG
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CTCGCTCGCTCACTGAGGCCGGGCGACCAAAGGTCGCCCGACGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGCAGCTG
CCTGCAG (SEQ ID NO: 80)

Exemplary Gene Therapy Vectors

The disclosure provides a gene therapy vector comprising a nucleic acid sequence which encodes APOE2 and may include inhibitory sequences of endogenous APOE4 expression, or in one embodiment may include another vector for expression of the inhibitory sequences or a composition having the inhibitory RNA sequences. Various aspects of the gene therapy vector(s) and method are discussed below. Although each parameter is discussed separately, the gene therapy vector and method comprise combinations of the parameters set forth below, e.g., to evoke protection against an APOE4 associated pathology. Accordingly, any combination of parameters can be used according to the gene therapy vector and the method.

A "gene therapy vector" is thus any molecule or composition that has the ability to carry a heterologous nucleic acid sequence into a suitable host cell where synthesis of the encoded

protein takes place. Typically, a gene therapy vector is a nucleic acid molecule that has been engineered, using recombinant DNA techniques that are known in the art, to incorporate the heterologous nucleic acid sequence, e.g., heterologous with respect to the other vector sequences such as the promoter or vector backbone sequences such as viral sequences. Desirably, the gene therapy vector is comprised of DNA. Examples of suitable DNA-based gene therapy vectors include plasmids and viral vectors. However, gene therapy vectors that are not based solely on nucleic acids, such as liposomes or nanoparticles, may also be employed. The gene therapy vector can be based on a single type of nucleic acid (e.g., a plasmid) or include non-nucleic acid molecules (e.g., a lipid or a polymer). The gene therapy vector can be integrated into the host cell genome, or can be present in the host cell in the form of an episome.

Gene or siRNA delivery vectors within the scope of the disclosure include, but are not limited to, isolated nucleic acid, e.g., plasmid-based vectors which may be extrachromosomally maintained, and viral vectors, e.g., recombinant adenovirus, retrovirus, lentivirus, herpesvirus, poxvirus, papilloma virus, or adeno-associated virus, including viral and non-viral vectors which are present in liposomes, e.g., neutral or cationic liposomes, such as DOSPA/DOPE, DOGS/DOPE or DMRIE/DOPE liposomes, and/or associated with other molecules such as DNA-anti-DNA antibody-cationic lipid (DOTMA/DOPE) complexes or natural or synthetic polymers. Exemplary viral gene delivery vectors are described below. Gene delivery vectors may be administered via any route including, but not limited to, intracranial, intrathecal, intramuscular, buccal, rectal, intravenous or intracoronary administration, and transfer to cells may be enhanced using electroporation and/or iontophoresis, and/or scaffolding such as extracellular matrix or hydrogels, e.g., a hydrogel patch.

In one embodiment, the gene therapy vector or the other vector is a viral vector. Suitable viral vectors include, for example, retroviral vectors, lentivirus vectors, herpes simplex virus (HSV)-based vectors, parvovirus-based vectors, e.g., adeno-associated virus (AAV)-based vectors, AAV-adenoviral chimeric vectors, and adenovirus-based vectors. These viral vectors can be prepared using standard recombinant DNA techniques described in, for example, Sambrook et al., *Molecular Cloning, a Laboratory Manual*, 3rd edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (2001), and Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing Associates and John Wiley & Sons, New York, N.Y. (1994).

Retroviral vectors

Retroviral vectors exhibit several distinctive features including their ability to stably and precisely integrate into the host genome providing long-term transgene expression. These vectors can be manipulated *ex vivo* to eliminate infectious gene particles to minimize the risk of systemic infection and patient-to-patient transmission. Pseudotyped retroviral vectors can alter

host cell tropism.

Lentiviruses

Lentiviruses are derived from a family of retroviruses that include human immunodeficiency virus and feline immunodeficiency virus. However, unlike retroviruses that only infect dividing cells, lentiviruses can infect both dividing and nondividing cells. Although lentiviruses have specific tropisms, pseudotyping the viral envelope with vesicular stomatitis virus yields virus with a broader range (Schnepp et al., Meth. Mol. Med., 69:427 (2002)).

Adenoviral vectors

Adenoviral vectors may be rendered replication-incompetent by deleting the early (E1A and E1B) genes responsible for viral gene expression from the genome and are stably maintained into the host cells in an extrachromosomal form. These vectors have the ability to transfect both replicating and nonreplicating cells. Adenoviral vectors have been shown to result in transient expression of therapeutic genes *in vivo*, peaking at 7 days and lasting approximately 4 weeks. In addition, adenoviral vectors can be produced at very high titers, allowing efficient gene therapy with small volumes of virus.

Adeno-associated virus vectors

Recombinant adeno-associated viruses (rAAV) are derived from nonpathogenic parvoviruses, evoke essentially no cellular immune response, and produce transgene expression lasting months in most systems. Moreover, like adenovirus, adeno-associated virus vectors also have the capability to infect replicating and nonreplicating cells.

AAV vectors include but are not limited to AAV1, AAV2, AAV5, AAV7, AAV8, AAV9 or AAVrh10, including chimeric viruses where the AAV genome is from a different source than the capsid.

Plasmid DNA vectors

Plasmid DNA is often referred to as "naked DNA" to indicate the absence of a more elaborate packaging system. Direct injection of plasmid DNA to myocardial cells *in vivo* has been accomplished. Plasmid-based vectors are relatively nonimmunogenic and nonpathogenic, with the potential to stably integrate in the cellular genome, resulting in long-term gene expression in postmitotic cells *in vivo*. Furthermore, plasmid DNA is rapidly degraded in the blood stream; therefore, the chance of transgene expression in distant organ systems is negligible. Plasmid DNA may be delivered to cells as part of a macromolecular complex, e.g., a liposome or DNA-protein complex, and delivery may be enhanced using techniques including electroporation.

Exemplary AAV Vectors

In an embodiment, the disclosure provides an adeno-associated virus (AAV) vector which comprises, consists essentially of, or consists of a nucleic acid sequence encoding APOE2. When the AAV vector consists essentially of a nucleic acid sequence encoding APOE2, additional components can be included that do not materially affect the AAV vector (e.g., genetic elements such as poly(A) sequences or restriction enzyme sites that facilitate manipulation of the vector *in vitro*). When the AAV vector consists of a nucleic acid sequence which encodes APOE2, the AAV vector does not comprise any additional components (i.e., components that are not endogenous to AAV and are not required to effect expression of the nucleic acid sequence).

Adeno-associated virus is a member of the Parvoviridae family and comprises a linear, single-stranded DNA genome of less than about 5,000 nucleotides. AAV requires co-infection with a helper virus (i.e., an adenovirus or a herpes virus), or expression of helper genes, for efficient replication. AAV vectors used for administration of therapeutic nucleic acids typically have approximately 96% of the parental genome deleted, such that only the terminal repeats (ITRs), which contain recognition signals for DNA replication and packaging, remain. This eliminates immunologic or toxic side effects due to expression of viral genes. In addition, delivering specific AAV proteins to producing cells enables integration of the AAV vector comprising AAV ITRs into a specific region of the cellular genome, if desired (see, e.g., U.S. Patents 6,342,390 and 6,821,511). Host cells comprising an integrated AAV genome show no change in cell growth or morphology (see, for example, U.S. Patent 4,797,368).

The AAV ITRs flank the unique coding nucleotide sequences for the non-structural replication (Rep) proteins and the structural capsid (Cap) proteins (also known as virion proteins (VPs)). The terminal 145 nucleotides are self-complementary and are organized so that an energetically intramolecular duplex forming a T-shaped hairpin may be formed. These hairpin structures function as an origin for viral DNA replication by serving as primers for the cellular DNA polymerase complex. The Rep genes encode the Rep proteins Rep78, Rep68, Rep52, and Rep40. Rep78 and Rep68 are transcribed from the p5 promoter, and Rep 52 and Rep40 are transcribed from the p19 promoter. The Rep78 and Rep68 proteins are multifunctional DNA binding proteins that perform helicase and nickase functions during productive replication to allow for the resolution of AAV termini (see, e.g., Im et al., Cell, 61:447 (1990)). These proteins also regulate transcription from endogenous AAV promoters and promoters within helper viruses (see, e.g., Pereira et al., J. Virol., 71:1079 (1997)). The other Rep proteins modify the function of Rep78 and Rep68. The cap genes encode the capsid proteins VP1, VP2, and VP3. The cap genes are transcribed from the p40 promoter.

The AAV vector may be generated using any AAV serotype known in the art. Several AAV serotypes and over 100 AAV variants have been isolated from adenovirus stocks or from

human or nonhuman primate tissues (reviewed in, e.g., Wu et al., Molecular Therapy, 14(3): 316 (2006)). Generally, the AAV serotypes have genomic sequences of significant homology at the nucleic acid sequence and amino acid sequence levels, such that different serotypes have an identical set of genetic functions, produce virions which are essentially physically and functionally equivalent, and replicate and assemble by practically identical mechanisms. AAV serotypes 1-5 and 7-9 are defined as "true" serotypes, in that they do not efficiently cross-react with neutralizing sera specific for all other existing and characterized serotypes. In contrast, AAV serotypes 6, 10 (also referred to as Rh10), and 11 are considered "variant" serotypes as they do not adhere to the definition of a "true" serotype. AAV serotype 2 (AAV2) has been used extensively for gene therapy applications due to its lack of pathogenicity, wide range of infectivity, and ability to establish long-term transgene expression (see, e.g., Carter, Hum. Gene Ther., 16:541 (2005); and Wu et al., *supra*). Genome sequences of various AAV serotypes and comparisons thereof are disclosed in, for example, GenBank Accession numbers U89790, J01901, AF043303, and AF085716; Chiorini et al., J. Virol., 71:6823 (1997); Srivastava et al., J. Virol., 45:555 (1983); Chiorini et al., J. Virol., 73:1309 (1999); Rutledge et al., J. Virol., 72:309 (1998); and Wu et al., J. Virol., 74:8635 (2000)).

AAV rep and ITR sequences are particularly conserved across most AAV serotypes. For example, the Rep78 proteins of AAV2, AAV3A, AAV3B, AAV4, and AAV6 are reportedly about 89-93% identical (see Bantel-Schaal et al., J. Virol., 73(2):939 (1999)). It has been reported that AAV serotypes 2, 3A, 3B, and 6 share about 82% total nucleotide sequence identity at the genome level (Bantel-Schaal et al., *supra*). Moreover, the rep sequences and ITRs of many AAV serotypes are known to efficiently cross-complement (e.g., functionally substitute) corresponding sequences from other serotypes during production of AAV particles in mammalian cells.

Generally, the cap proteins, which determine the cellular tropicity of the AAV particle, and related cap protein-encoding sequences, are significantly less conserved than Rep genes across different AAV serotypes. In view of the ability Rep and ITR sequences to cross-complement corresponding sequences of other serotypes, the AAV vector can comprise a mixture of serotypes and thereby be a "chimeric" or "pseudotyped" AAV vector. A chimeric AAV vector typically comprises AAV capsid proteins derived from two or more (e.g., 2, 3, 4, etc.) different AAV serotypes. In contrast, a pseudotyped AAV vector comprises one or more ITRs of one AAV serotype packaged into a capsid of another AAV serotype. Chimeric and pseudotyped AAV vectors are further described in, for example, U.S. Patent No. 6,723,551; Flotte, Mol. Ther., 13(1):1 (2006); Gao et al., J. Virol., 78:6381 (2004); Gao et al., Proc. Natl.

Acad. Sci. USA, 99:11854 (2002); De et al., Mol. Ther., 13:67 (2006); and Gao et al., Mol. Ther., 13:77 (2006).

In one embodiment, the AAV vector is generated using an AAV that infects humans (e.g., AAV2). Alternatively, the AAV vector is generated using an AAV that infects non-human primates, such as, for example, the great apes (e.g., chimpanzees), Old World monkeys (e.g., macaques), and New World monkeys (e.g., marmosets). In one embodiment, the AAV vector is generated using an AAV that infects a non-human primate pseudotyped with an AAV that infects humans. Examples of such pseudotyped AAV vectors are disclosed in, e.g., Cearley et al., Molecular Therapy, 13:528 (2006). In one embodiment, an AAV vector can be generated which comprises a capsid protein from an AAV that infects rhesus macaques pseudotyped with AAV2 inverted terminal repeats (ITRs). In a particular embodiment, the AAV vector comprises a capsid protein from AAV10 (also referred to as "AAVrh.10"), which infects rhesus macaques pseudotyped with AAV2 ITRs (see, e.g., Watanabe et al., Gene Ther., 17(8):1042 (2010); and Mao et al., Hum. Gene Therapy, 22:1525 (2011)).

In addition to the nucleic acid sequence encoding APOE2, the AAV vector may comprise expression control sequences, such as promoters, enhancers, polyadenylation signals, transcription terminators, internal ribosome entry sites (IRES), and the like, that provide for the expression of the nucleic acid sequence in a host cell, as well as, in one embodiment, sequences for APOE4 RNAi. Exemplary expression control sequences are known in the art and described in, for example, Goeddel, Gene Expression Technology: Methods in Enzymology, Vol. 185, Academic Press, San Diego, CA. (1990).

A large number of promoters, including constitutive, inducible, and repressible promoters, from a variety of different sources are well known in the art. Representative sources of promoters include for example, virus, mammal, insect, plant, yeast, and bacteria, and suitable promoters from these sources are readily available, or can be made synthetically, based on sequences publicly available, for example, from depositories such as the ATCC as well as other commercial or individual sources. Promoters can be unidirectional (i.e., initiate transcription in one direction) or bi-directional (i.e., initiate transcription in either a 3' or 5' direction). Non-limiting examples of promoters include, for example, the T7 bacterial expression system, pBAD (araA) bacterial expression system, the cytomegalovirus (CMV) promoter, the SV40 promoter, and the RSV promoter. Inducible promoters include, for example, the Tet system (U.S. Patent Nos. 5,464,758 and 5,814,618), the Ecdysone inducible system (No et al., Proc. Natl. Acad. Sci., 93:3346 (1996)), the T-REXTM system (Invitrogen, Carlsbad, CA), LACSWITCH™ System (Stratagene, San Diego, CA), and the Cre-ERT tamoxifen inducible recombinase system (Indra et

al., Nuc. Acid. Res., 27:4324 (1999); Nuc. Acid. Res., 28:e99 (2000); U.S. Patent No. 7,112,715; and Kramer & Fussenegger, Methods Mol. Biol., 308:123 (2005)).

The term "enhancer" as used herein, refers to a DNA sequence that increases transcription of, for example, a nucleic acid sequence to which it is operably linked. Enhancers can be located many kilobases away from the coding region of the nucleic acid sequence and can mediate the binding of regulatory factors, patterns of DNA methylation, or changes in DNA structure. A large number of enhancers from a variety of different sources are well known in the art and are available as or within cloned polynucleotides (from, e.g., depositories such as the ATCC as well as other commercial or individual sources). A number of polynucleotides comprising promoters (such as the commonly-used CMV promoter) also comprise enhancer sequences. Enhancers can be located upstream, within, or downstream of coding sequences. In one embodiment, the nucleic acid sequence encoding APOE2, is operably linked to a CMV enhancer/chicken beta-actin promoter (also referred to as a "CAG promoter") (see, e.g., Niwa et al., Gene, 108:193 (1991); Daly et al., Proc. Natl. Acad. Sci. U.S.A., 96:2296 (1999); and Sondhi et al., Mol. Ther., 15:481 (2007)).

Typically AAV vectors are produced using well characterized plasmids. For example, human embryonic kidney 293T cells are transfected with one of the transgene specific plasmids and another plasmid containing the adenovirus helper and AAV rep and cap genes (specific to AAVrh.10, 8 or 9 as required). After 72 hours, the cells are harvested and the vector is released from the cells by five freeze/thaw cycles. Subsequent centrifugation and benzonase treatment removes cellular debris and unencapsidated DNA. Iodixanol gradients and ion exchange columns may be used to further purify each AAV vector. Next, the purified vector is concentrated by a size exclusion centrifuge spin column to the required concentration. Finally, the buffer is exchanged to create the final vector products formulated (for example) in 1x phosphate buffered saline. The viral titers may be measured by TaqMan® real-time PCR and the viral purity may be assessed by SDS-PAGE.

Pharmaceutical Compositions and Delivery of the Vectors

The disclosure provides a composition comprising, consisting essentially of, or consisting of the above-described gene therapy vector and a pharmaceutically acceptable (e.g., physiologically acceptable) carrier, or a vector for expression of RNAi. When the composition consists essentially of the gene therapy vector and a pharmaceutically acceptable carrier, additional components can be included that do not materially affect the composition (e.g., adjuvants, buffers, stabilizers, anti-inflammatory agents, solubilizers, preservatives, etc.). When the composition consists of the gene therapy vector and the pharmaceutically acceptable carrier, the composition does not comprise any additional components. Any suitable carrier can be used

within the context of the disclosure, and such carriers are well known in the art. The choice of carrier will be determined, in part, by the particular site to which the composition may be administered and the particular method used to administer the composition. The composition optionally can be sterile with the exception of the gene therapy vector described herein. The composition can be frozen or lyophilized for storage and reconstituted in a suitable sterile carrier prior to use. The compositions can be generated in accordance with conventional techniques described in, e.g., *Remington: The Science and Practice of Pharmacy, 21st Edition*, Lippincott Williams & Wilkins, Philadelphia, PA (2001).

Suitable formulations for the composition include aqueous and non-aqueous solutions, isotonic sterile solutions, which can contain anti-oxidants, buffers, and bacteriostats, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, immediately prior to use. Extemporaneous solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. In one embodiment, the carrier is a buffered saline solution. In one embodiment, the gene therapy vector is administered in a composition formulated to protect the gene therapy vector from damage prior to administration. For example, the composition can be formulated to reduce loss of the gene therapy vector on devices used to prepare, store, or administer the gene therapy vector, such as glassware, syringes, or needles. The composition can be formulated to decrease the light sensitivity and/or temperature sensitivity of the gene therapy vector. To this end, the composition may comprise a pharmaceutically acceptable liquid carrier, such as, for example, those described above, and a stabilizing agent selected from the group consisting of polysorbate 80, L-arginine, polyvinylpyrrolidone, trehalose, and combinations thereof. Use of such a composition will extend the shelf life of the gene therapy vector, facilitate administration, and increase the efficiency of the method. Formulations for gene therapy vector-containing compositions are further described in, for example, Wright et al., *Curr. Opin. Drug Discov. Devel.*, *6*(2): 174-178 (2003) and Wright et al., *Molecular Therapy*, *12*: 171-178 (2005))

The composition also can be formulated to enhance transduction efficiency. In addition, one of ordinary skill in the art will appreciate that the gene therapy vector can be present in a composition with other therapeutic or biologically-active agents. For example, factors that control inflammation, such as ibuprofen or steroids, can be part of the composition to reduce swelling and inflammation associated with *in vivo* administration of the gene therapy vector. Immune system stimulators or adjuvants, e.g., interleukins, lipopolysaccharide, and double-

stranded RNA, can be administered to enhance or modify an immune response. Antibiotics, i.e., microbicides and fungicides, can be present to treat existing infection and/or reduce the risk of future infection, such as infection associated with gene therapy procedures.

Injectable depot forms are made by forming microencapsule matrices of the subject compounds in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissue.

In certain embodiments, a formulation comprises a biocompatible polymer selected from the group consisting of polyamides, polycarbonates, polyalkylenes, polymers of acrylic and methacrylic esters, polyvinyl polymers, polyglycolides, polysiloxanes, polyurethanes and co-polymers thereof, celluloses, polypropylene, polyethylenes, polystyrene, polymers of lactic acid and glycolic acid, polyanhydrides, poly(ortho)esters, poly(butyric acid), poly(valeric acid), poly(lactide-co-caprolactone), polysaccharides, proteins, polyhyaluronic acids, polycyanoacrylates, and blends, mixtures, or copolymers thereof.

The composition can be administered in or on a device that allows controlled or sustained release, such as a sponge, biocompatible meshwork, mechanical reservoir, or mechanical implant. Implants (see, e.g., U.S. Patent No. 5,443,505), devices (see, e.g., U.S. Patent No. 4,863,457), such as an implantable device, e.g., a mechanical reservoir or an implant or a device comprised of a polymeric composition, are particularly useful for administration of the gene therapy vector. The composition also can be administered in the form of sustained-release formulations (see, e.g., U.S. Patent No. 5,378,475) comprising, for example, gel foam, hyaluronic acid, gelatin, chondroitin sulfate, a polyphosphoester, such as bis-2-hydroxyethyl-terephthalate (BHET), and/or a polylactic-glycolic acid.

Delivery of the compositions comprising the gene therapy vectors may be intracerebral (including but not limited to intraparenchymal, intraventricular, or intracisternal), intrathecal (including but not limited to lumbar or cisterna magna), or systemic, including but not limited to intravenous, or any combination thereof, using devices known in the art. Delivery may also be via surgical implantation of an implanted device.

The dose of the gene therapy vector in the composition administered to the mammal will depend on a number of factors, including the size (mass) of the mammal, the extent of any side-effects, the particular route of administration, and the like. In one embodiment, the method comprises administering a "therapeutically effective amount" of the composition comprising the gene therapy vector described herein. A "therapeutically effective amount" refers to an amount

effective, at dosages and for periods of time necessary, to achieve a desired therapeutic result. The therapeutically effective amount may vary according to factors such as the extent of pathology, age, sex, and weight of the individual, and the ability of the gene therapy vector to elicit a desired response in the individual. The dose of gene therapy vector in the composition required to achieve a particular therapeutic effect typically is administered in units of vector genome copies per cell (gc/cell) or vector genome copies/per kilogram of body weight (gc/kg). One of ordinary skill in the art can readily determine an appropriate gene therapy vector dose range to treat a patient having a particular disease or disorder, based on these and other factors that are well known in the art. The therapeutically effective amount may be between 1×10^{10} genome copies to 1×10^{13} genome copies. The therapeutically effective amount may be between 1×10^{11} genome copies to 1×10^{14} genome copies. The therapeutically effective amount may be between 1×10^{12} genome copies to 1×10^{15} genome copies. The therapeutically effective amount may be from 1×10^{13} genome copies (gc) to 1×10^{16} gc, e.g., from 1×10^{13} gc to 1×10^{14} gc, 1×10^{14} gc to 1×10^{15} gc, or 1×10^{15} gc to 1×10^{14} gc. Assuming a 70 kg human, the dose ranges may be from 1.4×10^{8} gc/kg to 1.4×10^{11} gc/kg, 1.4×10^{9} gc/kg to 1.4×10^{12} gc/kg, 1.4×10^{10} gc/kg to 1.4×10^{13} gc/kg, or 1.4×10^{11} gc/kg to 1.4×10^{14} gc/kg.

In one embodiment, the composition is administered once to the mammal. It is believed that a single administration of the composition will result in expression of APOE2, and suppression of APOE4 expression, in the mammal with minimal side effects. However, in certain cases, it may be appropriate to administer the composition multiple times during a therapeutic period to ensure sufficient exposure of cells to the composition. For example, the composition may be administered to the mammal two or more times (e.g., 2, 3, 4, 5, 6, 6, 8, 9, or 10 or more times) during a therapeutic period.

The present disclosure thus provides pharmaceutically acceptable compositions which comprise a therapeutically-effective amount of gene therapy vector comprising a nucleic acid sequence which encodes an APOE2 and a sequence which inhibits APOE4 expression.

Subjects

The subject may be any animal, including a human and non-human animal. Non-human animals include all vertebrates, e.g., mammals and non-mammals, such as non-human primates, sheep, dogs, cats, cows, horses, chickens, amphibians, and reptiles, although mammals are envisioned as subjects, such as non-human primates, sheep, dogs, cats, cows and horses. The subject may also be livestock such as, cattle, swine, sheep, poultry, and horses, or pets, such as dogs and cats.

In one embodiment, subjects include human subjects suffering from or at risk for the medical diseases and disorders described herein. The subject is generally diagnosed with the condition by skilled artisans, such as a medical practitioner.

The methods described herein can be employed for subjects of any species, gender, age, ethnic population, or genotype. Accordingly, the term subject includes males and females, and it includes elderly, elderly-to-adult transition age subject adults, adult-to-pre-adult transition age subjects, and pre-adults, including adolescents, children, and infants.

Examples of human ethnic populations include Caucasians, Asians, Hispanics, Africans, African Americans, Native Americans, Semites, and Pacific Islanders. The methods may be more appropriate for some ethnic populations such as Caucasians, especially northern European populations, as well as Asian populations.

The term subject also includes subjects of any genotype or phenotype as long as they are in need of treatment, as described above. In addition, the subject can have the genotype or phenotype for any hair color, eye color, skin color or any combination thereof. The term subject includes a subject of any body height, body weight, or any organ or body part size or shape.

Exemplary Nanoparticle Formulations

Biodegradable nanoparticles, e.g., comprising the gene therapy vector or isolated nucleic acid or a vector for RNAi expression, may include or may be formed from biodegradable polymeric molecules which may include, but are not limited to polylactic acid (PLA), polyglycolic acid (PGA), co-polymers of PLA and PGA (i.e., polyactic-co-glycolic acid (PLGA)), poly-ε-caprolactone (PCL), polyethylene glycol (PEG), poly(3-hydroxybutyrate), poly(p-dioxanone), polypropylene fumarate, poly(orthoesters), polyol/diketene acetals addition polymers, poly-alkyl-cyano-acrylates (PAC), poly(sebacic anhydride) (PSA), poly(carboxybiscarboxyphenoxyphenoxy hexone (PCPP) poly[bis (p-carboxypheonoxy)methane](PCPM), copolymers of PSA, PCPP and PCPM, poly(amino acids), poly(pseudo amino acids), polyphosphazenes, derivatives of poly[(dichloro)phosphazenes] and poly[(organo)phosphazenes], poly-hydroxybutyric acid, or S-caproic acid, elastin, or gelatin. (See, e.g., Kumari et al., Colloids and Surfaces B: Biointerfaces 75 (2010) 1-18; and U.S. Pat. Nos. 6,913,767; 6,884,435; 6,565,777; 6,534,092; 6,528,087; 6,379,704; 6,309,569; 6,264,987; 6,210,707; 6,090,925; 6,022,564; 5,981,719; 5,871,747; 5,723,269; 5,603,960; and 5,578,709; and U.S. Published Application No. 2007/0081972; and International Application Publication Nos. WO 2012/115806; and WO 2012/054425; the contents of which are incorporated herein by reference in their entireties).

The biodegradable nanoparticles may be prepared by methods known in the art. (See, e.g., Nagavarma et al., Asian J. of Pharma. And Clin. Res., Vol 5, Suppl 3, 2012, pages 16-23;

Cismaru et al., Rev. Roum. Chim., 2010, 55(8), 433-442; and International Application Publication Nos. WO 2012/115806; and WO 2012/054425; the contents of which are incorporated herein by reference in their entireties). Suitable methods for preparing the nanoparticles may include methods that utilize a dispersion of a preformed polymer, which may include but are not limited to solvent evaporation, nanoprecipitation, emulsification/solvent diffusion, salting out, dialysis, and supercritical fluid technology. In some embodiments, the nanoparticles may be prepared by forming a double emulsion (e.g., water-in-oil-in-water) and subsequently performing solvent-evaporation. The nanoparticles obtained by the disclosed methods may be subjected to further processing steps such as washing and lyophilization, as desired. Optionally, the nanoparticles may be combined with a preservative (e.g., trehalose).

Typically, the nanoparticles have a mean effective diameter of less than 1 micron, e.g., the nanoparticles have a mean effective diameter of between about 25 nm and about 500 nm, e.g., between about 50 nm and about 250 nm, about 100 nm to about 150 nm, or about 450 nm to 650 nm. The size of the particles (e.g., mean effective diameter) may be assessed by known methods in the art. which may include but are not limited to transmission electron microscopy (TEM), scanning electron microscopy (SEM), Atomic Force Microscopy (AFM), Photon Correlation Spectroscopy (PCS), Nanoparticle Surface Area Monitor (NSAM), Condensation Particle Counter (CPC), Differential Mobility Analyzer (DMA), Scanning Mobility Particle Sizer (SMPS), Nanoparticle Tracking Analysis (NTA), X-Ray Diffraction (XRD), Aerosol Time of Flight Mass Spectroscopy (ATFMS), and Aerosol Particle Mass Analyzer (APM).
The biodegradable nanoparticles may have a zeta-potential that facilitates uptake by a target cell. Typically, the nanoparticles have a zeta-potential greater than 0. In some embodiments, the nanoparticles have a zeta-potential between about 5 mV to about 45 mV, between about 15 mV to about 35 mV, or between about 20 mV and about 40 mV. Zeta-potential may be determined via characteristics that include electrophoretic mobility or dynamic electrophoretic mobility. Electrokinetic phenomena and electroacoustic phenomena may be utilized to calculate zeta-potential.

In one embodiment, a non-viral delivery vehicle comprises polymers including but not limited to poly(lactic-co-glycolic acid) (PLGA), polylactic acid (PLA), linear and/or branched PEI with differing molecular weights (e.g., 2, 22 and 25 kDa), dendrimers such as polyamidoamine (PAMAM) and polymethoacrylates; lipids including but not limited to cationic liposomes, cationic emulsions, DOTAP, DOTMA, DMRIE, DOSPA, distearoylphosphatidylcholine (DSPC), DOPE, or DC-cholesterol; peptide based vectors including but not limited to Poly-L-lysine or protamine; or poly(β-amino ester), chitosan, PEI-polyethylene glycol, PEI-mannose-dextrose, DOTAP-cholesterol or RNAiMAX.

In one embodiment, the delivery vehicle is a glycopolymer-based delivery vehicle, poly(glycoamidoamine)s (PGAAs), that have the ability to complex with various polynucleotide types and form nanoparticles. These materials are created by polymerizing the methylester or lactone derivatives of various carbohydrates (D-glucarate (D), *meso*-galactarate (G), D-mannarate (M), and L-tartarate (T)) with a series of oligoethyleneamine monomers (containing between 1-4 ethylenamines (Liu and Reineke, 2006). A subset composed of these carbohydrates and four ethyleneamines in the polymer repeat units yielded exceptional delivery efficiency.

In one embodiment, the delivery vehicle comprises polyethyleneimine (PEI), Polyamidoamine (PAMAM), PEI-PEG, PEI-PEG-mannose, dextran-PEI, OVA conjugate, PLGA microparticles, or PLGA microparticles coated with PAMAM, or any combination thereof. The disclosed cationic polymer may include, but are not limited to, polyamidoamine (PAMAM) dendrimers. Polyamidoamine dendrimers suitable for preparing the presently disclosed nanoparticles may include 3rd-, 4th-, 5th-, or at least 6th-generation dendrimers.

In one embodiment, the delivery vehicle comprises a lipid, e.g., *N*-[1-(2,3-dioleoyloxy)propel]-*N*,*N*,*N*-trimethylammonium (DOTMA), 2,3-dioleyloxy-*N*-[2-spermine carboxamide] ethyl-*N*,*N*-dimethyl-1-propanammonium trifluoracetate (DOSPA, Lipofectamine); 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP); *N*-[1-(2,3-dimyristloxy) propyl]; *N*,*N*-dimethyl-*N*-(2-hydroxyethyl) ammonium bromide (DMRIE), 3-β-[*N*-(*N*,*N*'-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol); dioctadecyl amidoglyceryl spermine (DOGS, Transfectam); or imethyldioctadeclyammonium bromide (DDAB). The positively charged hydrophilic head group of cationic lipids usually consists of monoamine such as tertiary and quaternary amines, polyamine, amidinium, or guanidinium group. A series of pyridinium lipids have been developed (Zhu et al., 2008; van der Woude et al., 1997; Ilies et al., 2004). In addition to pyridinium cationic lipids, other types of heterocyclic head group include imidazole, piperizine and amino acid. The main function of cationic head groups is to condense negatively charged nucleic acids by means of electrostatic interaction to slightly positively charged nanoparticles, leading to enhanced cellular uptake and endosomal escape.

Lipids having two linear fatty acid chains, such as DOTMA, DOTAP and SAINT-2, or DODAC, may be employed as a delivery vehicle, as well as tetraalkyl lipid chain surfactant, the dimer of *N*,*N*-dioleyl-*N*,*N*- dimethylammonium chloride (DODAC). All the *trans*-orientated lipids regardless of their hydrophobic chain lengths ($C_{16:1}$, $C_{18:1}$ and $C_{20:1}$) appear to enhance the transfection efficiency compared with their *cis*-orientated counterparts.

The structures of cationic polymers useful as a delivery vehicle include but are not limited to linear polymers such as chitosan and linear poly(ethyleneimine), branched polymers such as branch poly(ethyleneimine) (PEI), circle-like polymers such as cyclodextrin, network

(crosslinked) type polymers such as crosslinked poly(amino acid) (PAA), and dendrimers. Dendrimers consist of a central core molecule, from which several highly branched arms 'grow' to form a tree-like structure with a manner of symmetry or asymmetry. Examples of dendrimers include polyamidoamine (PAMAM) and polypropylenimine (PPI) dendrimers.

DOPE and cholesterol are commonly used neutral co-lipids for preparing cationic liposomes. Branched PEI-cholesterol water-soluble lipopolymer conjugates self-assemble into cationic micelles. Pluronic (poloxamer), a non-ionic polymer and SP1017, which is the combination of Pluronics L61 and F127, may also be used.

In one embodiment, PLGA particles are employed to increase the encapsulation frequency although complex formation with PLL may also increase the encapsulation efficiency. Other cationic materials, for example, PEI, DOTMA, DC-Chol, or CTAB, may be used to make nanospheres.

In one embodiment, complexes are embedded in or applied to a material including but not limited to hydrogels of poloxamers, polyacrylamide, poly(2-hydroxyethyl methacrylate), carboxyvinyl-polymers (e.g., Carbopol 934, Goodrich Chemical Co.), cellulose derivatives, e.g., methylcellulose, cellulose acetate and hydroxypropyl cellulose, polyvinyl pyrrolidone or polyvinyl alcohols, or combinations thereof.

In some embodiments, a biocompatible polymeric material is derived from a biodegradable polymeric such as collagen, e.g., hydroxylated collagen, fibrin, polylactic-polyglycolic acid, or a polyanhydride. Other examples include, without limitation, any biocompatible polymer, whether hydrophilic, hydrophobic, or amphiphilic, such as ethylene vinyl acetate copolymer (EVA), polymethyl methacrylate, polyamides, polycarbonates, polyesters, polyethylene, polypropylenes, polystyrenes, polyvinyl chloride, polytetrafluoroethylene, N-isopropylacrylamide copolymers, poly(ethylene oxide)/poly(propylene oxide) block copolymers, poly(ethylene glycol)/poly(D,L-lactide-co-glycolide) block copolymers, polyglycolide, polylactides (PLLA or PDLA), poly(caprolactone) (PCL), or poly(dioxanone) (PPS).

In another embodiment, the biocompatible material includes polyethyleneterephalate, polytetrafluoroethylene, copolymer of polyethylene oxide and polypropylene oxide, a combination of polyglycolic acid and polyhydroxyalkanoate, gelatin, alginate, poly-3-hydroxybutyrate, poly-4-hydroxybutyrate, and polyhydroxyoctanoate, and polyacrylonitrilepolyvinylchlorides.

In one embodiment, the following polymers may be employed, e.g., natural polymers such as starch, chitin, glycosaminoglycans, e.g., hyaluronic acid, dermatan sulfate and chrondrotin sulfate, and microbial polyesters, e.g., hydroxyalkanoates such as hydroxyvalerate

and hydroxybutyrate copolymers, and synthetic polymers, e.g., poly(orthoesters) and polyanhydrides, and including homo and copolymers of glycolide and lactides (e.g., poly(L-lactide, poly(L-lactide-co-D,L-lactide), poly(L-lactide-co-glycolide, polyglycolide and poly(D,L-lactide), pol(D,L-lactide-coglycolide), poly(lactic acid colysine) and polycaprolactone.

In one embodiment, the biocompatible material is derived from isolated extracellular matrix (ECM). ECM may be isolated from endothelial layers of various cell populations, tissues and/or organs, e.g., any organ or tissue source including the dermis of the skin, liver, alimentary, respiratory, intestinal, urinary or genital tracks of a warm blooded vertebrate. ECM employed in the invention may be from a combination of sources. Isolated ECM may be prepared as a sheet, in particulate form, gel form and the like.

The biocompatible scaffold polymer may comprise silk, elastin, chitin, chitosan, poly(d-hydroxy acid), poly(anhydrides), or poly(orthoesters). More particularly, the biocompatible polymer may be formed polyethylene glycol, poly(lactic acid), poly(glycolic acid), copolymers of lactic and glycolic acid, copolymers of lactic and glycolic acid with polyethylene glycol, poly(E-caprolactone), poly(3-hydroxybutyrate), poly(p-dioxanone), polypropylene fumarate, poly(orthoesters), polyol/diketene acetals addition polymers, poly(sebacic anhydride) (PSA), poly(carboxybiscarboxyphenoxyphenoxy hexone (PCPP) poly[bis (p-carboxypheonoxy) methane] (PCPM), copolymers of SA, CPP and CPM, poly(amino acids), poly(pseudo amino acids), polyphosphazenes, derivatives of poly[(dichloro)phosphazenes] or poly[(organo) phosphazenes], poly-hydroxybutyric acid, or S-caproic acid, polylactide-co-glycolide, polylactic acid, polyethylene glycol, cellulose, oxidized cellulose, alginate, gelatin or derivatives thereof.

Thus, the polymer may be formed of any of a wide range of materials including polymers, including naturally occurring polymers, synthetic polymers, or a combination thereof. In one embodiment, the scaffold comprises biodegradable polymers. In one embodiment, a naturally occurring biodegradable polymer may be modified to provide for a synthetic biodegradable polymer derived from the naturally occurring polymer. In one embodiment, the polymer is a poly(lactic acid) ("PLA") or poly(lactic-co-glycolic acid) ("PLGA"). In one embodiment, the scaffold polymer includes but is not limited to alginate, chitosan, poly(2-hydroxyethylmethacrylate), xyloglucan, co-polymers of 2-methacryloyloxyethyl phosphorylcholine, poly(vinyl alcohol), silicone, hydrophobic polyesters and hydrophilic polyester, poly(lactide-co-glycolide), N-isoproylacrylamide copolymers, poly(ethylene oxide)/poly(propylene oxide), polylactic acid, poly(orthoesters), polyanhydrides, polyurethanes, copolymers of 2-hydroxyethylmethacrylate and sodium methacrylate, phosphorylcholine, cyclodextrins, polysulfone and polyvinylpyrrolidine, starch, poly-D,L-lactic acid-para-

dioxanone-polyethylene glycol block copolymer, polypropylene, poly(ethylene terephthalate), poly(tetrafluoroethylene), poly-epsilon-caprolactone, or crosslinked chitosan hydrogels.

Alternatively, the nucleic acids or vectors, can be administered in dosages of at least about 0.0001 mg/kg to about 1 mg/kg, of at least about 0.001 mg/kg to about 0.5 mg/kg, at least about 0.01 mg/kg to about 0.25mg/kg or at least about 0.01 mg/kg to about 0.25 mg/kg of body weight, although other dosages may provide beneficial results.

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Exemplary Embodiments

In one embodiment, a gene therapy vector is provided comprising a promoter operably linked to a nucleic acid sequence comprising an open reading frame encoding APOE2 and a 3' untranslated region (3' UTR), and a nucleotide sequence having RNAi sequences corresponding to APOE4 for inhibition of APOE4 mRNA. In one embodiment, the vector comprises the nucleotide sequence. In one embodiment, the nucleotide sequence is 5' or 3' to the open reading frame. In one embodiment, the nucleotide sequence is 5' and 3' to the open reading frame. In one embodiment, the nucleotide sequence is on a different vector. In one embodiment, the vector is a viral vector. In one embodiment, the viral vector is an AAV, adenovirus, lentivirus, herpesvirus or retrovirus vector. In one embodiment, the AAV is AAV5, AAV9 or AAVrh10. In one embodiment, the APOE4 is human APOE4. In one embodiment, the APOE2 is human APOE2. In one embodiment, the nucleotide sequence is linked to a second promoter. In one embodiment, the second promoter is a PolIII promoter. In one embodiment, the RNAi comprises miRNA including a plurality of miRNA sequences. In one embodiment, the RNAi comprises siRNA including a plurality of siRNA sequences. In one embodiment, the open reading frame comprises a plurality of silent nucleotide substitutions. In one embodiment, at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the codons have a silent nucleotide substitution. In one embodiment, the open reading frame further comprises a peptide tag. In one embodiment, the tag comprises HA, histidine tag, AviTag, maltose binding tag, Strep-tag, FLAG-tag, V5-tag, Myc-tag, Spot-tag, T7 tag, or NE-tag.

Also provided is a host cell or mammal comprising the vector. In one embodiment, the cell is a mammalian cell. In one embodiment, the cell is a human cell. In one embodiment, the mammal is a non-human primate. In one embodiment, the mammal is a human.

Further provided is a method to prevent, inhibit or treat Alzheimer's disease in a mammal, comprising: administering to the mammal an effective amount of a composition

comprising the gene therapy vector.

A method to prevent, inhibit or treat a disease associated with APOE4 expression in a mammal is provided comprising: administering to the mammal an effective amount of a composition comprising the gene therapy vector.

In one embodiment, a composition comprises liposomes comprising the vector. In one embodiment, the composition comprises nanoparticles comprising the nucleic acid. In one embodiment, the gene therapy vector comprises a viral vector. In one embodiment, the mammal is a E2/E4 heterozygote. In one embodiment, the mammal is a E4/E4 homozygote. In one embodiment, the composition is systemically administered. In one embodiment, the composition is orally administered. In one embodiment, the composition is intravenously administered. In one embodiment, the composition is locally administered. In one embodiment, the composition is injected. In one embodiment, the composition is administered to the central nervous system. In one embodiment, the composition is administered to the brain. In one embodiment, the composition is a sustained release composition. In one embodiment, the mammal is a human. In one embodiment, the RNAi sequences comprise a plurality of miRNA sequences, e.g., identical miRNA sequences.

Example A

Alzheimer's disease (AD) affects 5 million Americans and is rapidly increasing in prevalence. Existing drugs have little impact on underlying disease processes and no preventative therapies are currently available. Inheritance of the APOE4 allele represents a high risk for development of disease while inheritance of the APOE2 allele is protective, reducing the risk of developing AD by ≥50% and delaying age of onset. Adeno-associated virus (AAV) delivery of the human APOE2 gene to murine models of AD expressing human APOE4 (homozygous expression) demonstrated reduced amyloid-β peptide and amyloid burden. The odds ratio of developing AD is reduced in E2/E4 heterozygotes compared with E4/E4 homozygotes (2.6 vs. 14.9). Suppression of APOE4, e.g., via delivery of an AAV vector, while simultaneously expressing human APOE2 may reduce the risk for AD even further. In one embodiment, gene therapy such as AAV therapy is designed to deliver both the human APOE2 gene coding sequence and artificial RNAs such as microRNA(s) (miRNA) targeted to the endogenous APOE4. The combination of knockdown of detrimental endogenous APOE4 expression with the expression of the beneficial APOE2 allele may provide enhanced protection from AD development for individuals with homozygous for the APOE4 allele.

In one embodiment, siRNA interacts with mRNA to silence translation. To express an siRNA from a DNA sequence, such as a gene therapy expression vector, the targeting sequence must be embedded in a small hairpin RNA (shRNA) or miRNA scaffold. The vector-expressed

artificial miRNAs are similar to endogenous RNAi's and undergo two processing steps. Since miRNAs are expressed at lower levels they are less likely to induce liver and CNS toxicity upon delivery by gene therapy vectors.

In one embodiment, knockdown with miRNA against all isoforms of endogenous APOE may be accomplished using multiple miRNAs targeting different sections of APOE mRNA, thereby enhancing silencing. In one embodiment, vector-derived human APOE2 may contain silent mutations in the coding sequence to prevent silencing.

As shown in Figure 3, the miRNA having the RNAi sequences to inhibit APOE4 expression may be inserted into 5' non-coding sequences, e.g., an intron, and/or 3' non-coding sequences. Multiple miRNAs can be placed in tandem for enhanced silencing of, e.g., APOE4. It was found that the level of hAPOE2-HA and miRNA expression were similar. There was a lower level of miRNA expression (compared with an U6 promoter) which in turn results in fewer off-target effects and lower potential for toxicity.

In one embodiment (see Figure 4), a constitutive promoter such as CAG drives hAPOE2-HA and an U6 promoter (an exemplary Pol III promoter) promoter drives miRNA. In one embodiment, multiple miRNAs are placed in tandem to enhance silencing of APOE4, e.g., 2, 3 4 or more miRNAs. In one embodiment, Pol III promoters are used for transcription of rRNA, tRNA, and/or miRNA. In one embodiment, a vector may have a defined terminator, e.g., no poly A is needed since PolIII transcription is terminated by an oligo (dT) stretch in the non-template strand (dA in the template strand)). In one embodiment, a two vector system may be employed where the second vector includes a stuffer sequence, e.g., for a reporter gene, to maintain length and track expression.

Thus, the disclosure provides for a vector, e.g., a viral vector such as an AAV vector, delivering both the human APOE2 gene and artificial miRNAs targeting human APOE4. These gene therapy vectors can be used to mitigate the risk of AD development in APOE4 homozygous individuals (as well as E2/E4 heterozygotes) by tipping the balance toward the expression of the beneficial APOE2 allele.

In one embodiment, the vector is useful in disorders or diseases which may benefit from increased APOE2 and/or decreased APOE4. In one embodiment, the vector is delivered to a mammal such as human at risk of AD. AD currently affects 5 million people in the US and worldwide prevalence is expected to rise to 65 million by 2030. Global prevalence of the APOE4 allele is 15% and about 50% of AD patients carry at least one APOE4 allele. Detrimental APOE4 gene is targeted for decreased expression while protective APOE2 expression is provided and the risk related to APOE4 is further reduced compared to a gene therapy that only delivers APOE2.

Example B

Figure 5 shows a system where miRNA knocks down all APOE isoform expression and where the vector derived APOE2 is resistant to miRNA. For example, by using a CAG promoter, the level of hApoE2-HA and miRNA expression were similar and a lower level of miRNA expression (compared with U6 promoter) may mean less silencing, however, there are also fewer off-targets and toxicity. In one embodiment, miRNA can be inserted in the CAG intron or 3' untranslated region.

Figure 6 depicts testing of APOE knockdown efficiency by siRNAs in U87 cells. Four different siRNAs targeting the coding sequence of APOE were generated based on a comparison of multiple siRNA design algorithms. siRNAs were transfected into U87 cells (astroglioma cell line), and APOE mRNA copies were quantified by RT-qPCR. The identified sequences were as follows:

1. GGUGGAGCAAGCGGUGGAGuu (SEQ ID NO:1)
2. GGAGUUGAAGGCCUACAAAuu (SEQ ID NO:2)
3. GGAAGACAUGCAGCGCCAGuu (SEQ ID NO:3)
4. GCGCGCGGAUGGAGGAGAUuu (SEQ ID NO:4)

Non-targeting siRNA is GTAGCGACTAAACACATCAuu (SEQ ID NO:5)

Other sequences for siRNAs include:

GCCGATGACCTGCAGAAGCuu (SEQ ID NO:20)

GCGCGCGGATGGAGGAGATuu (SEQ ID NO:21)

GTAAGCGGCTCCTCCGCGAuu (SEQ ID NO:22)

The sequence from one siRNA (#2 above) was converted into a miRNA. A scaffold based on a modified version of mir155 (Fowler et al. 2015 Nucl. Acids Res., 44:e48, the disclosure of which is incorporated by reference herein) was employed. Mismatches in the passenger strand were chosen for GC content and position. However, any miRNA backbone may be employed, e.g., mir21, mir30 or mir33.

For example, for a miR from siRNA#2, the following may be employed:

CTGGAGGCTTGCTGAAGGCTGTATGCTGATTTGTAGGCCTTCAACTCCTGTTTTGGCC
ACTGACTGACAGGAGTGAGGCCTACAAATCAGGACACAAGGCCTGTTACTAGCACT
CACATGGAACAAATGGCC (SEQ ID NO:23);

CTGGAGGCTTGCTTTGGGCTGTATGCTGATTTGTAGGCCTTCAACTCCTGTTTTGGCC
ACTGACTGACAGGAGTTGAAGTCACAAATCAGGACACAAGGCCCTTTATCAGCACT
CACATGGAACAAATGGCCACCGTGGGAGGATGACAA (SEQ ID NO:24); or

CTGGAGGCTTGCTTTGGGCTGTATGCTGTTCCGATTTGTAGGCCTTCAAGTTTTGGCC
ACTGACTGACTTGAAGTCACAAATCGGAACAGGACACAAGGCCCTTTATCAGCACTC

ACATGGAACAAATGGCCACCGTGGGAGGATGACAA (SEQ ID NO:25)

In one embodiment, the miRNA has one or more of: a U or A at guide position 1 relative to the 5' microprocessor cleavage site, U or A at positions 2-7, 10-14 and 17, and G or C at positions 19-21, and/or G/C content of 36.4% to 45.5%, and/or a guide strand that is 2 nucleotides longer than passenger strand, and/or mismatches in 1) a loop where 3 to 5 adjacent nucleotides of the guide strand are not base paired with target strand, 2) a 3 bp spaced mismatch where 2 single guide strand nucleotide mismatches are separated by 3 guide/passenger base pairs and/or 3) a 4 bp spaced mismatch where 2 single guide strand nucleotide mismatches are separated by 4 guide/passenger base pairs. Mismatches in the passenger strand were chosen for optimal GC content and position. Mfold was used to predict miRNA hairpin secondary structures. Two tandem copies of the miRNA were cloned into either the CAG intron or the 3' untranslated region of the pAAV expression cassette. Up to four copies of the miRNA (of that length) can be inserted within the AAV size limits.

Vector derived APOE2 was modified to be resistant to silencing by the targeting miRNA mentioned above (see underlined sequence below). Silent changes were made in the nucleotide sequence of the miRNA targeting region (red/bold).

APOE2 from vector:

ATGAAGGTTCTGTGGGCTGCGTTGCTGGTCACATTCCTGGCAGGATGCCAGGC
CAAGGTGGAGCAAGCGGTGGAGACAGAGCCGGAGCCCGAGCTGCGCCAGCAGACC
GAGTGGCAGAGCGGCCAGCGCTGGGAACTGGCACTGGGTCGCTTTTGGGATTACCT
GCGCTGGGTGCAGACACTGTCTGAGCAGGTGCAGGAGGAGCTGCTCAGCTCCCAGG
TCACCCAGGAACTGAGGGCGCTGATGGACGAGACCATGAAGGAGTTGAAGGCCTAC
AAATCGGAACTGGAGGAACAACTGACCCCGGTGGCGGAGGAGACGCGGGCACGGC
TGTCCAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGACATGGAGGACGTG
TGCGGCCGCCTGGTGCAGTACCGCGGCGAGGTGCAGGCCATGCTCGGCCAGAGCAC
CGAGGAGCTGCGGGTGCGCCTCGCCTCCCACCTGCGCAAGCTGCGTAAGCGGCTCCT
CCGCGATGCCGATGACCTGCAGAAGTGCCTGGCAGTGTACCAGGCCGGGGCCCGCG
AGGGCGCCGAGCGCGGCCTCAGCGCCATCCGCGAGCGCCTGGGGCCCCTGGTGGAA
CAGGGCCGCGTGCGGGCCGCCACTGTGGGCTCCCTGGCCGGCCAGCCGCTACAGGA
GCGGGCCCAGGCCTGGGGCGAGCGGCTGCGCGCGCGGATGGAGGAGATGGGCAGC
CGGACCCGCGACCGCCTGGACGAGGTGAAGGAGCAGGTGGCGGAGGTGCGCGCCA
AGCTGGAGGAGCAGGCCCAGCAGATACGCCTGCAGGCCGAGGCCTTCCAGGCCCGC
CTCAAGAGCTGGTTCGAGCCCCTGGTGGAAGACATGCAGCGCCAGTGGGCCGGGCT
GGTGGAGAAGGTGCAGGCTGCCGTGGGCACCAGCGCCGCCCCTGTGCCCAGCGACA
ATCAC (SEQ ID NO:6)

Modified APOE2:

ATGAAGGTTCTGTGGGCTGCGTTGCTGGTCACATTCCTGGCAGGATGCCAGGC
CAAGGTGGAGCAAGCGGTGGAGACAGAGCCGGAGCCCGAGCTGCGCCAGCAGACC
GAGTGGCAGAGCGGCCAGCGCTGGGAACTGGCACTGGGTCGCTTTTGGGATTACCT
GCGCTGGGTGCAGACACTGTCTGAGCAGGTGCAGGAGGAGCTGCTCAGCTCCCAGG
TCACCCAGGAACTGAGGGCGCTGATGGACGAGACCATGAAAGAACTCAAAGCTTAT
AAGAGCGAGCTGGAGGAACAACTGACCCCGGTGGCGGAGGAGACGCGGGCACGGC
TGTCCAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGACATGGAGGACGTG
TGCGGCCGCCTGGTGCAGTACCGCGGCGAGGTGCAGGCCATGCTCGGCCAGAGCAC
CGAGGAGCTGCGGGTGCGCCTCGCCTCCCACCTGCGCAAGCTGCGTAAGCGGCTCCT
CCGCGATGCCGATGACCTGCAGAAGTGCCTGGCAGTGTACCAGGCCGGGGCCCGCG
AGGGCGCCGAGCGCGGCCTCAGCGCCATCCGCGAGCGCCTGGGGCCCCTGGTGGAA
CAGGGCCGCGTGCGGGCCGCCACTGTGGGCTCCCTGGCCGGCCAGCCGCTACAGGA
GCGGGCCCAGGCCTGGGGCGAGCGGCTGCGCGCGCGGATGGAGGAGATGGGCAGC
CGGACCCGCGACCGCCTGGACGAGGTGAAGGAGCAGGTGGCGGAGGTGCGCGCCA
AGCTGGAGGAGCAGGCCCAGCAGATACGCCTGCAGGCCGAGGCCTTCCAGGCCCGC
CTCAAGAGCTGGTTCGAGCCCCTGGTGGAAGACATGCAGCGCCAGTGGGCCGGGCT
GGTGGAGAAGGTGCAGGCTGCCGTGGGCACCAGCGCCGCCCCTGTGCCCAGCGACA
ATCAC (SEQ ID NO:7)

The sequence above changes all possible nucleotides silently while considering codon usage within the composite recognition site for the 3 miRNAs derived from siRNA#2. However, other examples are shown below:

Modified APOE:

ATGAAGGTTCTGTGGGCTGCGTTGCTGGTCACATTCCTGGCAGGATGCCAGGCCAAG
GTGGAGCAAGCGGTGGAGACAGAGCCGGAGCCCGAGCTGCGCCAGCAGACCGAGT
GGCAGAGCGGCCAGCGCTGGGAACTGGCACTGGGTCGCTTTTGGGATTACCTGCGCT
GGGTGCAGACACTGTCTGAGCAGGTGCAGGAGGAGCTGCTCAGCTCCCAGGTCACC
CAGGAACTGAGGGCGCTGATGGACGAGACCATGAAAGAACTTAAAGCATATAAGA
GTGAGCTGGAGGAACAACTGACCCCGGTGGCGGAGGAGACGCGGGCACGGCTGTC
CAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGACATGGAGGACGTGTGCG
GCCGCCTGGTGCAGTACCGCGGCGAGGTGCAGGCCATGCTCGGCCAGAGCACCGAG
GAGCTGCGGGTGCGCCTCGCCTCCCACCTGCGCAAGCTGCGTAAGCGGCTCCTCCGC
GATGCCGATGACCTGCAGAAGTGCCTGGCAGTGTACCAGGCCGGGGCCCGCGAGGG
CGCCGAGCGCGGCCTCAGCGCCATCCGCGAGCGCCTGGGGCCCCTGGTGGAACAGG
GCCGCGTGCGGGCCGCCACTGTGGGCTCCCTGGCCGGCCAGCCGCTACAGGAGCGG

GCCCAGGCCTGGGGCGAGCGGCTGCGCGCGCGGATGGAGGAGATGGGCAGCCGGA
CCCGCGACCGCCTGGACGAGGTGAAGGAGCAGGTGGCGGAGGTGCGCGCCAAGCTG
GAGGAGCAGGCCCAGCAGATACGCCTGCAGGCCGAGGCCTTCCAGGCCCGCCTCAA
GAGCTGGTTCGAGCCCCTGGTGGAAGACATGCAGCGCCAGTGGGCCGGGCTGGTGG
AGAAGGTGCAGGCTGCCGTGGGCACCAGCGCCGCCCCTGTGCCCAGCGACAATCAC
(SEQ ID NO:26);
Modified APOE:
ATGAAGGTTCTGTGGGCTGCGTTGCTGGTCACATTCCTGGCAGGATGCCAGGCCAAG
GTGGAGCAAGCGGTGGAGACAGAGCCGGAGCCCGAGCTGCGCCAGCAGACCGAGT
GGCAGAGCGGCCAGCGCTGGGAACTGGCACTGGGTCGCTTTTGGGATTACCTGCGCT
GGGTGCAGACACTGTCTGAGCAGGTGCAGGAGGAGCTGCTCAGCTCCCAGGTCACC
CAGGAACTGAGGGCGCTGATGGACGAGACCATGAAAGAACTCAAAGCATATAAGA
GTGAGCTGGAGGAACAACTGACCCCGGTGGCGGAGGAGACGCGGGCACGGCTGTC
CAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGACATGGAGGACGTGTGCG
GCCGCCTGGTGCAGTACCGCGGCGAGGTGCAGGCCATGCTCGGCCAGAGCACCGAG
GAGCTGCGGGTGCGCCTCGCCTCCCACCTGCGCAAGCTGCGTAAGCGGCTCCTCCGC
GATGCCGATGACCTGCAGAAGTGCCTGGCAGTGTACCAGGCCGGGGCCCGCGAGGG
CGCCGAGCGCGGCCTCAGCGCCATCCGCGAGCGCCTGGGGCCCCTGGTGGAACAGG
GCCGCGTGCGGGCCGCCACTGTGGGCTCCCTGGCCGGCCAGCCGCTACAGGAGCGG
GCCCAGGCCTGGGGCGAGCGGCTGCGCGCGCGGATGGAGGAGATGGGCAGCCGGA
CCCGCGACCGCCTGGACGAGGTGAAGGAGCAGGTGGCGGAGGTGCGCGCCAAGCTG
GAGGAGCAGGCCCAGCAGATACGCCTGCAGGCCGAGGCCTTCCAGGCCCGCCTCAA
GAGCTGGTTCGAGCCCCTGGTGGAAGACATGCAGCGCCAGTGGGCCGGGCTGGTGG
AGAAGGTGCAGGCTGCCGTGGGCACCAGCGCCGCCCCTGTGCCCAGCGACAATCAC
(SEQ ID NO:27);
Modified APOE:
ATGAAGGTTCTGTGGGCTGCGTTGCTGGTCACATTCCTGGCAGGATGCCAGGCCAAG
GTGGAGCAAGCGGTGGAGACAGAGCCGGAGCCCGAGCTGCGCCAGCAGACCGAGT
GGCAGAGCGGCCAGCGCTGGGAACTGGCACTGGGTCGCTTTTGGGATTACCTGCGCT
GGGTGCAGACACTGTCTGAGCAGGTGCAGGAGGAGCTGCTCAGCTCCCAGGTCACC
CAGGAACTGAGGGCGCTGATGGACGAGACCATGAAAGAACTTAAAGCTTATAAGA
GTGAGCTGGAGGAACAACTGACCCCGGTGGCGGAGGAGACGCGGGCACGGCTGTC
CAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGACATGGAGGACGTGTGCG
GCCGCCTGGTGCAGTACCGCGGCGAGGTGCAGGCCATGCTCGGCCAGAGCACCGAG
GAGCTGCGGGTGCGCCTCGCCTCCCACCTGCGCAAGCTGCGTAAGCGGCTCCTCCGC

GATGCCGATGACCTGCAGAAGTGCCTGGCAGTGTACCAGGCCGGGGCCCGCGAGGG
CGCCGAGCGCGGCCTCAGCGCCATCCGCGAGCGCCTGGGGCCCCTGGTGGAACAGG
GCCGCGTGCGGGCCGCCACTGTGGGCTCCCTGGCCGGCCAGCCGCTACAGGAGCGG
GCCCAGGCCTGGGGCGAGCGGCTGCGCGCGCGGATGGAGGAGATGGGCAGCCGGA
CCCGCGACCGCCTGGACGAGGTGAAGGAGCAGGTGGCGGAGGTGCGCGCCAAGCTG
GAGGAGCAGGCCCAGCAGATACGCCTGCAGGCCGAGGCCTTCCAGGCCCGCCTCAA
GAGCTGGTTCGAGCCCCTGGTGGAAGACATGCAGCGCCAGTGGGCCGGGCTGGTGG
AGAAGGTGCAGGCTGCCGTGGGCACCAGCGCCGCCCCTGTGCCCAGCGACAATCAC
(SEQ ID NO:28);

Modified APOE:

ATGAAGGTTCTGTGGGCTGCGTTGCTGGTCACATTCCTGGCAGGATGCCAGGCCAAG
GTGGAGCAAGCGGTGGAGACAGAGCCGGAGCCCGAGCTGCGCCAGCAGACCGAGT
GGCAGAGCGGCCAGCGCTGGGAACTGGCACTGGGTCGCTTTTGGGATTACCTGCGCT
GGGTGCAGACACTGTCTGAGCAGGTGCAGGAGGAGCTGCTCAGCTCCCAGGTCACC
CAGGAACTGAGGGCGCTGATGGACGAGACCATGAAAGAACTTAAAGCATATAAGA
GCGAGCTGGAGGAACAACTGACCCCGGTGGCGGAGGAGACGCGGGCACGGCTGTC
CAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGACATGGAGGACGTGTGCG
GCCGCCTGGTGCAGTACCGCGGCGAGGTGCAGGCCATGCTCGGCCAGAGCACCGAG
GAGCTGCGGGTGCGCCTCGCCTCCCACCTGCGCAAGCTGCGTAAGCGGCTCCTCCGC
GATGCCGATGACCTGCAGAAGTGCCTGGCAGTGTACCAGGCCGGGGCCCGCGAGGG
CGCCGAGCGCGGCCTCAGCGCCATCCGCGAGCGCCTGGGGCCCCTGGTGGAACAGG
GCCGCGTGCGGGCCGCCACTGTGGGCTCCCTGGCCGGCCAGCCGCTACAGGAGCGG
GCCCAGGCCTGGGGCGAGCGGCTGCGCGCGCGGATGGAGGAGATGGGCAGCCGGA
CCCGCGACCGCCTGGACGAGGTGAAGGAGCAGGTGGCGGAGGTGCGCGCCAAGCTG
GAGGAGCAGGCCCAGCAGATACGCCTGCAGGCCGAGGCCTTCCAGGCCCGCCTCAA
GAGCTGGTTCGAGCCCCTGGTGGAAGACATGCAGCGCCAGTGGGCCGGGCTGGTGG
AGAAGGTGCAGGCTGCCGTGGGCACCAGCGCCGCCCCTGTGCCCAGCGACAATCAC
(SEQ ID NO:29);

Modified APOE:

ATGAAGGTTCTGTGGGCTGCGTTGCTGGTCACATTCCTGGCAGGATGCCAGGCCAAG
GTGGAGCAAGCGGTGGAGACAGAGCCGGAGCCCGAGCTGCGCCAGCAGACCGAGT
GGCAGAGCGGCCAGCGCTGGGAACTGGCACTGGGTCGCTTTTGGGATTACCTGCGCT
GGGTGCAGACACTGTCTGAGCAGGTGCAGGAGGAGCTGCTCAGCTCCCAGGTCACC
CAGGAACTGAGGGCGCTGATGGACGAGACCATGAAAGAACTCAAAGCTTATAAGA
GTGAGCTGGAGGAACAACTGACCCCGGTGGCGGAGGAGACGCGGGCACGGCTGTC

CAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGACATGGAGGACGTGTGCG
GCCGCCTGGTGCAGTACCGCGGCGAGGTGCAGGCCATGCTCGGCCAGAGCACCGAG
GAGCTGCGGGTGCGCCTCGCCTCCCACCTGCGCAAGCTGCGTAAGCGGCTCCTCCGC
GATGCCGATGACCTGCAGAAGTGCCTGGCAGTGTACCAGGCCGGGGCCCGCGAGGG
CGCCGAGCGCGGCCTCAGCGCCATCCGCGAGCGCCTGGGGCCCCTGGTGGAACAGG
GCCGCGTGCGGGCCGCCACTGTGGGCTCCCTGGCCGGCCAGCCGCTACAGGAGCGG
GCCCAGGCCTGGGGCGAGCGGCTGCGCGCGCGGATGGAGGAGATGGGCAGCCGGA
CCCGCGACCGCCTGGACGAGGTGAAGGAGCAGGTGGCGGAGGTGCGCGCCAAGCTG
GAGGAGCAGGCCCAGCAGATACGCCTGCAGGCCGAGGCCTTCCAGGCCCGCCTCAA
GAGCTGGTTCGAGCCCCTGGTGGAAGACATGCAGCGCCAGTGGGCCGGGCTGGTGG
AGAAGGTGCAGGCTGCCGTGGGCACCAGCGCCGCCCCTGTGCCCAGCGACAATCAC
(SEQ ID NO:30)

Modified APOE:

ATGAAGGTTCTGTGGGCTGCGTTGCTGGTCACATTCCTGGCAGGATGCCAGGCCAAG
GTGGAGCAAGCGGTGGAGACAGAGCCGGAGCCCGAGCTGCGCCAGCAGACCGAGT
GGCAGAGCGGCCAGCGCTGGGAACTGGCACTGGGTCGCTTTTGGGATTACCTGCGCT
GGGTGCAGACACTGTCTGAGCAGGTGCAGGAGGAGCTGCTCAGCTCCCAGGTCACC
CAGGAACTGAGGGCGCTGATGGACGAGACCATGAAAGAACTCAAAGCATATAAGA
GCGAGCTGGAGGAACAACTGACCCCGGTGGCGGAGGAGACGCGGGCACGGCTGTC
CAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGACATGGAGGACGTGTGCG
GCCGCCTGGTGCAGTACCGCGGCGAGGTGCAGGCCATGCTCGGCCAGAGCACCGAG
GAGCTGCGGGTGCGCCTCGCCTCCCACCTGCGCAAGCTGCGTAAGCGGCTCCTCCGC
GATGCCGATGACCTGCAGAAGTGCCTGGCAGTGTACCAGGCCGGGGCCCGCGAGGG
CGCCGAGCGCGGCCTCAGCGCCATCCGCGAGCGCCTGGGGCCCCTGGTGGAACAGG
GCCGCGTGCGGGCCGCCACTGTGGGCTCCCTGGCCGGCCAGCCGCTACAGGAGCGG
GCCCAGGCCTGGGGCGAGCGGCTGCGCGCGCGGATGGAGGAGATGGGCAGCCGGA
CCCGCGACCGCCTGGACGAGGTGAAGGAGCAGGTGGCGGAGGTGCGCGCCAAGCTG
GAGGAGCAGGCCCAGCAGATACGCCTGCAGGCCGAGGCCTTCCAGGCCCGCCTCAA
GAGCTGGTTCGAGCCCCTGGTGGAAGACATGCAGCGCCAGTGGGCCGGGCTGGTGG
AGAAGGTGCAGGCTGCCGTGGGCACCAGCGCCGCCCCTGTGCCCAGCGACAATCAC
(SEQ ID NO:31); or

Modified APOE:

ATGAAGGTTCTGTGGGCTGCGTTGCTGGTCACATTCCTGGCAGGATGCCAGGCCAAG
GTGGAGCAAGCGGTGGAGACAGAGCCGGAGCCCGAGCTGCGCCAGCAGACCGAGT
GGCAGAGCGGCCAGCGCTGGGAACTGGCACTGGGTCGCTTTTGGGATTACCTGCGCT

GGGTGCAGACACTGTCTGAGCAGGTGCAGGAGGAGCTGCTCAGCTCCCAGGTCACC
CAGGAACTGAGGGCGCTGATGGACGAGACCATGAAAGAACTTAAAGCTATAAGA
GCGAGCTGGAGGAACAACTGACCCCGGTGGCGGAGGAGACGCGGGCACGGCTGTC
CAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGACATGGAGGACGTGTGCG
GCCGCCTGGTGCAGTACCGCGGCGAGGTGCAGGCCATGCTCGGCCAGAGCACCGAG
GAGCTGCGGGTGCGCCTCGCCTCCCACCTGCGCAAGCTGCGTAAGCGGCTCCTCCGC
GATGCCGATGACCTGCAGAAGTGCCTGGCAGTGTACCAGGCCGGGGCCCGCGAGGG
CGCCGAGCGCGGCCTCAGCGCCATCCGCGAGCGCCTGGGGCCCCTGGTGGAACAGG
GCCGCGTGCGGGCCGCCACTGTGGGCTCCCTGGCCGGCCAGCCGCTACAGGAGCGG
GCCCAGGCCTGGGGCGAGCGGCTGCGCGCGCGGATGGAGGAGATGGGCAGCCGGA
CCCGCGACCGCCTGGACGAGGTGAAGGAGCAGGTGGCGGAGGTGCGCGCCAAGCTG
GAGGAGCAGGCCCAGCAGATACGCCTGCAGGCCGAGGCCTTCCAGGCCCGCCTCAA
GAGCTGGTTCGAGCCCCTGGTGGAAGACATGCAGCGCCAGTGGGCCGGGCTGGTGG
AGAAGGTGCAGGCTGCCGTGGGCACCAGCGCCGCCCCTGTGCCCAGCGACAATCAC
(SEQ ID NO:32).

Vectors may be tested in non-human animals such as mice. In one embodiment, the vector includes sequences from the AAV9-CAG-APOE2 vector (AAV9-APOE2), the adeno-associated viral vector serotype 9 expressing the APOE2 behind the chicken β actin promoter or from the AAVrh.10-CAG-APOE2 vector (AAVrh.10-APOE2), the rhesus adeno-associated viral vector serotype 10 expressing the APOE2 transgene behind the chicken β actin promoter.

The AAVrh.10 and AAV9 vectors may be produced and purified as described previously (Sondhi et al., 2007, 2012; Zolotukhin et al., 2002). Briefly, the vectors are produced by cotransfection of HEK293T cells with an expression cassette plasmid and adenoviral helper plasmids. The packaging cell line, HEK293T, is maintained in Dulbecco's modified Eagles medium, supplemented with 5% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and maintained at 37°C with 5% CO_2. The cells are plated at 30%-40% confluence in CellSTACKS (Corning, Tewksbury, MA) for 24 hours (or when at 70%-80% confluence) followed by transfection with plasmids using the PEIpro procedure. The cells are incubated at 37°C for 3 days before harvesting and lysing by 5 freeze/thaw cycles. The resulting cell lysate is treated with 50 U/mL of Benzonase at 37°C for 30 minutes. For AAVrh.10 vectors, the cell lysate is purified by iodixanol density gradient followed by Q-HP ion-exchange chromatography. For AAV9 vectors, the cell lysate is precipitated in PEG (final concentration of PEG: 8%) overnight. After centrifugation, the supernatant is discarded and the pellet is resuspended in 15 mL lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.5). The sample is purified by

centrifugation through 1.37 g/mL CsCl in a 38.5 mL polyallomer tube using an SW28 rotor at 24,000 rpm (182,000g), at 20°C for 24 hours. A 21-gauge needle (Hamilton, Reno, NV) is inserted through the bottom side of the centrifuge tube and 1 mL fractions are collected. Vector-containing fractions are determined by dot blot with a ^{32}P-labeled probe containing the sequence of the vector constructs. The positive fractions are subsequently pooled and diluted with 1.37 g/mL CsCl, and the samples are loaded into a 13.5 mL Quick-Seal tube and centrifuged in an ultra-centrifuge (Beckman LE-80K; Beckman Coulter, Fullerton, CA) 90 Ti rotor, at 67,000 rpm (384,000g), 20°C for 16-20 hours. Fractions (0.5 mL) are collected, and the positive fractions were pooled. The purified AAVrh.10 or AAV9 vectors are concentrated in phosphate-buffered saline (PBS). Vector genome titer is determined by Taq-Man quantitative polymerase chain reaction. The purified vectors are sterile filtered; tested 14 days for growth on medium supporting the growth of aerobic bacteria, anaerobic bacteria, or fungi; tested for endotoxin; and demonstrated to be mycoplasma free.

. The AAV preparation (2 mL, 1.0 x 10^{10} vg or at another dose) may be injected using, e.g., a 33-gauge needle (Hamilton) and a syringe pump (KD Scientific, Holliston, MA) at a rate of 0.2 mL/min.

The invention will be described by the following non-limiting examples.

Example 1

AAV mediated APOE4 Gene Silencing to Treat Homozygous APOE4-associated Alzheimer's Disease

The homozygous APOE4 genotype is the major risk factor for the development of early Alzheimer's disease. Genetic studies in mouse models of human APOE4 dependent pathology have established that reduction of APOE4 expression can rescue the phenotype. There have been 3 attempts to suppress central nervous system (CNS) APOE4 in murine models using anti-sense 4 oligomers or small interfering RNAs, but these approaches require repetitive CNS administration due to the short half-life of these drugs. It was hypothesized that APOE4 could be persistently suppressed in the CNS of APOE4 homozygotes using adeno-associated virus (AAV) expression of microRNAs (miRNA) designed to hybridize to and suppress human APOE mRNA. To accomplish this, an initial screening of human APOE suppression by 9 different miRNAs was conducted following transfection in 293 and Huh7 cells. Optimal suppression in both cell types was obtained with miRNA2A (targeting coding region nt330-351, 66% suppression in 293 cells, 69% suppression in Huh cells) and miRNA N4 (3' untranslated region nt1142-1162, 57% suppression in 293, 82% suppression in HuH). miRNA expression cassettes were designed for delivery by AAV gene transfer vectors with two copies each of two different miRNAs (mir2A and mirN4) co-expressed from the CAG promoter with a mCherry reporter transgene. A

neurotropic AAVrh.10 variant with an engineered capsid (AAV.S2) was used to deliver the suppression cassette to the hippocampus of TRE4 mice (murine ApoE knockout, human APOE4 knockin at the murine apoE locus). Two weeks after intra-hippocampus injection into TRE4 mice, 3 mm coronal tissue slices were collected. Regional expression of miRNA at the injection site was demonstrated both by direct assessment of miRNA level relative to an endogenous reference and by assessing mCherry level as a proxy. The AAV.S2 engineered capsid provided 2.31 ± 0.37-fold higher expression of miRNA over that provided by wildtype rh.10 ($p<0.05$). In the targeted region, a single intra-hippocampus administration suppressed hippocampal APOE4 mRNA levels by 76.5 ± 3.9% compared to 41.3 ± 3.3% with the same cassette delivered by the wildtype rh.10 capsid ($p<0.0001$). It was concluded that an expression cassette with 2 different miRNAs targeting APOE4 delivered by engineered AAV capsid generated significant suppression of CNS APOE4. Since APOE4 heterozygote humans have lower risk of Alzheimer's disease than APOE4 homozygotes, this level of APOE4 suppression may be therapeutic.

Example 2

It is described herein below, the utility of capsid S2 with enhanced transduction of the brain. AAVrh.10 variants such as S2 may be selected in vivo and/or capsid variants with no interaction with the primary AAV receptor may be identified which will allow other homing peptides to be coupled and redirect delivery to other tissues.

Enhanced transduction of brain by capsid S2

Nonhuman primate serotype AAVrh.10 has been demonstrated to effectively transfer genes to the CNS following direct administration (Sondhi D et al, Sci Trans Med 2020; 12: eabb5413) or via cerebrospinal fluid (Rosenberg JB et al, Hum Gene Ther Clin Dev 2018; 29:24). It was hypothesized that, for a given dose, CNS distribution of AAVrh.10 could be further improved by genetic modification of capsid loop IV with sequences designed to broaden distribution. Following initial screening of multiple variants, the AAVrh.10S2 capsid was selected, where amino acids 452-459 in loop IV were replaced with DGAATKQ, a sequence modified from AAV9 CAP-B10 (Goertsen D et al, Nat Neurosci 2022; 25:106). AAVrh.10S2 coding for mCherry was assessed *in vitro* in cell lines U87 (glioma), SVGp12 (glial) and SH-SY5Y (neuroblastoma) and *in vivo* following direct intrahippocampal administration of AAVrh.10S2mCherry to C57Bl/6 mice. In all neural cell lines, expression of mCherry was significantly greater (average 5.8-fold all cell lines) for AAVrh.10S2mCherry than for the same dose (10^4 gc/cell) of the wildtype AAVrh.10mCherry control ($p<0.05$, all cell types). CNS expression of mCherry was assessed 4 weeks following intrahippocampal administration of $2.5x10^{10}$ gc of AAVrh.10S2mCherry compared to AAVrh.10mCherry. With the same dose, mCherry mRNA levels in the hippocampus and cortex were higher with AAVrh.10S2 mCherry

compared to AAVrh.10mCherry (hippocampus 55-fold, cortex 7.6-fold; $p<0.05$ by ANOVA). Consistent with the mRNA data, protein expression with AAVrh.10mCherry in the hippocampus was 17.7± 2.6 ng/µg protein and 5.9± 0.5 ng/µg in the cortex. In contrast, with the same dose, hippocampus mCherry levels with AAVrh.10S2mCherry were 39.8 ± 1.8 ng/µg and in the cortex were 17.7± 2.2 ng/µg, >2-fold higher than with the unmodified AAVrh.10 vector ($p<0.001$ by ANOVA). In summary, the modified AAVrh.10S2 capsid has enhanced CNS distribution *in vivo*, providing greater brain transduction compared to unmodified AAVrh.10 capsid following direct intraparenchymal administration. Pending assessment in larger species, the AAVrh.10S2 capsid may be useful for treating diffuse neurological diseases.

Selection of cardiotropic AAV rh.10 variants by serial *in vivo* selection

This procedure is designed to select AAVrh.10 capsids with improved trafficking to the heart. A library of mixed AAVs is created in which each AAV particle encodes the capsid gene which encloses itself. Random AAV genomes encoding AAVrh.10-like capsids with many variants of loops IV and VIII are made cloning capsid genes formed by annealing of oligonucleotides with mixed bases in the relevant regions. These mixed plasmids are used to make AAV pools that are injected into mice. The capsids which preferentially transduce heart are assumed to express heart tropic capsids and therefore these sequences can be recovered by RT-PRC of heart mRNA. They are recloned back into the capsid gene and the process is repeated to select increasingly cardiotropic variants.

Following three rounds of screening and recovery, 22 capsid sequences were amplified and sequenced (Table 1).

Table 1.

Capsid Name	**Substitution at 452-458**	**Insertion at position 588**
104	--	QLTTRDNHQ (SEQ ID NO:42)
106	--	TTRNDFLPQ (SEQ ID NO:43)
9D	--	TTRGDRP (SEQ ID NO:44)
9G	--	NHTNKTQ (SEQ ID NO:45)
9M31	--	ANFVSYDTA (SEQ ID NO:46)
AB2	GVSLGGG (SEQ ID NO:47)	--
AB3	GVSLKQG (SEQ ID NO:48)	--
AB4	--	LTTPRGD (SEQ ID NO:49)
AB5	TTPRGDL (SEQ ID NO:50)	--
B	--	RGDLSTP (SEQ ID NO:51)
C	--	RGDLNQY (SEQ ID NO:52)

E	--	RTTTTRP (SEQ ID NO:53)
F	--	RGDTGDR (SEQ ID NO:54)
GRP	--	QRPPREP (SEQ ID NO:55)
H	--	GNRVDDF (SEQ ID NO:56)
NY1	DEEETKR (SEQ ID NO:57)	--
NY2	DGNNTKR (SEQ ID NO:58)	--
NY3	DGNNTKRQ (SEQ ID NO:59)	--
NY4	DVSLTRQ (SEQ ID NO:60)	--
NY5	DGARGDQ (SEQ ID NO:61)	--
NY7	QGTRRIQ (SEQ ID NO:62)	--
WPP	--	WPPKTTS (SEQ ID NO:63)

Initially these were uncharacterized except for their recovery from the *in vivo* selection. To identify potentially superior capsid, they were all used to encapsulate mCherry expression cassette and transduction was assessed in human cardiomyocyte-like cell lines (T0539, AC16) and human embryonic cardiomyocytes. On that basis, a number of quantitatively superior variants were identified including previously described variant (9D, 30) and variants derived from *in vivo* selection AB3, NY2, NY3 (Table 2).

Table 2.

	AAV mediated expression of luciferase under standard assay conditions (Light unit x 10^6 /µl)					
Capsid variant	T0539 cardiomyocyte -like cell line	Human AC16 cardiomyocyte -like cell line	Human Primary Cardiomyocytes	HL-1 (mouse cardiomyocyte)	Hela AAVR+	Hela AAVR-
9D	1596	1253	748	41	1069	7
30	583	1842	946	101	390	19
G	409	799	1088	122	687	14
NY3	590	1325	2204	30	133	1
37	322	1122	680	50	242	13
NY2	550	944	1686	27	114	1
D	427	1111	645	21	132	9
DB22	794	619	905	13	50	7
AB3	422	663	1078	20	111	2
GB10	316	373	544	46	59	8
56	296	782	943	16	55	2
GB22	253	365	430	40	52	9
82.5	257	1128	625	20	75	1
16	132	344	388	18	80	4
30.22	137	333	210	27	131	18

M3.B22	184	434	92	9	79	34
M2	228	809	504	20	69	1
S2	231	641	378	19	61	1
M3.B10	231	278	121	6	56	35
9G	272	218	215	12	38	19
18 n	674	349	584	11	13	3
DB10	510	295	596	9	14	2
M3	128	329	68	12	58	19
rh.10 (Original)	188	560	200	13	45	1

AAVR independent capsids.

Most serotypes of AAV enter cells through the universal AAVR receptor encoded by the gene KIAA0319L. The known exceptions include AAV4 and a hybrid serotype AAV32.33 (PMID: 29343568). The engineered capsids were assessed to determine which ones were AAVR dependent. A HeLa cell line is available which is deleted for AAVR (see Figure 18D).

It follows that an AAVR independent vector would infect both HeLa wildtype and HeLa-AAVR-KO equally well. AAVR dependence was highly variable with the original AAVrh.10 having a transduction ratio (AAVR+/AAVR-) of 32.6 and some capsids had apparently higher AAVR dependence such as 9D (AAVR+/AAVR- ratio 213). But three capsids stood out as being AAVR independent with AAVR+/AAVR- ratio of close to 1 (Table 2)

Table 3. Differential transduction of cell lines with or without AAVR.

Capsid	Substitution in loop VI	Insert in loop VIII	AAVR+/AAVR- ratio
9D	--	TTRGDRP (SEQ ID NO:44)	213
rh.10	--	--	32.6
36.10	IDGAATK (SEQ ID NO:64)	ENHTKND (SEQ ID NO:65)	1.20
106	--	TTRNDFLPQ (SEQ ID NO:43)	1.20
82.22	IDGQSSK (SEQ ID NO:85)	KTTRLSIVP (SEQ ID NO:66)	1.05

AAVR independent capsids would be of use in the context of grafting alternate cellular recognition sequence that target to a specific cells type. For example, cyclic RGD peptides have been used in homing to tumor cells (PMID: 12727103). Removing the capacity to bind AAVR would improve delivery to tumors. Similarly, peptide libraries have been screened to identify

homing peptides for muscle (PMID: 19474807) and vascular bed (PMID: 16387552) which would also have improved performance with AAV capsid with no competition from AAVR.

Example 2

An AAV expression cassette was developed that suppresses expression of the endogenous APOE4 gene and simultaneously provides expression of APOE2. APOE4 mice are used to demonstrate a significant shift from APOE4 to APOE2 *in vivo* that is different than that with APOE2 alone.

shRNAs /miRNAs were identified that suppress endogenous APOE expression (Figure 7). Combining multiple different miRNAs may result in more efficient suppression. shRNAs/miRNAs were shown to suppress *in vitro* expression of APOE4 and a modified APOE2 was resistant to suppression by miRNAs. In one embodiment, miRNAs and silencing resistant APOE2 are combined in one vector and *in vivo* efficacy is shown.

Exemplary miRNAs N1-4 series are in non-coding regions and so do not need codon changes while the K series of miRNAs are used in combination with silencing-resistant APOE2 (Figure 8).

miRNAs are based on the mouse mir155 backbone (designed as recommended by Fowler (PMID: 26582923) (Figure 9).

HEK293 and HuH7 cells were transfected with 1 μg pmCherry or pmCherry with mir N1, 2, 3, or 4. 48 h later, cells were harvested and RNA isolated for relative quantification analysis using multiplex qPCR (APOE + GAPDH) (Figure 10). mirN4 gave highest inhibition of APOE mRNA in 2 cell types.

Figure 11 shows target sequences.

HEK293 cells were transfected with 1 μg pmCherry, pmCherry-mir2A, or pmCherry-mir2A with mir K1, 3, 7, or 8. 48 hours later, cells were harvested and RNA isolated for APOE mRNA quantification (Figure 12) using TaqMan (absolute quantification). A combination of mirK8 and 2A provided for additional silencing.

Figure 13 depicts vectors with different exemplary miRNA combinations.

Two exemplary targets are within the coding region of APOE and they do not overlap the common APOE alleles (E2,E4) (Figure 14). A silencing resistant APOE cDNA may be used to silence E4 and augment E2.

Figure 15A shows a design for an APOE2 gene to circumvent silencing by mirK and mir2A. A Kozak sequence was added before the ATG in the open reading frame of the native gene. The APOE2 allele was converted by making codon changes to give a mismatch with mir2A and mirK targets and silent changes in codons were made to decrease or eliminate CpG. See sequence below for this exemplary allele.

ATGAAGGTTCTGTGGGCTGCGTTGCTGGTCACATTCCTGGCAGGATGCCAGGCCAAG
GTGGAGCAAGCGGTGGAGACAGAGCCGGAGCCCGAGCTGCGCCAGCAGACCGAGT
GGCAGAGCGGCCAGCGCTGGGAACTGGCACTGGGTCGCTTTTGGGATTACCTGCGCT
GGGTGCAGACGTTAAGCGAACAGGTGCAGGAGGAGCTGCTCAGCTCCCAGGTCACC
CAGGAACTGAGGGCGCTGATGGACGAGACCATGAAAGAACTCAAAGCTTATAAGAG
CGAGCTGGAGGAACAACTGACCCCGGTGGCGGAGGAGACGCGGGCACGGCTGTCCA
AGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGACATGGAGGACGTGTGCGGC
CGCCTGGTGCAGTACCGCGGCGAGGTGCAGGCCATGCTCGGCCAGAGCACCGAGGA
GCTGCGGGTGCGCCTCGCCTCCCACCTGCGCAAGCTGCGTAAGCGGCTCCTCCGCGA
TGCCGATGACCTGCAGAAGTGCCTGGCAGTGTACCAGGCCGGGGCCCGCGAGGGCG
CCGAGCGCGGCCTCAGCGCCATCCGCGAGCGCCTGGGGCCCCTGGTGGAACAGGGC
CGCGTGCGGGCCGCCACTGTGGGCTCCCTGGCCGGCCAGCCGCTACAGGAGCGGGC
CCAGGCCTGGGGCGAGCGGCTGCGCGCGCGGATGGAGGAGATGGGCAGCCGGACCC
GCGACCGCCTGGACGAGGTGAAGGAGCAGGTGGCGGAGGTGCGCGCCAAGCTGGA
GGAGCAGGCCCAGCAGATACGCCTGCAGGCCGAGGCCTTCCAGGCCCGCCTCAAGA
GCTGGTTCGAGCCCCTGGTGGAAGACATGCAGCGCCAGTGGGCCGGGCTGGTGGAG
AAGGTGCAGGCTGCCGTGGGCACCAGCGCCGCCCCTGTGCCCAGCGACAATCACTA
A (SEQ ID NO:67)

Other exemplary alleles are as follows:

atgaaggttctgtgggctgcgttgctggtcacattcctggcaggatgccaggccaaggtggagcaagcggtggagacagagccggagcc
cgagctgcgccagcagaccgagtggcagagcggccagcgctgggaactggcactgggtcgcttttgggattacctgcgctgggtgcaga
cactgtctgagcaggtgcaggaggagctgctcagctcccaggtcacccaggaactgagggcgctgatggacgagaccatgaaggagttg
aaggcctacaaatcggaactggaggaacaactgaccccggtggcggaggagacgcgggcacggctgtccaaggagctgcaggcggc
gcaggcccggctgggcgcggacatggaggacgtgtgcggccgcctggtgcagtaccgcggcgaggtgcaggccatgctcggccaga
gcaccgaggagctgcgggtgcgcctcgcctcccacctgcgcaagctgcgtaagcggctcctccgcgatgccgatgacctgcagaagcg
cctggcagtgtaccaggccggggcccgcgagggcgccgagcgcggcctcagcgccatccgcgagcgcctggggcccctggtggaac
agggccgcgtgcgggccgccactgtgggctccctggccggccagccgctacaggagcgggcccaggcctggggcgagcggctgcgc
gcgcggatggaggagatgggcagccggacccgcgaccgcctggacgaggtgaaggagcaggtggcggaggtgcgcgccaagctgg
aggagcaggcccagcagatacgcctgcaggccgaggccttccaggcccgcctcaagagctggttcgagccctggtggaagacatgca
gcgccagtgggccgggctggtggagaaggtgcaggctgccgtgggcaccagcgccgccccctg

TTCCTTCGGATAAcCAtTAGAc (SEQ ID NO:68);

Atgaaggttctgtgggctgcgttgctggtcacattcctggcaggatgccaggccaaggtggagcaagcggtggagacagagccggagcc
cgagctgcgccagcagaccgagtggcagagcggccagcgctgggaactggcactgggtcgcttttgggattacctgcgctgggtgcaga
cactgtctgagcaggtgcaggaggagctgctcagctcccaggtcacccaggaactgagggcgctgatggacgagaccatgaaggagttg
aaggcctacaaatcggaactggaggaacaactgaccccggtggcggaggagacgcgggcacggctgtccaaggagctgcaggcggc
gcaggcccggctgggcgcggacatggaggacgtgtgcggccgcctggtgcagtaccgcggcgaggtgcaggccatgctcggccaga
gcaccgaggagctgcgggtgcgcctcgcctcccacctgcgcaagctgcgtaagcggctcctccgcgatgccgatgacctgcagaagcg
cctggcagtgtaccaggccggggcccgcgagggcgccgagcgcggcctcagcgccatccgcgagcgcctggggcccctggtggaac
agggccgcgtgcgggccgccactgtgggctccctggccggccagccgctacaggagcgggcccaggcctggggcgagcggctgcgc
gcgcggatggaggagatgggcagccggacccgcgaccgcctggacgaggtgaaggagcaggtggcggaggtgcgcgccaagctgg

aggagcaagcgcaacaaatcagactccaggccgaggccttccaggcccgcctcaagagctggttcgagcccctggtggaagacatgca gcgccagtgggccgggctggtggagaaggtgcaggctgccgtgggcaccagcgccgcccctgtgcccagcgacaatcactga (SEQ ID NO:69); and

Atgaaggttctgtgggctgcgttgctggtcacattcctggcaggatgccaggccaaggtggagcaagcggtggagacagagccggagcc cgagctgcgccagcagaccgagtggcagagcggccagcgctgggaactggcactgggtcgcttttgggattacctgcgctgggtgcaga cactgtctgagcaggtgcaggaggagctgctcagctcccaggtcacccaggaactgagggcgctgatggacgagaccatgaaggagttg aaggcctatAAGAGTGAGTTAGAAGAGCAactgaccccggtggcggaggagacgcgggcacggctgtccaaggag ctgcaggcggcgcaggcccggctgggcgcggacatggaggacgtgtgcggccgcctggtgcagtaccgcggcgaggtgcaggccat gctcggccagagcaccgaggagctgcgggtgcgcctcgcctcccacctgcgcaagctgcgtaagcggctcctccgcgatgccgatgac ctgcagaagcgcctggcagtgtaccaggccggggcccgcgagggcgccgagcgcggcctcagcgccatccgcgagcgcctggggcc cctggtggaacagggccgcgtgcgggccgccactgtgggctccctggccggccagccgctacaggagcgggcccaggcctggggcg agcggctgcgcgcgcggatggaggagatgggcagccggacccgcgaccgcctggacgaggtgaaggagcaggtggcggaggtgcg cgccaagctggaggagcaggcccagcagatacgcctgcaggccgaggccttccaggcccgcctcaagagctggttcgagcccctggtg gaagacatgcagcgccagtgggccgggctggtggagaaggtgcaggctgccgtgggcaccagcgccgcccctgtgcccagcgacaat cactga (SEQ ID NO:70). See Figures 15B-D.

Figure 16 illustrates mRNA expression from an engineered APOE cDNA. Wild-type hAPOE2, silencing resistant hAPOE2 and a negative control
were transfected into HuH7 cells and 48 hours later, cells were harvested and cDNA prepared. Human APOE2 mRNA was quantified (absolute) by qRT-PCR with specific primes and probes. At least at mRNA level, CpG-free gene is expressed equally to the wild-type gene.

Figure 17 illustrates *in vivo* experiments.

Example 3

Methods

Design and Assessment of shRNAs

To suppress endogenous APOE4 in the CNS of APOE4 homozygotes, potential targets were identified in the APOE mRNA. Complementary shRNAs were designed using Vector Builder (Chicago, IL) and the Genetic Perturbation Platform (GPP) web portal of the Broad Institute (https://portals.broadinstitute.org/gpp/public/). Initially these shRNAs were cloned into plasmids using expression from the RNA polymerase III dependent U6 promoter. Assessment of the ability of these shRNAs to suppress human APOE was first tested using plasmids transfected in triplicate into human embryonic kidney HEK293T and human liver HuH7 cells and total RNA collected. APOE mRNA level was determined after 48 hours by TaqMan PCR using a FAM-MGB probe and primer combination for human APOE (Hs00171168_m1, Thermofisher) and a commercial VIC- probe/primer for the GAPDH as reference (4310884E, Thermofisher).

Design and Assessment of AAV Genomes with miRNAs Targeting APOE4

After identifying effective shRNAs that inhibited APOE expression in vitro, the designs were adapted to generate miRNAs in an optimized mouse miR-155 backbone. Two copies of the same miRNA were cloned into either the 5' intron or the 3' untranslated region of an AAV-CAG-mCherry plasmid, switching transcription from RNA polymerase III to RNA polymerase II. The miRNAs 2A, N1, N2, N3, N4, K3, K7, K8, and K13 (Table 4) were assessed in vitro for APOE mRNA suppression in human cell lines by TaqMan gene expression assay. The cells lines tested included HEK293T (human embryonic kidney cells, ATCC: CRL-3216) and HuH7 (human hepatocytes from liver tumor (RRID:CVCL 0336). After identifying effective combinations of two miRNAs, additional constructs were made with 4 miRNAs, 2 copies of one miRNA in the 5' intron and 2 copies with a different miRNA in the 3' untranslated region.

AAV Vectors with Modified Capsids

Based on the known structure of AAV vectors complexed to the primary receptor and precedents of capsid modification in other AAV serotypes (Goertsen et al., 2022; Kunze et al., 2018; Acharya et al., 2020; Chen et al., 2009; Shi & Bartlett, 2003; Vőlkner et al., 2021), modified AAVrh.10 capsids included peptide insertions in loop IV, substitutions in loop VIII, or both (Table 4I). The goal of these modifications was to enhance transduction and spatial distribution of vectors in the CNS. Capsid engineering was performed by PCR amplification of full-length AAVrh.10 rep/cap plasmid to linearize the backbone. Oligonucleotide sequences reverse-translated from peptides of interest were obtained from ThermoFisher Scientific (Waltham, MA). Purified oligonucleotides were ligated with the open-ended AAVrh.10 rep/cap plasmid by one-step NEBuilder Hi-Fi assembly method (NEB, Catalog E2621) that removes 3' and 5'-end mismatch sequences and ligates fragments. Assembled AAVrh.10 rep/cap plasmid were transformed and modified pAAVrh.10 plasmids generated. AAV expression plasmids contained a CMV/β-actin hybrid promoter with mCherry transgene surrounded by AAV2 inverted terminal repeats (ITRs). For production of capsid modified AAV vectors, HEK293T cells were transfected using PEImax with the 3-plasmid method using AAV rep/cap plasmid, pDeltaF4 helper plasmid and the mCherry transgene-containing plasmid. After 72 hours, HEK cells were harvested and AAVs purified on iodixanol gradients, followed by 1X phosphate buffered saline, pH 7.4 (PBS) washes containing 0.01% pluronic acid (P1300, Sigma-Aldrich) and concentrated using Vivapsin columns (Cytiva Life Sciences). Unmodified AAVrh.10 vector was generated using pPAK-MArh.10 containing adenovirus 5 E2, VA, and E4 helper genes supplying the required adenovirus and AAV functions with mCherry expression cassette. Vector preparations were assessed for titer by TaqMan real-time polymerase chain reaction to determine genome copies (Rosenberg et al., 2014; Rosenberg et al., 2018) and purity was assessed using SDS-PAGE.

Transduction Assays

Four human CNS-derived cell lines were obtained from ATCC and chosen to test vector transduction *in vitro*, including U87 (human glioblastoma, HTB-14), SVGp12 (human fetal glia, CRL-8621), SH-SY5Y (human neuroblastoma, CRL-2266) and HMC3 (human microglia, CRL-3304). Cells were plated in a 96-well plate (10^4/well) in triplicates. At >70% confluency, cells were infected with AAV expressing mCherry packaged in unmodified AAVrh.10 or modified AAVrh.10 capsids at multiplicity of infection 10^4 viral genomes/cell. Three days post-infection, fluorescent images were obtained using an Olympus IX71 microscope with comparable image settings between vector groups. Hoechst 33342 nuclear counterstain (62249, ThermoFisher Scientific) served as a proxy for total cells in a region of interest. For image quantification, mCherry positive cells were counted manually following the ImageJ pipeline. Multiple images were randomly chosen based on stained Hoechst cells. For each vector, transduction efficiency was reported as percent mCherry positive cells over total cells. Two-way repeated measures ANOVA with post-hoc pairwise comparison with the Tukey correction was used to identify the AAV.S2 as the best modified capsid with highest transduction of CNS derived cells compared to all other capsids.

Vector Administration

For CNS biodistribution comparisons between AAVrh.10 and AAV.S2 capsid, 7-week-old C57Bl/6 male mice (Jackson Labs, Bar Harbor, ME) received unilateral AAV administrations into the hippocampus. Mice were anesthetized with isoflurane and placed on a stereotaxic frame (Harvard Apparatus, Holliston, MA). Following a skin incision, burr holes the size of the injection needle were generated using a high-speed drill. Mice (4 to 5/group) were administered either with PBS alone, AAVrh.10 or the modified AAV.S2 at a dose of $2.5x10^{10}$ gc in 2 µl PBS using a using a 33-gauge needle (Hamilton, Reno NV) and a microprocessor-controlled infusion pump (KD Scientific, Holliston, MA) via stereotactic catheters in the hippocampus of the mouse brain at a rate of 0.2 µl/minute. Stereotactic coordinates used for the hippocampus included: - 1.7 mm antero-posterior (AP) from bregma; ± 1.2 mm medio-lateral (ML) from bregma and -1.7 mm dorso-ventral (DV) below the dura. After each vector administration, the needle was left in place for 3 minutes to minimize backflow and then slowly withdrawn. Health checks were performed 3 times/week for the first two weeks, and then weekly afterwards on all surviving mice, with observation for any visible external abnormalities around the head region and for unusual or altered behavior. After 4 weeks, brains were harvested, and 3 mm coronal sections made using a brain matrix (Harvard Apparatus, Holliston, MA). Vector biodistribution, mRNA level and mCherry expression was evaluated across the anterior-posterior axis. A second series of mice received bilateral hippocampus injection and one hemisphere preserved for molecular analysis

and the other fixed with paraformaldehyde for tissue sectioning and immunohistochemistry (IHC).

Vector Genome Quantification

Brains were homogenized using a TissueLyser instrument with 5 mm steel beads (both from Qiagen) in a lysis buffer containing 10 mM HEPES KOH, pH 7.4, 5 mM mannitol, 1 % Triton X10. Genomic DNA was isolated from tissue homogenate using Qiagen DNeasy blood and tissue kit following manufacturer's protocol and stored at -80°C until use. DNA quality was assessed using UV spectrophotometry (Nanodrop ND1000) for qPCR assay using a standard curve method in QuantStudio6 real time PCR instrument. TaqMan qPCR was performed with a primer and probe set targeting the CMV enhancer in the CAG promoter (27 and DNA standard using AAV-CAG-mCherry plasmid spanning 101 to 108 genome copies was used to generate the standard curve. The total amount of the vector genome was expressed per µg of input DNA.

Transgene mRNA Expression

mCherry mRNA expression was assessed from mice brain following intrahippocampal administration. Total RNA was isolated from brain homogenate using an RNeasy kit (Qiagen, Valencia, CA). Briefly, 200 µl of brain homogenate was mixed with 500 to 700 µl of TRIzol reagent (Thermofisher). Chloroform (150 to 200 µl) was added to trizol homogenate and centrifuged at 13,000 rpm for 15 minutes to separate the top clear layer containing RNA. Ethanol (90%, 500 µl) was added to the collected top RNA layer, mixed well, and was loaded onto a RNeasy mini column which was washed according to the manufacturer's protocol. RNA was eluted with 15 to 20 µl RNase free water. RNA was quantified by UV spectrophotometry (Nanodrop ND v1000) and stored at -80°C until use. cDNA using 1 µg of total RNA was prepared using a reverse transcription kit (Applied Biosystem, Waltham, MA) containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.75 mM MgCl2, 500 µM dNTP, 2.5 µM random hexamer, 1/µL RNase inhibitor and 2.5U/µL multiscribe reverse transcriptase in a final volume of 20 µl.

Reaction parameters for reverse transcription included: 25°C for 10 minutes; 37°C for 30 minutes and 95°C for 5 minutes. mCherry mRNA levels were assessed using a FAM dye labeled mCherry-specific primer-probe set (Life Technologies). The standard curve was generated with the relevant AAV-CAG-mCherry plasmid as the standard. The total amount of transgene mRNA was normalized to 1 µg of total input RNA.

Transgene Protein Levels

Brain homogenate of mice administered modified or unmodified AAVrh.10 vectors expressing the mCherry reporter gene were assessed for mCherry protein levels using an mCherry ELISA kit (Abcam) following manufacturer's protocol. Samples were diluted to be within the linear range of detection. Total protein concentration in brain homogenate was

measured using micro-BCA kit (Thermo Fisher Scientific, Rockford, IL).

Immunofluorescence Analytics

Mouse brains were fixed in formalin and paraffin embedded to create 5 μm thick sagittal slices on glass slides for immune histochemistry (Histowiz, New York, NY). Slides were deparaffinized and rehydrated first with (xyelene/cyanol) histoclear and then through a series of alcohol steps. To expose antigens on tissue slices for better antibody penetration, slides were boiled in a citrate buffer of pH 6.0-based antigen retrieval solution (Abcam), cooled, and washed with 1X PBS. Sections were bordered with a water-resistant PAP-pen to prevent leakage of reagents (ab2601, Abcam). Slides were blocked with 5% normal goat serum (ThermoFisher Scientific) for 2 hours at room temperature and primary antibody in blocking buffer was added for 1 hour at room temperature. Anti-mCherry Ab (Abcam, Boston, MA) was used at a dilution of 1:500. Other primary antibodies used in colocalization experiments were anti-NeuN (ab104225, Abcam) for mature neurons (dilution, 1:500) and anti-GFAP (ab4674, Abcam) to identify mature astrocytes (dilution, 1:500). All slides were washed 3x for at least 10 min each with 1x TBS-T. Goat anti-chicken or anti-mouse secondary antibodies with IgY H&L (Alexa Fluor 488, ab150169, Abcam, Waltham MA), were used at a dilution of 1:1,000 in blocking buffer at room temperature for 1 hour to achieve single or dual antibody staining. Slides were washed, stained with DAPI, and mounted with DAKO aqueous mounting medium (DAKO) and covered with 22 mm x 40 mm coverglass (Corning, Glendale, AZ). Cells were imaged using EVOS FL Auto automated scanning microscope (Life technologies, Carlsbad, CA). Images were analyzed and region of interest evaluated using ImageJ28.

In Vivo Assessment of AAV Vectors with miRNAs

Suppression of APOE4 expression in vivo was assessed in TRE4 mice (provided by Patrick Sullivan at Duke University (Bales et al., 2009)), in which the mouse ApoE gene has been deleted and the human APOE4 gene inserted under the control of the murine ApoE locus. Five-month-old TRE4 mice received a unilateral administration into the right hippocampus of $2.5x10^{10}$ genome copies of AAV vectors expressing 2 copies each of miRNAs N4 and 2A (referred to as "mirAPOE"). The expression cassette was driven by the CAG promoter and included an mCherry gene flanked by two copies of miRNA 2A in the 5' intron and 2 copies of the miRNA-N4 in the following 3' untranslated region. This expression cassette was packaged either in the AAVrh.10 capsid or the modified AAV.S2 capsid. Control mice (n=5/group) received either PBS or the same vectors but lacking the miRNAs (i.e., AAVrh.10-CAGmCherry and AAV.S2-CAGmCherry). Two weeks after vector administration, mice were sacrificed, and the brain was cut into 3 mm coronal sections and bisected. APOE4 and mCherry mRNA levels were determined by TaqMan PCR relative to the mouse Tfrc reference gene (4458366,

Thermofisher). Gene expression quantitation was validated by using a constant amount of RNA input and absolute copy quantitation with a reference plasmid standard. APOE levels were measured with a human APOE-specific ELISA (ab108813, Abcam, UK) and normalized to total protein.

microRNA quantification

miRNA 2A and miRNA N4 levels were quantified in mice brains by qPCR using specific custom-designed primer/probe sets for each miRNA (ThermoFisher Assay design for mir2A: CT7DP3N; assay design for mir N4: CTNKTC7). A ubiquitously expressed miRNA (ThermoFisher commercial assay: hsa-mir361-5p; A25576) was assessed from the same tissue samples serving as endogenous control. Briefly, total RNA was extracted using TRIzol-chloroform method. A total of 100 µl aqueous phase RNA mixed in 100% ethanol (1:1 ratio) was transferred to mirVana miRNA isolation spin columns (Thermofisher AM1561). Samples in column were washed in 700 µl of wash solution 1 followed by wash solution 2 (2x) as per manufacturer's protocol. miRNA was eluted from column in 80 µl of warm elution buffer. Total miRNA was quantified in Qubit and 5 ng of total miRNA was used for cDNA synthesis using mir2A- or mirN4- specific primers. Specific miRNA products were quantified by qPCR by TaqMan.

Statistics

Data analysis was performed in GraphPad Prism v9.0 and Microsoft excel. Groups with two categorical variables were evaluated with 2-way ANOVA in all immunohistochemistry experiments comparing vector biodistribution by brain structure. All other analyses used either 1-way ANOVA with post hoc tests or Student unpaired t-test. Standard curve evaluation and unknown interpolations from ELISA was performed in GraphPad Prism using either linear or 4PL-regression that assumes symmetry around the inflection point with a minima and maxima.

Results

miRNAs that Effectively Suppresses APOE Expression *In Vitro*

Multiple targets within the human APOE4 mRNA were identified (Figure 18A, Table 4). Plasmids were designed expressing shRNAs targeting these sites expressed using the U6 promoter. Those effective in suppression of endogenous APOE4 expression in vitro were engineered into miRNAs based on the mouse mir155 backbone (Fowler et al., 2016) and inserted in the 3'untranslated region of a CAG-mCherry expression cassette. Plasmids expressing 9 different miRNAs were assessed under standard conditions for suppression of endogenous APOE mRNA levels following transfection into HEK293T and HuH7 cells (Table 4). Plasmids with miRNAs decreased endogenous APOE expression to between 1.5- and 2.9-fold in 293 cells and between 2.5- and 8.3-fold in Huh cells. APOE suppression was an average of 1.5±0.1 times

greater in the HuH7 liver-derived cell relative to HEK293T cells ($p<0.01$, paired t test). Two miRNAs (2A and N4) giving the highest average suppression in the two cells lines were chosen for follow up.

Based on the literature, use of two independent miRNAs provides better overall suppression of the target gene than one. With this background, the best performing miRNAs were tested in combination with each other. For example, the expression of APOE following transfection of a plasmid expressing mir2A in 293T cells was assessed and the impact of four other miRNAs (N1, N2, N3 N4) both alone and in combination with mir2A was determined (Figure 18 B,C). In 293T cells, the plasmid with 2 copies of mir2A in the intron of the CAG-mCherry expression cassette reduced APOE expression by 2.4 ± 0.1-fold relative to the control transfected with the same CAGmCherry plasmid with no mir. Moving those 2 copies of mir2A to the 3' untranslated region increased their impact, with a 2.8 ± 0.4-fold decrease ($p<0.01$ compared to location in the intron). In all cases, the combination of two miRNAs provided greater suppression of APOE expression than either alone. In 293 cells, mirN4 provided the most effective suppression in partnership with mir2A (Figure 18B). While the plasmid with mirN4 alone reduced APOE expression 2.2 ± 0.3-fold, when combined with mir2A the overall suppression was 3.5±0.4-fold (mir2A/mirN4 combination $p<0.005$ compared to other combinations with mir2A). A similar pattern of combining two different miRNAs was seen in the Huh7 cell line (Figure 18C). While the combination of 2 miRNAs provides further suppression beyond each alone, they did not achieve the product of their individual effects implying they are not truly independent.

AAV Capsid Optimization

APOE is produced in the CNS primarily by astrocytes and to a lesser extent by microglia and under stress can be produced by neurons (Lanfranco et al., 2021). Widespread APOE suppression needs an AAV capsid with broad CNS distribution and tropism to several cell types, including astrocytes and neurons. AAV serotype rh.10 was used as a base, which has the properties of broad distribution in the CNS, including neurons, and to a lesser extent, astrocytes (Cearley & Wolfe, 2006). Using existing literature precedents (Goertsen et al., 2022; Kunse et al., 2018; Fischer et al., 2022; Hanlon et al., 2019; Acharya et al., 2020; Chen et al., 2009; Shi & Barlett, 2003; Vőlkner et al., 2021) combined with molecular modelling, we designed 12 novel rh.10 variants (Table 4) by replacing residues 451 to 459 in loop IV and/or inserting short peptides into position 588 in loop VIII, with some capsids, S4, S5 and S6 having two modifications from different sources.

The modified capsids were assessed for transduction of 4 human CNS cell lines in vitro. An mCherry expression cassette was packaged into each capsid allowing transduction efficiency

to be assessed by cell counting. The results (Figure 19, Table 5) demonstrated that most of the modified capsids showed enhanced transduction of multiple cell types at the same multiplicity of infection as the AAVrh.10 control. The most striking example was the S2 modification which provided the best transduction averaged over 4 cell lines including glioblastoma (U87; 5.3 ± 0.1-fold better than rh.10), fetal glia (SVGp12; 5.3 ± 0.1-fold), neuroblastoma (SH-SY5Y; 6.7 ± 0.3-fold) and microglia (HMC3; 6.2 ± 0.3-fold). On this basis, the AAV.S2 variant was the focus of the subsequent studies.

Distribution of Transgene Expression

To quantify the spread of vector and transgene expression from the AAV.S2 vector compared to the AAVrh.10 parent capsid, intra-hippocampal administration was carried out. Upon sacrifice at 28 days post-administration, the brains were cut into serial 3 mm coronal slices (Figure 20A) and vector DNA, mCherry mRNA and mCherry protein quantified (Figure 20B-D). Relative to the AAVrh.10 vector, the AAV.S2 vector genome and mRNA data provided higher expression, with an increase in amplitude and shift in the peak expression towards a more posterior sections tailing into the cerebellum (Figure 20B, C). The integrated area under the curve was 3.9 ± 0.7 times higher for genome copies and 4.5 ±0.2-fold higher for mRNA in the AAV.S2 mice compared to the AAVrh.10 mice. The mCherry protein expression demonstrated a similar pattern to the vector genome and mRNA with the same overall higher level but with a shift toward more expression in posterior sections and in the anterior sections representing the olfactory lobe (Figure 20D). Like the DNA and mRNA levels, the integrated area of protein expression was 3.7 ± 0.5-fold higher.

The superior performance of capsid S2 was also evident by morphological assessment (Figure 21). After hippocampus administration at a dose where AAVrh.10-mediated transduction of 55.5±5.2% of cells in the hippocampus and 45.4±4.1% of the cells in the cortex, AAV.S2 achieved significantly higher transduction at 72.0±2.5 and 70.0±4.3 % respectively (Figure 21C). The superior performance of AAV.S2 was also evident in the level of expression (Figure 21D) which was 1.6±0.2 times higher in AAV.S2 than in rh.10 in the hippocampus ($p<0.05$) and 2.5±0.3 times higher in the cortex ($p<0.01$).

To determine if the enhanced transduction mediated by capsid AAV.S2 was specific to cell type, co-immunofluorescence studies were conducted. Neuronal colocalization was assessed using NeuN as a marker. In the hippocampus, there was extensive co-localization of AAV-derived mCherry with neurons and it was more evident in the AAV.S2 transduced brains than in the AAVrh.10 controls (Figure 22A,B). In the hippocampus, the percentage of mCherry positive cells also positive for NeuN increased from 2.8 ± 0.5% in AAVrh.10-treated mice to 12.8 ± 1.2 in AAV.S2-treated mice (Figure 22D). Astrocyte transduction was assessed using GFAP as an

astrocyte-specific marker (Figure 22C). ImageJ analysis established that the percentage of mCherry positive cells also positive for GFAP increased from 0.8 ± 0.2% in AAVrh.10 treated mice to 4.6 ± 0.4% in S2 treated mice (Figure 22D). Overall, the data suggests that the AAV.S2 capsid provides greater transduction and spread through the CNS compared to the rh.10 parent capsid, while retaining the ability to transduce astrocytes.

In Vivo Assessment of AAV with miRNAs Targeting APOE

The ability of artificial miRNAs to suppress APOE expression *in vitro* combined with a modified capsid with greater capacity to transfect CNS cells provides an opportunity to assess the *in vivo* suppression of APOE4 expression in brain. In the TRE4 mouse, the human APOE4 gene is inserted into the mouse ApoE locus and therefore reagents intended to suppress human APOE4 expression can be evaluated. The vectors used included an expression cassette with 2 copies of mir2A in the CAG intron and 2 copies of mirN4 in the 3'untranslated region of a CAG-mCherry (referred to as AAV-mirAPOE) (Figure 23A). The control consisted of the CAG-mCherry cassette with no miRNAs (AAV-mCherry). The two expression cassettes were delivered in either the original AAVrh.10 capsid or the optimized AAV.S2 capsid.

Two weeks following unilateral hippocampus administration, the brain was collected and cut into coronal slices (A to D) where sections C and D represent the administration site in the hippocampus. Two methods were used to assess expression of the miRNAs that suppress APOE2 expression (Figure 23B). Since the anti-APOE miRNAs 2A and N4 are co-transcribed with the mCherry transgene, the mCherry mRNA is an indirect indication of miRNA expression. In the injected sections C and D, the vectors with or without the included miRNAs both expressed mCherry. Consistent with the data in Figure 3, the AAV.S2-mirAPOE vector expressed 4.8 times more mRNA than the AAVrh.10-mirAPOE vector in sections C and D where the vector was administered ($p<0.05$). Direct determination of the mir2A expression level relative to an internal miRNA standard gave a similar result with the AAV.S2 capsid providing a 2.6-fold higher normalized level of mir2A than the AAVrh.10 capsid ($p<0.05$, Figure 23B).

The impact of mir2A and mirN4 on the levels of endogenous APOE4 mRNA (Figure 23C) was assessed. In the both the AAVrh.10 and AAV.S2 treated mice, the mCherry cassette with no miRNAs did not impact APOE mRNA level in any section of the injected hemisphere ($p>0.1$). Addition of 2 copies each of mir2A and mirN4 resulted in a local reduction of APOE expression level by a maximum of 2.4 ± 0.3-fold in sections C and D. Overall the same expression cassette with mirAPOE delivered by the AAV.S2 capsid reduced APOE levels by a total of 3.4 ± 0.4-fold (Figure 23C). The specific of this effect was verified by assessing regional APOE protein level by ELISA. For the mirAPOE cassette with the AAV.S2 capsid there was a clear suppression of APOE level in the coronal sections at the sites of vector administration

which was not seen with the mCherry or PBS controls (Figure 23D). Specificity of the suppression of the APOE by the miRNA was also confirmed by determining the correlation of mir2A and APOE expression across all sections for all mice that received the mCherry-mirAPOE expression cassette (Figure 23E). Overall, there was a strong negative correlation with high significance (r^2 =0.56, p<0.001).

Table 4. Human APOE miRNA Targets1

Name	APOE target sequence[1]	Location in endogenous APOE mRNA/type[2]	Inhibition in 293T cells[3](% decrease)	Inhibition in HuH7 cells[3] (% decrease)
2A	Ggagttgaaggcctacaaatc (SEQ ID NO:33)	330-351/coding	66	69
N1	Aatcacaggcaggaagatgaa (SEQ ID NO:34)	54-74/overlap with start codon	36	60
N2	Ctggccaatcacaggcaggaa (SEQ ID NO:35)	48-68/(5'UTR)	35	75
N3	Agtttaataaagattcaccaa (SEQ ID NO:36)	1136-1156/3'UTR	35	64
N4	Ataaagattcaccaagtttca (SEQ ID NO:37)	1142 – 1162/3'UTR	57	82
K3	Gcccagcgacaatcactgaacg (SEQ ID NO:38)	1005 – 1026/overlap with stop codon	52	72
K7	Acaaatcggaactggaggaaca (SEQ ID NO:39)	344-365/coding	57	74
K8	gcagacactgtctgagcaggtg (SEQ ID NO:40)	43-263/coding	53	82
K13	Aggcccagcagatacgcctgca (SEQ ID NO:41)	860-881/coding	n.d.[4]	88

[1]Targets were chosen based on online design utilities including VectorBuilder and the Broad Institute Genetic Perturbation Platform (https://portals.broadinstitute.org/gpp/public/).
[2]Using canonical mRNA coordinates (GenBank NM_000041.4).
[3]In standard transfection conditions with 0.5µg of plasmid co-expressing miRNA with mCherry driven by the CAG promoter.
[4]n.d. - Not determined.

Table 5. AAVrh.10 Variants

Capsid	Substitution at 451[1]	Insert at 588[2]
AAVrh.10	–	–
AAVrh.10.M2		NNPTPSR (SEQ ID NO:86)
AAVrh.10.M3	–	CDCRGDCFC (SEQ ID NO:87)
AAVrh.10.M4	-	NVVRSSS (SEQ ID NO:88)

AAVrh.10.M6	-	FVVGQSY (SEQ ID NO:89)
AAVrh.10.M8–	-	CRGGKRSSC (SEQ ID NO:71)
AAVrh.10.P1	-	RGDLGLS (SEQ ID NO:72)
AAVrh.10.S1	DGAATKN (SEQ ID NO:73)	-
AAV.S2	DGAATK**Q** (SEQ ID NO:74)	-
AAVrh.10.S3	**E**GQSSKS-(SEQ ID NO:75)	-
AAVrh.10.S4	**DGAATKN** (SEQ ID NO:76)	**CDCRGDCFC** (SEQ ID NO:77)
AAVrh.10.S5	**DGQSSKS** (SEQ ID NO:78)	**CDCRGDCFC** (SEQ ID NO:77)
AAVrh.10.S6	**EGQSSKS** (SEQ ID NO:79)	**CDCRGDCFC** (SEQ ID NO:77)

[1-2] Residues in *non-bold* type represent published sequences; residues in **bold** represent novel sequences based on molecular modelling. S4,5,6 represent novel combinations.

Table 6. Expression of AAVrh.10 Modified Capsids in Human Neurologic Cell Lines[1]

	U87		**SVGp12**		**SH-SY5Y**		**HMC3**	
Capsid	**%[2]**	**P[3]**	**%[4]**	**p**	**%[4]**	**p**	**%[4]**	**p**
AAV.M2	14.7 ± 1.9	n.s	16.7 ± 1.0	*	27.2 ± 1.4	***	35.9 ± 1.3	***
AAV.M3	47.6 ± 8.7	*	69.0 ± 1.7	***	26.4 ± 2.7	**	43.0 ± 1.6	***
AAV.M4	27.3 ± 4.7	n.s	20.1 ± 2.3	*	8.7 ± 1.4	n.s	-	
AAV.M5	16.1 ± 1.3	n.s	26.5 ± 6.0	n.s	10.6 ± 2.7	n.s	-	
AAV.M6	46.0 ± 0.1	**	42.3 ± 0.1	***	57.9 ± 0.1	***	49.9 ± 0.5	***
AAV.M8	37.4 ± 2.1	*	41.2 ± 6.5	*	11.1 ± 0.6	**	61.7 ± 7	**
AAV.P1	49.0 ± 1.8	**	65.8 ± 3.3	***	35.4 ± 1.7	***	-	
AAV.S1 39.1 ± 5.3	*	24.3 ± 4.2	*	22.8 ± 4.7	*	34.6 ± 4.9	*	
AAV.S2	78.6 ± 5.0	**	60.6 ± 3.8	***	40.3 ± 2.0	***	66.8 ± 2.9	***
AAV.S3	46.7 ± 6.8	*	19.8 ± 4.8	n.s	16.0 ± 2.6	*	45.2[4]	
AAV.S4	35.7 ± 8.6	n.s	30.6 ± 4.2	*	19.7 ± 4.0	*	29.6[4]	
AAV.S5	35.7 ± 6.2	*	17.4 ± 0.4	*	18.7 ± 3.5	*	32.2[4]	
AAV.S6	22.0 ± 4.5	n.s	25.6 ± 2.9	*	38.1 ± 1.8	***	78.24	

[1] - not determined
[2] % of cells positive for mCherry
[3] p values are derived from t-test comparing mean of cell line expression in capsids to that of AAVrh.10. n.s = not significant; * p<0.05; ** p<0.005; *** p<0.0005
[4] Mean of n=2 determinations

Discussion

The link between the E4 variant of the APOE gene and risk for Alzheimer's disease provides opportunities for therapeutic intervention (Wolters et al., 2019; Chen et al., 2021). This study explores AAV-mediated delivery of interfering RNAs to suppress APOE4 expression in the brain. The credibility of this approach is provided by a recent report of six APOE4 subjects with heterozygous loss of function mutations in the second APOE allele who did not develop early Alzheimer's disease (Chemparathy et al., 2023), i.e., reducing APOE4 expression by 50% may be protective.

RNA interference has previously been used to suppress specific mRNAs with significant impacts on disease progression in several diseases including spinocerebellar ataxia type 133, Charcot-Marie-Tooth disease type 1A (Stavrou et al., 2022), facioscapulohumeral muscular dystrophy (Wallace et al., 2011) and Huntington disease (Thomson et al., 2023). Methods for design and optimization of shRNA and artificial microRNAs targeting a gene of interest are well developed (Fowler et al., 2016; Kotowska-Zimmer et al., 2021). APOE mRNA levels are high throughout the brain (https://www.proteinatlas.org/ENSG00000130203-APOE/summary/rna). A combination of 2 miRNAs targeting different regions of the mRNA was needed to obtain effective of APOE4 level. Use of two miRNA has also been used for effective suppression of the PABPN1 gene as a potential therapy for oculopharyngeal muscular dystrophy (Strings-Ufombah et al., 2021).

While anti-sense oligonucleotides have the potential to achieve gene suppression (Hill & Meisler, 2021; Lauffer et al., 2024) they require repeated administration while AAV delivery requires only a one-time administration of a vector for persistent RNA interference (Borel et al., 2014; Borel & Mueller, 2019; Wong et al., 2023). Due to the blood brain barrier, the systemic and brain APOE pool are largely separate16 and therefore AAV delivery of antisense constructs to the brain is optimal for protection against Alzheimer's disease. APOE is expressed throughout the brain43, so we sought AAV with both high expression level and maximum spread following a single administration would be effective.

Suppression of APOE4 expression calls for an AAV vector that transduces astrocytes and provides widespread transduction following administration by clinically accessible methods. Serotype rh.10 ranks highly in head-to-head comparisons of brain transduction (Cearley &Wolfe, 2006), but we sought to enhance its utility by adding capsid modifications known to be useful in the context of other AAV serotypes. *In vitro* and in vivo assessments of multiple possible capsid modifications identified AAV.S2 which contains a substitution of peptide DGAATKQ (SEQ ID NO: 90) at position 451 of the AAVrh.10 capsid. This is a novel variant from AAV9.CAP-

B1018, an AAV9 variant originally selected for CNS transduction following intravenous administration. AAV9.CAP-B10 was assessed following intravenous administration to marmosets and found to deliver a 6-fold higher mRNA level in brain compared to the AAV9 basal vector (Goertsen et al., 2022). This is comparable to the observation of a 4.5-fold increase in mRNA level obtained from a similar peptide inserted into the AAVrh.10 backbone following direct administration of vector into the brain parenchyma.

The utility of capsid AAV.S2 was demonstrated by its capacity to suppress APOE4 expression following direct injection into hippocampus of TRE4 mice expressing human APOE4. The better spread and higher level of reporter gene expression of AAV.S2 at the same dose was also manifest in the more effective suppression of endogenous APOE4 expression in TRE4 mice. In the context of the complexity and expense of making AAV vector at clinical scale, this additional efficacy per unit dose may be a major asset.

The data show the capacity of AAV mediated CNS delivery of miRNAs to suppress endogenous CNS APOE4 expression by a significant amount. Based on the report of APOE4 heterozygous, Alzheimer's disease-free older adults with a null second allele this alone could be an effective therapy to reduce risk.

In summary, with the goal of developing a therapeutic that will lower APOE4 levels in the CNS of APOE4 homozygotes, it was hypothesized that this could be achieved using adeno-associated virus (AAV) expression of microRNAs (miRNA) designed to hybridize to and suppress expression of human APOE mRNA. Two strategies were employed. First, miRNAs were designed that targeted various regions in the human APOE coding sequence and these miRNAs were tested *in vitro* and the ability of these miRNAs to suppress human APOE4 in the CNS of mice with the human APOE4 coding sequence replacing the mouse APOE determined. Second, effective suppression of APOE4 also requires a delivery system to provide maximum spread throughout the brain parenchyma. Since systemic and CNS pools of APOE are largely independent (Huynh et al., 2019) and because hepatic APOE does not affect CNS amyloid pathology, enhanced delivery of the APOE4 silencing expression cassette to the brain is desirable. To achieve this, AAVrh.10 provides widespread gene expression following direct administration to the CNS. To enhance the delivery capacity of AAVrh.10, a variety of peptide insertions in the capsid loops IV and VIII were used to generate 2nd generation AAVrh.10 variants with enhanced CNS distribution. These studies identified a variant AAVS2 with much higher expression and wider spatial reach than AAVrh.10. By combining this capsid with an enhanced expression cassette delivering miRNAs to suppress APOE4 expression, highly effective suppression of APOE mRNA was achieved following direct administration into hippocampus of the TRE4 mouse which expresses the human APOE4 gene.

Example 4

Variants of APOE, a 299 amino acid lipid transport protein that functions as a major carrier of cholesterol in the CNS, are the major genetic risk factors for sporadic late-onset Alzheimer's disease (AD). APOE has 3 common variants: APOE3 (C112/R158), APOE4 (R112/R158) and APOE2 (C112/C158). Epidemiologic, clinical and experimental evidence demonstrates that, compared to the most common APOE3 allele, APOE4 is pathogenic, conveying a high risk for AD and APOE2 is protective, reducing risk. Inheritance of the APOE4 allele is linked to abnormal lipid transport in the brain leading to neuroinflammation, cognitive impairment, and accumulation of tau and amyloid, the key histological hallmarks of AD. In the context that APOE4 is toxic while APOE2 is protective, a therapy for APOE4 homozygotes is to reduce CNS levels of APOE4 and to substitute APOE4 by addition of APOE2. A "silence and replace" therapy was developed, where suppression of CNS APOE4 is achieved with microRNAs (miRNA) designed to hybridize to and "silence" the endogenous human APOE4 mRNA, together with simultaneous "replacement" with an APOE2 gene resistant to the suppressing miRNA. APOE2 transcripts expressed from a vector with those elements can evade silencing by the miRNA that inhibits the endogenous mRNA.

Design. Multiple microRNAs were screened to determine which ones inhibited APOE mRNA levels. One example, mir2A, is an engineered mouse mir155 derivative with the recognition sequence GGAGTTGAAGGCCTACAAATC (SEQ ID NO:33) corresponding to nucleotides 330-351 of the APOE mRNA (GenBank NM_000041.4). To create a replacement APOE2 that evades silencing, the redundancy of the genetic code was exploited to change codons in a way that preserves the encoded amino acid sequence while making the replacement gene that is unrecognizable to mir2A.

```
                    MetLysGluLeuLysAlaTyrLysSer
APOE-SR     GACCATGAAAGAACTCAAAGCTTATAAGAGCGAGCTGG...
            ||||||||| ||   | || || || ||  ||||||||
NATIVE      GACCATGAAGGAGTTGAAGGCCTACAAATCGGAACTGG
                    MetLysGluLeuLysAlaTyrLysSer
```

MetLysGluLeuLysAlaTyrLysSer (SEQ ID NO:81)
GACCATGAAAGAACTCAAAGCTTATAAGAGCGAGCTGG (SEQ ID NO:82)
GACCATGAAGGAGTTGAAGGCCTACAAATCGGAACTGG (SEQ ID NO:83)
MetLysGluLeuLysAlaTyrLysSer (SEQ ID NO:84)
GAACTCAAAGCTTATAAGA (SEQ ID NO:85)
GGAGTTGAAGGCCTACAAAT (SEQ ID NO:86)

In vivo verification. The silencing resistant APOE2-SR gene has been delivered to the hippocampus of mice expressing APOE4 by AAV vectors with and without concomitant expression of miRNA2A. The functionality of APOE-SR in avoiding silencing is clear in that the total APOE expression level is the same with and without the miRNA when assessed at the protein level or at the mRNA level with probes that detect all APOE variants.

Sequence of APOE2-SR

>APOE2-SR (354 bp)
ATGAAGGTTCTGTGGGCTGCGTTGCTGGTCACATTCCTGGCAGGATGCCAGGCCAAG
GTGGAGCAAGCGGTGGAGACAGA
GCCGGAGCCCGAGCTGCGCCAGCAGACCGAGTGGCAGAGCGGCCAGCGCTGGGAA
CTGGCACTGGGTCGCTTTTGGGATT
ACCTGCGCTGGGTGCAGACACTGTCTGAGCAGGTGCAGGAGGAGCTGCTCAGCTCC
CAGGTCACCCAGGAACTGAGGGCG
CTGATGGACGAGACCATGAAAGAACTCAAAGCTTATAAGAGCGAGCTGGAGGAACAA
CTGACCCCGGTGGCGGAGGAGAC
GCGGGCACGGCTGTCCAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGAC
ATGGAGGACGTGTGCGGCCGCCTGG
TGCAGTACCGCGGCGAGGTGCAGGCCATGCTCGGCCAGAGCACCGAGGAGCTGCG
GGTGCGCCTCGCCTCCCACCTGCGC
AAGCTGCGTAAGCGGCTCCTCCGCGATGCCGATGACCTGCAGAAGTGCCTGGCAGT
GTACCAGGCCGGGGGCCCGCGAGGG
CGCCGAGCGCGGCCTCAGCGCCATCCGCGAGCGCCTGGGGCCCCTGGTGGAACAG
GGCCGCGTGCGGGCCGCCACTGTGG
GCTCCCTGGCCGGCCAGCCGCTACAGGAGCGGGCCCAGGCCTGGGGCGAGCGGCT
GCGCGCCGCGGATGGAGGAGATGGGC
AGCCGGACCCGCGACCGCCTGGACGAGGTGAAGGAGCAGGTGGCGGAGGTGCGCG
CCAAGCTGGAGGAGCAGGCCCAGCA
GATACGCCTGCAGGCCGAGGCCTTCCAGGCCCGCCTCAAGAGCTGGTTCGAGCCCC
TGGTGGAAGACATGCAGCGCCAGT
GGGCCGGGCTGGTGGAGAAGGTGCAGGCTGCCGTGGGCACCAGCGCCGCCCCTGT
GCCCAGCGACAATCACTGA
(SEQ ID NO:87)

Results

Endogenous APOE4 suppression was achieved by identification of miRNAs that suppress human APOE4 expression in TRE4 mice with humanized APOE4 gene when delivered with AAV.S2, a neurotropic AAVrh.10 variant that has broad CNS distribution following intraparenchymal CNS administration. By combining 2 different APOE-directed miRNAs (2 copies of mir2A, which is in the intron before the APOE-SR, and mirN4 wich is after the APOE2-SR but before polyA site), over 70% suppression of endogenous APOE4 mRNA level could be obtained (Karan et al., Hum. Gene Ther., 35:904 (2024)). A silencing resistant (SR) APOE2 gene (APOE2-SR) was engineered that was expressed at high levels even in that suppress endogenous APOE4 expression.

AAV.S2 vectors expressing APOE2-SR with and without the miRNAs were administered to the hippocampus of human APOE4 (TRE4) mice and APOE protein levels assessed 2 weeks later by ELISA in coronal sections of the brain. Relative to endogenous APOE4 levels in TRE4

mice PBS control, both APOE2-SR vectors increased total APOE protein by 2.0±1.1 fold for APOE-SR and 2.3±1.0 fold for APOE-SR with miRNAs ($p<0.05$ compared to PBS control, $p>0.5$ comparing APOE2-SR with or without the miRNAs) (Figures 24-25). TaqMan allelic discrimination assay was used to assess APOE2 to APOE4 ratio. Both vectors with APOE2-SR increased the E2/E4 ratio relative to controls by 3.5±0.6 fold for APOE2-SR and 6.3±0.4 fold for APOE2-SR+miRNAs ($p<0.05$ comparing APOE2-SR with and without miRNA). Thus, a single AAV vector can deliver a cassette that has the dual functions of suppressing expression of the toxic APOE4 variant while also delivering the protective APOE2 variant. This dual approach may provide a more potent gene therapy for APOE4-dependent Alzheimer's disease than either alone.

Example 5

In one embodiment, a gene therapy vector is provided comprising i) two or more distinct RNAi nucleic acid sequences for inhibition of APOE4 expression, wherein optionally one RNAi nucleic acid sequence targets a non-coding APOE sequence and another RNAi nucleic acid sequence targets a APOE coding sequence; or ii) one or more copies of a RNAi nucleic acid sequence comprising SEQ ID NO:33 for inhibition of APOE4 expression, e.g., one or more RNAi nucleic acid sequences that inhibit APOE transcripts comprising SEQ ID NO:83 or 84 but not APOE transcripts comprising SEQ ID NO:82 or 85. In one embodiment, the two or more RNAi nucleic acid sequences correspond to different sequences in an APOE coding region. In one embodiment, the two or more RNAi nucleic acid sequences correspond to different sequences in an APOE non-coding region. In one embodiment, the RNAi nucleic acid sequences are inserted 5' or 3' to an open reading frame in the vector. In one embodiment, the RNAi nucleic acid sequences are inserted 5' and 3' to an open reading frame in the vector. In one embodiment, one of the RNAi nucleic acid sequences corresponds to a sequence in an APOE coding region and another of the RNAi nucleic acid sequences corresponds to a sequence in an APOE non-coding region. In one embodiment, there are from 1 to 5 copies of the RNAi sequences. In one embodiment, there are from 2 to 5 copies of the RNAi sequences. In one embodiment, the gene therapy vector is a viral vector, e.g., an AAV, adenovirus, lentivirus, herpesvirus or retrovirus vector. In one embodiment, the AAV genome is an AAV2, AAV5, AAV9 or AAVrh10 genome. In one embodiment, the APOE4 is human APOE4. In one embodiment, the vector further comprises a coding region for APOE2 that expresses a mRNA that does not bind RNA transcribed from the one or more of the RNAi nucleic acid sequences. In one embodiment, the APOE2 is human APOE2. In one embodiment, one of the RNAi sequences comprises one of SEQ ID Nos. 33-41 or the complement thereof. In one embodiment, the RNAi comprises miRNA, and optionally a plurality of miRNA sequences. In

one embodiment, the open reading frame for APOE2 comprises a plurality of silent nucleotide substitutions relative to SEQ ID NO:6. In one embodiment, the APOE coding region comprises SEQ ID NO:82, 85 or 87. Also provided is a composition comprising the gene therapy vector and optionally a pharmaceutically acceptable carrier.

Further provided is a method to prevent, inhibit or treat a neurological disease in a mammal, comprising: administering to the mammal an effective amount of a composition comprising the gene therapy vector, and a method to prevent, inhibit or treat a disease associated with APOE4 expression in a mammal, comprising: administering to the mammal an effective amount of a composition comprising the gene therapy vector. In one embodiment, the mammal is a E2/E4 heterozygote. In one embodiment, the mammal is a E4/E4 homozygote. In one embodiment, the composition is systemically administered. In one embodiment, the composition is orally administered. In one embodiment, the composition is intravenously administered. In one embodiment, the composition is locally administered. In one embodiment, the composition is injected. In one embodiment, the composition is administered to the central nervous system. In one embodiment, the composition is administered to the brain. In one embodiment, the composition is a sustained release composition. In one embodiment, the mammal is a human. In one embodiment, the RNAi sequences comprise a plurality of miRNA sequences each comprising the one or more RNAi nucleic acid sequences for inhibition of APOE4 mRNA. In one embodiment, one of the miRNA sequences in the vector is inserted 5' to the open reading frame and another is inserted 3' to the open reading frame. In one embodiment, the RNAi sequences comprise a miRNA sequence comprising one or more of SEQ ID Nos. 33-41. In one embodiment, the miRNA sequence in the vector is inserted 5' to the open reading frame. In one embodiment, the miRNA sequence in the vector is inserted 3' to the open reading frame. In one embodiment, the vector is a rAAV comprising a capsid other than an AAV9 or AAVrh10 capsid.

In one embodiment, a gene therapy vector comprising a first promoter operably linked to a nucleic acid sequence comprising a 5' untranslated region, an open reading frame encoding APOE2 and a 3' untranslated region, and an isolated nucleotide sequence comprising one or more RNAi nucleic acid sequences for inhibition of APOE4 mRNA inserted 5' and/or 3' of the open reading frame, wherein the one or more RNAi nucleic acid sequences comprise SEQ ID NO:33. In one embodiment the gene therapy vector is a rAAV vector which optionally comprises a capsid comprising one of SEQ ID Nos. 42-66, 71-79 or 85-89. In one embodiment, the capsid comprises SEQ ID NO:74.

Also provided is a method to prevent, inhibit or treat a neurological disease in a mammal, comprising: administering to the mammal an effective amount of a composition comprising the gene therapy vector, wherein the amount increases total APOE protein in the mammal by at least

1.25, 1.5 fold, 2 fold, 2.5 fold or more, and/or increases E2/E4 ratio in the mammal by at least 2 fold, 2.5 fold, 3 fold, 3.5 fold or more relative to a corresponding mammal not administered the composition, and a method to prevent, inhibit or treat a disease associated with APOE4 expression in a mammal, comprising: administering to the mammal an effective amount of a composition comprising the gene therapy vector, wherein the amount increases total APOE protein by at least 1.25 fold, 1.5 fold, 2 fold, 2.5 fold or more and/or increases E2/E4 ratio by at least 2 fold, 2.5 fold, 3 fold, 3.5 fold or more relative to a corresponding mammal not administered the composition. In one embodiment, the gene therapy vector is a rAAV vector. In one embodiment, the AAV capsid is not an AAV9 or AAVrh10 capsid. In one embodiment, the AAV capsid comprises one of SEQ ID Nos.42 to 66, 71 to 79 or 85 to 89. In one embodiment, the mammal is a E2/E4 heterozygote. In one embodiment, the mammal is a E4/E4 homozygote. In one embodiment, the composition is systemically administered. In one embodiment, the composition is orally administered. In one embodiment, the composition is intravenously administered. In one embodiment, the composition is locally administered. In one embodiment, the composition is injected. In one embodiment, the composition is administered to the central nervous system. In one embodiment, the composition is administered to the brain.

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All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification this invention has been described in relation to certain embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

WHAT IS CLAIMED IS:

1. A gene therapy vector comprising i) two or more distinct RNAi nucleic acid sequences for inhibition of APOE4 expression, wherein optionally one RNAi nucleic acid sequence targets a non-coding APOE sequence and another RNAi nucleic acid sequence targets a APOE coding sequence; or ii) one or more copies of a RNAi nucleic acid sequence comprising SEQ ID NO:33 for inhibition of APOE4 expression.

2. The vector of claim 1i) wherein the two or more RNAi nucleic acid sequences correspond to different sequences in an APOE coding region.

3. The vector of claim 1 or 2 wherein RNAi nucleic acid sequences are inserted 5' or 3' to an open reading frame in the vector.

4. The vector of claim 1 or 2 wherein RNAi nucleic acid sequences are inserted 5' and 3' to an open reading frame in the vector.

5. The vector of claim 1i) wherein one of the RNAi nucleic acid sequences corresponds to a sequence in an APOE coding region and another of the RNAi nucleic acid sequences corresponds to a sequence in an APOE non-coding region.

6. The vector of any one of claims 1 to 5 wherein there are from 1 to 5 copies of the RNAi sequences.

7. The vector of any one of claims 1 to 5 wherein there are from 2 to 5 copies of the RNAi sequences.

8. The vector of any one of claims 1 to 6 wherein the gene therapy vector is a viral vector.

9. The vector of claim 8 wherein the viral vector is an AAV, adenovirus, lentivirus, herpesvirus or retrovirus vector.

10. The vector of claim 9 wherein the AAV genome is an AAV2, AAV5, AAV9 or AAVrh10 genome.

11. The vector of any one of claims 1 to 10 wherein the APOE4 is human APOE4.

12. The vector of any one of claims 1 to 11 further comprising a coding region for APOE2 that expresses a mRNA that does not bind RNA transcribed from the one or more of the RNAi nucleic acid sequences.

13. The vector of claim 12 wherein the APOE2 is human APOE2.

14. The vector of any one of claims 1 to 13 which comprises at least two different RNAi sequences.

15. The vector of claim 14 wherein one of the RNAi sequences comprises one of SEQ ID Nos. 33-41 or the complement thereof.

16. The vector of any one of claims 1 to 14 wherein the RNAi comprises miRNA including a plurality of miRNA sequences.

17. The vector of any one of claims 12 to 16 wherein the open reading frame for APOE2 comprises a plurality of silent nucleotide substitutions relative to SEQ ID NO:6.

18. The vector of claim 17 wherein the APOE coding region comprises SEQ ID NO:82, 85 or 87.

19. A composition comprising the gene therapy vector of any one of claims 1 to 18 and optionally a pharmaceutically acceptable carrier.

20. A method to prevent, inhibit or treat a neurological disease in a mammal, comprising: administering to the mammal an effective amount of a composition comprising the gene therapy vector of any one of claims 1 to 18 or the composition of claim 19.

21. A method to prevent, inhibit or treat a disease associated with APOE4 expression in a mammal, comprising: administering to the mammal an effective amount of a composition comprising the gene therapy vector of any one of claims 1 to 18 or the composition of claim 19.

22. The method of any one of claims 20 to 21 wherein the mammal is a E2/E4 heterozygote.

23. The method of any one of claims 20 to 21 wherein the mammal is a E4/E4 homozygote.

24. The method of any one of claims 20 to 23 wherein the composition is systemically administered.

25. The method of any one of claims 20 to 23 wherein the composition is orally administered.

26. The method of any one of claims 20 to 23 wherein the composition is intravenously administered.

27. The method of any one of claims 20 to 23 wherein the composition is locally administered.

28. The method of any one of claims 20 to 23 wherein the composition is injected.

29. The method of any one of claims 20 to 23 wherein the composition is administered to the central nervous system.

30. The method of any one of claims 20 to 23 wherein the composition is administered to the brain.

31. The method of any one of claims 20 to 30 wherein the composition is a sustained release composition.

32. The method of any one of claims 20 to 31 wherein the mammal is a human.

33. The method of any one of claims 20 to 32 wherein the RNAi sequences comprise a plurality of miRNA sequences each comprising the one or more RNAi nucleic acid sequences for inhibition of APOE4 mRNA.

34. The method of claim 33 wherein one of the miRNA sequences in the vector is inserted 5' to the open reading frame and another is inserted 3' to the open reading frame.

35. The method of any one of claims 20 to 32 wherein the RNAi sequences comprise a miRNA sequence comprising one or more of SEQ ID Nos. 33-41.

36. The method of claim 35 wherein the miRNA sequence in the vector is inserted 5' to the open reading frame.

37. The method of claim 35 wherein the miRNA sequence in the vector is inserted 3' to the open reading frame.

38. The method of any one of claims 20 to 37 wherein the vector is a rAAV comprising a capsid other than an AAV9 or AAVrh10 capsid.

39. A gene therapy vector comprising a first promoter operably linked to a nucleic acid sequence comprising a 5' untranslated region, an open reading frame encoding APOE2 and a 3' untranslated region, and an isolated nucleotide sequence comprising one or more RNAi nucleic acid sequences for inhibition of APOE4 mRNA inserted 5' and/or 3' of the open reading frame, wherein the one or more RNAi nucleic acid sequences comprise SEQ ID NO:33.

40. A method to prevent, inhibit or treat a neurological disease in a mammal, comprising: administering to the mammal an effective amount of a composition comprising the gene therapy vector of any one of claims 1 to 18 or 39 or the composition of claim 19, wherein the amount increases total APOE protein in the mammal by at least 1.5 fold and/or increases E2/E4 ratio in the mammal by at least 3 fold relative to a corresponding mammal not administered the composition.

41. A method to prevent, inhibit or treat a disease associated with APOE4 expression in a mammal, comprising: administering to the mammal an effective amount of a composition comprising the gene therapy vector of any one of claims 1 to 18 or 39 or the composition of claim 19, wherein the amount increases total APOE protein by at least 1.5 fold and/or increases E2/E4 ratio by at least 3 fold relative to a corresponding mammal not administered the composition.

42. The method of claim 40 or 41 wherein the gene therapy vector is a rAAV vector.

43. The method of claim 42 wherein the AAV capsid is not an AAV9 or AAVrh10 capsid.

44. The method of claim 38 or 42 wherein the AAV capsid comprises one of SEQ ID Nos.42 to 66, 71 to 79 or 85 to 89.

45. The method of any one of claims 40 to 43 wherein the mammal is a E2/E4 heterozygote.

46. The method of any one of claims 40 to 43 wherein the mammal is a E4/E4 homozygote.

47. The method of any one of claims 40 to 46 wherein the composition is systemically administered.

48. The method of any one of claims 40 to 46 wherein the composition is orally administered.

49. The method of any one of claims 40 to 46 wherein the composition is intravenously administered.

50. The method of any one of claims 40 to 46 wherein the composition is locally administered.

51. The method of any one of claims 40 to 46 wherein the composition is injected.

52. The method of any one of claims 40 to 46 wherein the composition is administered to the central nervous system.

53. The method of any one of claims 40 to 46 wherein the composition is administered to the brain.