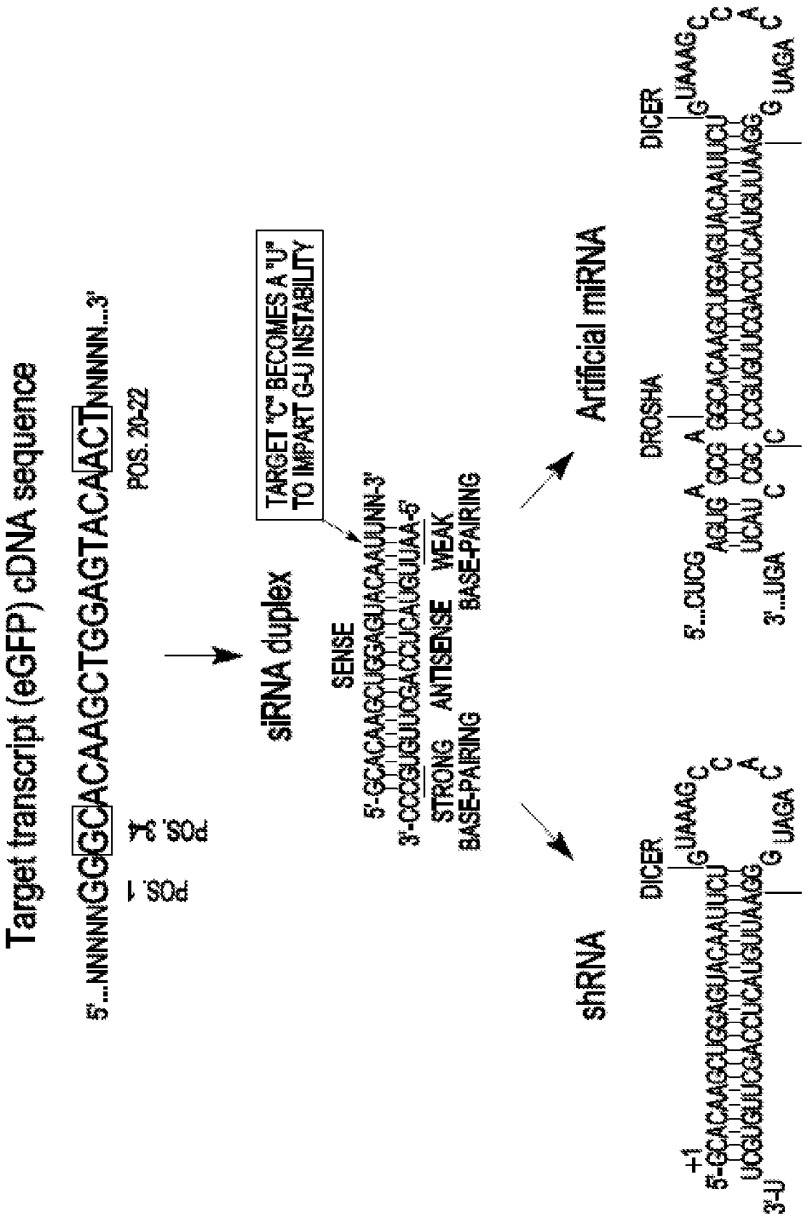

Target transcript (eGFP) cDNA sequence
5'...NNNNNGGGCACAAGCTGGAGTACAACTNNNNN...3'
POS. 1
POS. X
POS. 20-22
siRNA duplex
TARGET "C" BECOMES A "U" TO IMPART G-U INSTABILITY
SENSE
5'-GCACAAGCUGGAGUACAAUUNN-3'
3'-CCGUGUUCGACCUCAUGUUAA-5'
STRONG ANTISENSE WEAK
BASE-PAIRING BASE-PAIRING
shRNA
DICER
+1
5-GCACAAGCUGGAGUACAAUUCU
UCGUGUUCGACCUCAUGUUAAGG
3-U
G UAGA
C A C
UAAAG
Artificial miRNA
DROSHA
5'...CUCG
AGUG GCG GGCACAAGCUGGAGUACAAUUCU
UCAU CGC CCGUGUUCGACCUCAUGUUAAGG
3'...UGA C C G UAGA
C A C
UAAAG
DICER

FIG. 1

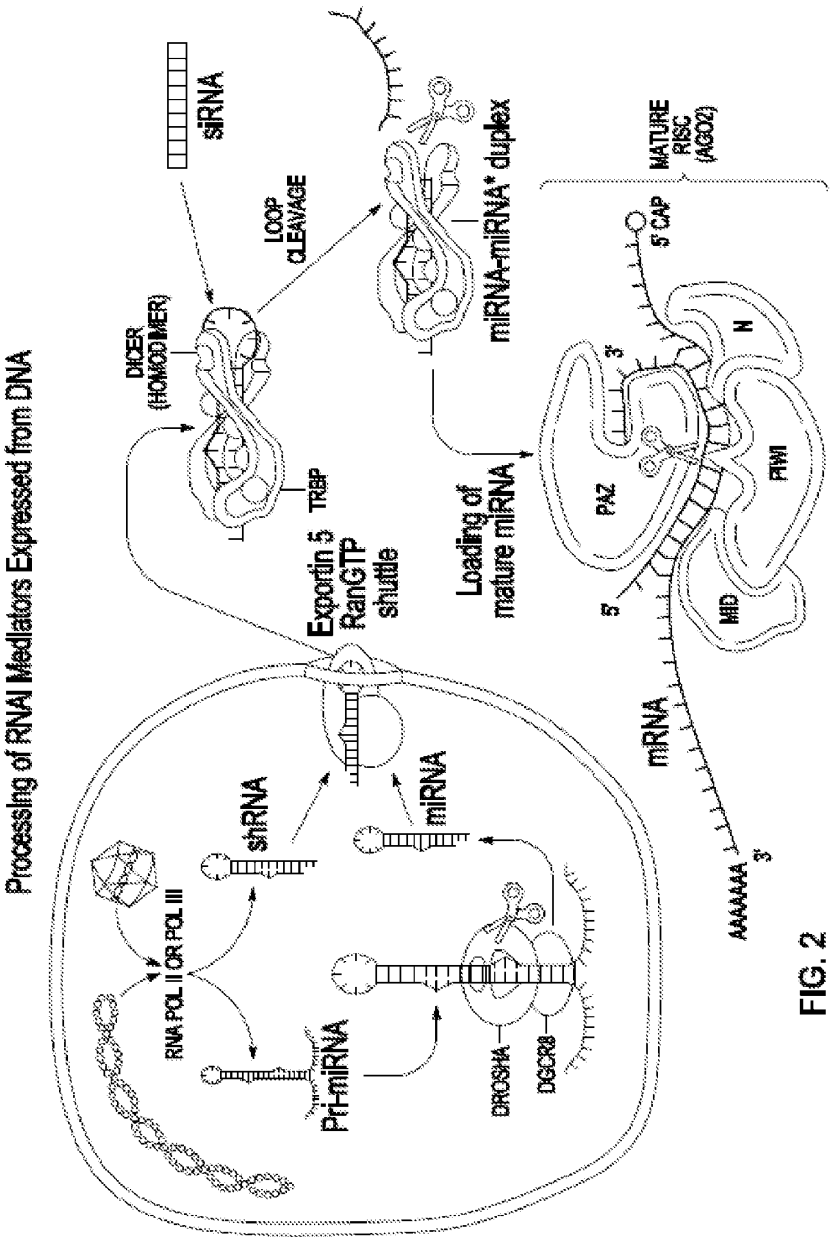

Processing of RNAI Mediators Expressed from DNA
RNA POL II OR POL III
shRNA
miRNA
Pri-miRNA
DROSHA
DGCR8
Exportin 5
RanGTP
shuttle
TRBP
DICER
(HOMODIMER)
LOOP
CLEAVAGE
siRNA
miRNA-miRNA* duplex
Loading of
mature miRNA
MATURE
RISC
(AGO2)
5' CAP
3'
N
PAZ
PIWI
MID
5'
mRNA
AAAAAA
3'

FIG. 2

FIG. 3

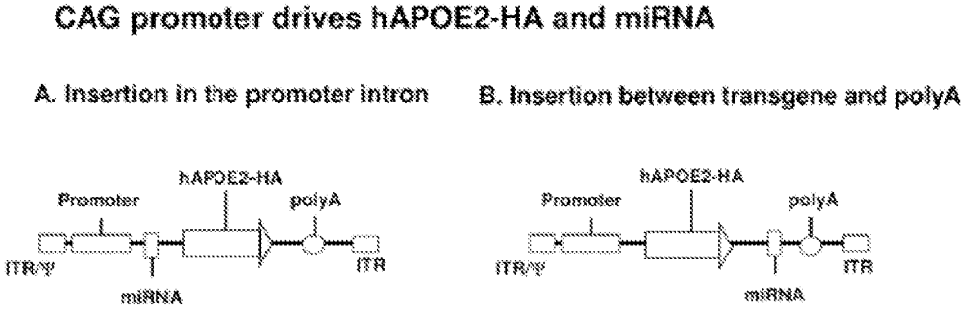


FIG. 3A

FIG. 3B

FIG. 4

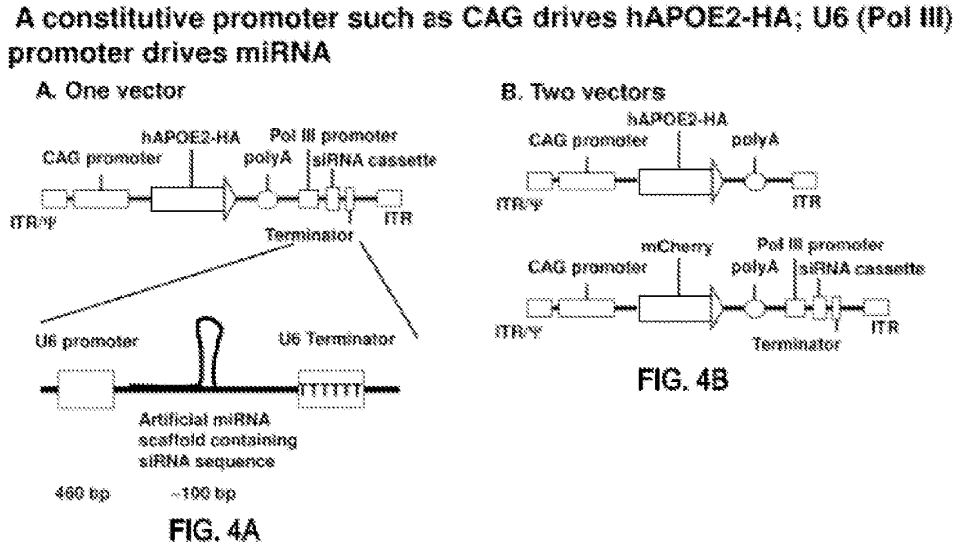


FIG. 4A

FIG. 4B

Total length: ~4000 bp

FIG. 5

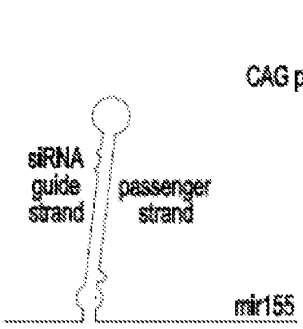


FIG. 5A

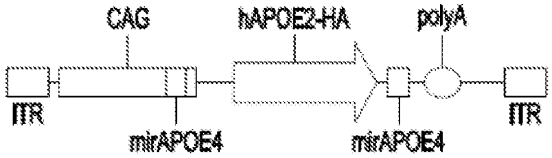


FIG. 5B

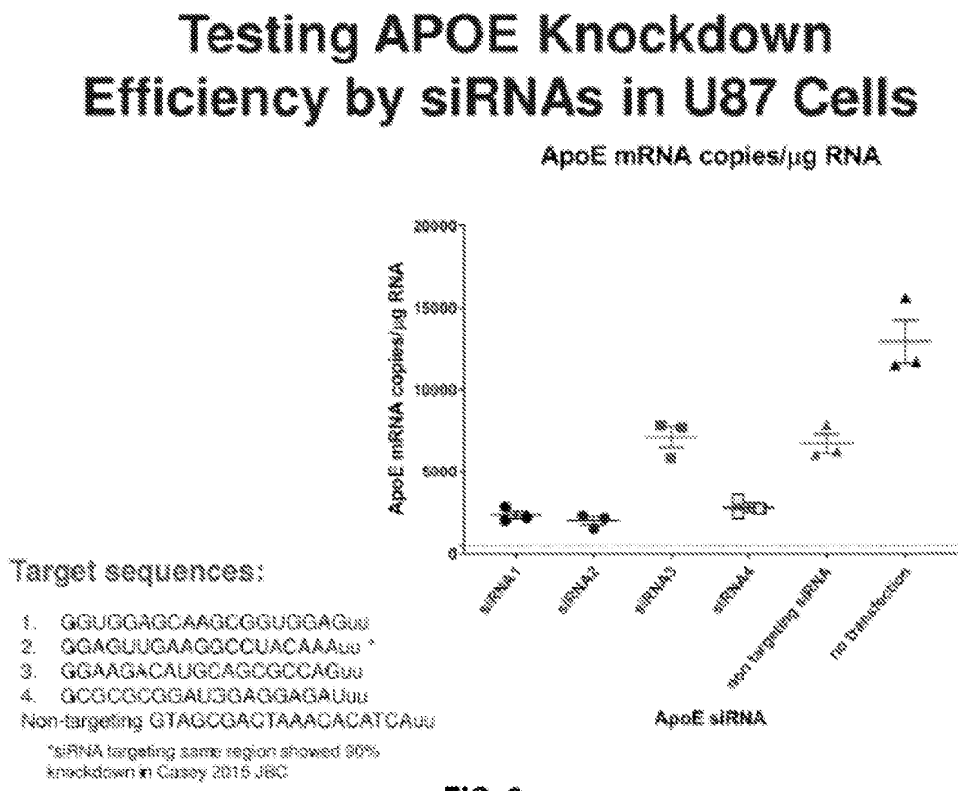
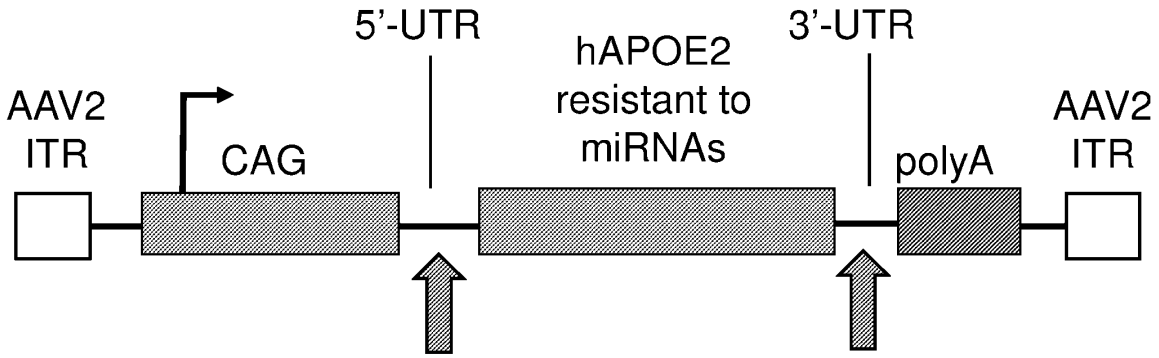

Testing APOE Knockdown Efficiency by siRNAs in U87 Cells
ApoE mRNA copies/µg RNA
20000
15000
10000
5000
0
siRNA1
siRNA2
siRNA3
siRNA4
non targeting siRNA
no transfection
ApoE siRNA
Target sequences:
1. GGUGGAGCAAGCGGUGGAGuu
2. GGAGUUGAAGGCCUACAAAuu *
3. GGAAGACAUGCAGCGCCAGuu
4. GCGCGCCGAUGGAGGAGAUuu
Non-targeting GTAGCGACTAAACACATCAuu
*siRNA targeting same region showed 90% knockdown in Casey 2015 JBC

FIG. 6

Figure 7.

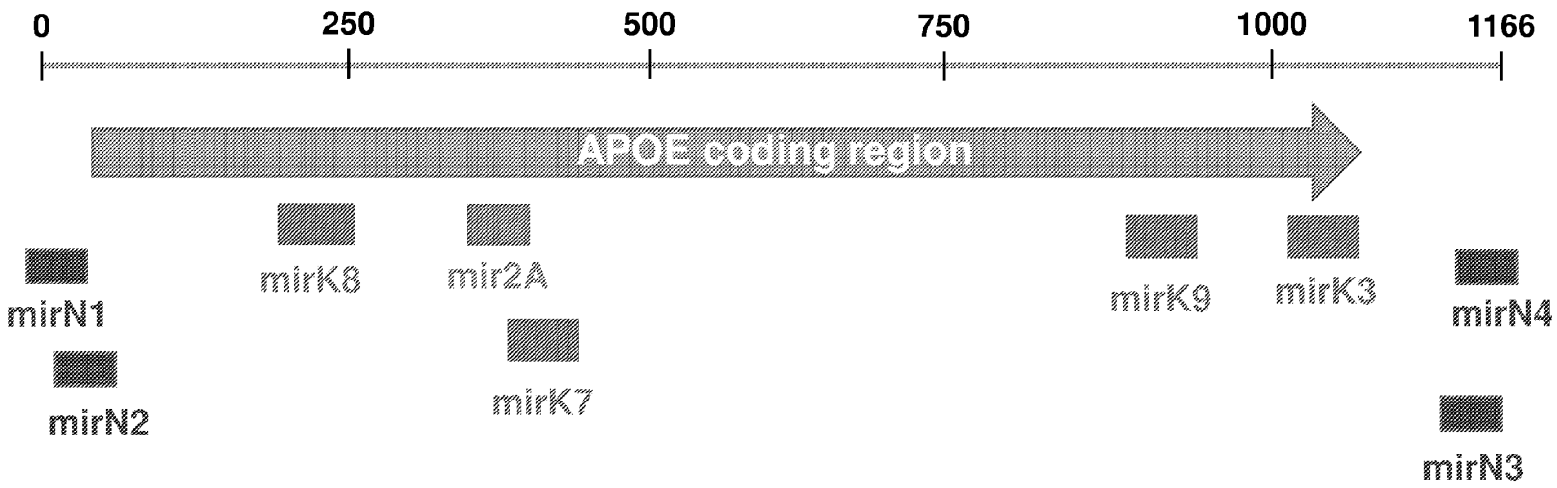
Constructs

- 2 to 4 copies of 2 -3 different miRNAs and APOE2 gene resistant to silencing



Insert multiple miRNAs against APOE

Figure 8.miRNA Targets



- 2A
- **N1-4** series are in non-coding regions – do not need codon changes
- K series– silencing-resistant APOE2 constructed

Figure 9

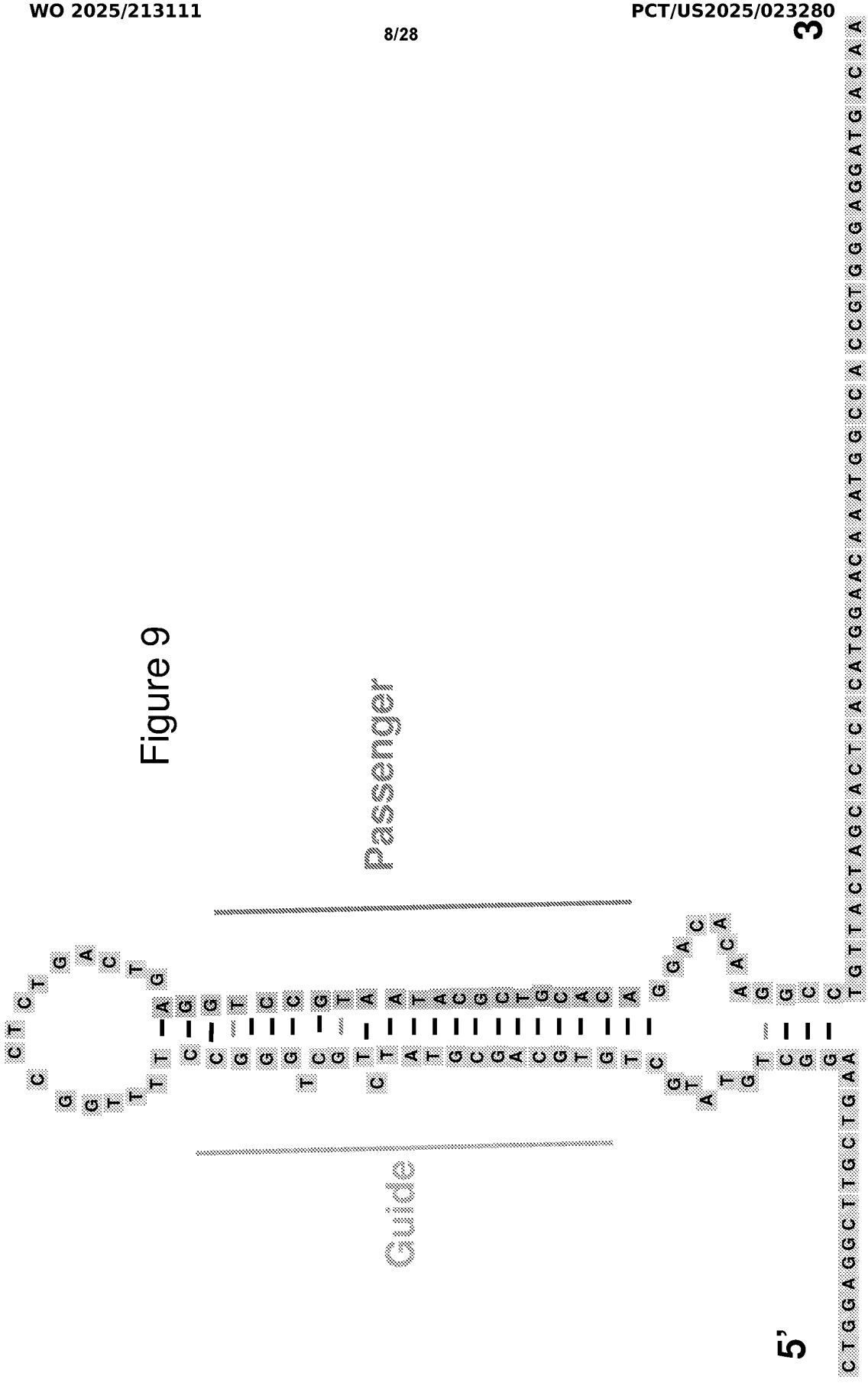


Figure 10. In Vitro Assessment of miRNAs in 2 Cell Types

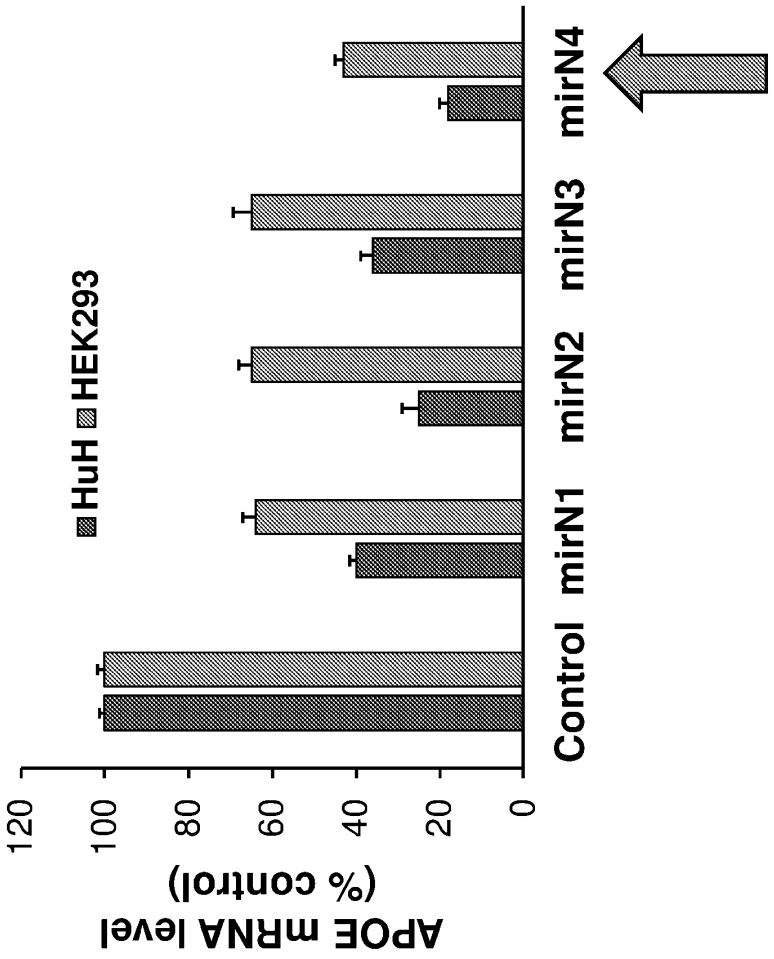


Figure 11. *In Vitro* Suppression of APOE mRNA by Single miRNAs

miR	Target nt in cDNA	Inhibition in 293 cells (%)	Inhibition in HuH cells (%)
2A	ggagttgaaggcctacaaatc	66	69
N1	aatcacaggcaggaagatgaa	36	60
N2	ctggccaatcacaggcaggaa	35	75
N3	agtttaataaagattcaccaa	35	64
N4	ataaagattcaccaagtttca	57	82
K3	gcccagcgacaatcactgaacg	52	72
K7	acaaatcggaactggaggaaca	57	74
K8	gcagacactgtctgagcaggtg	53	82
K13	aggcccagcagatacgcctgca		88

Figure 12. Combination of 2 miRNAs Provides Additional Silencing

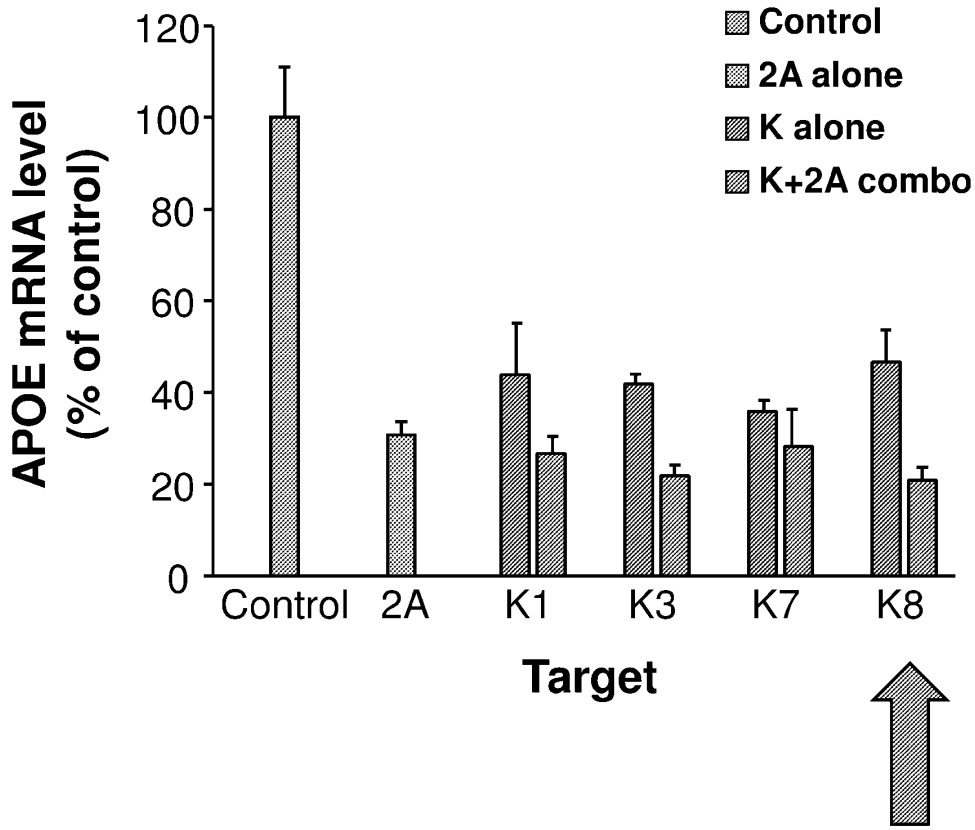
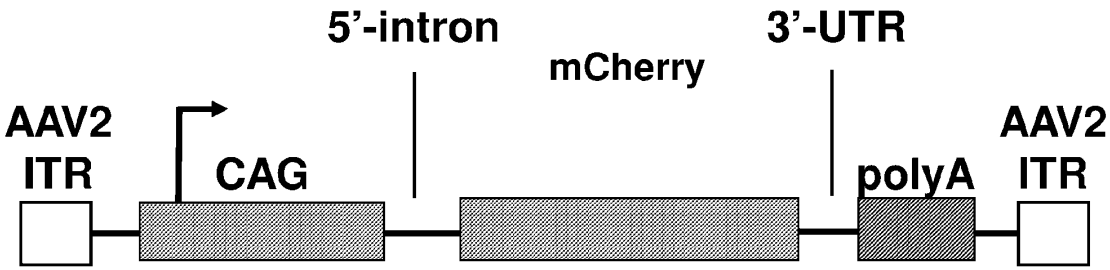


Figure 13. miRNA Combinations in a Single Vector



	5' intron	3' UTR	Complete
1	mir2A X 2	mirN4 X 2	✓*
2	mir2A X 2	mirK8 X 2	✓
3	mirK8 X 2	mir2A X 2	✓
4	mir2A X 4	mirK8 X 2	X
5	mir2A X 4	mirK8 X 4	X
6	mirN4 X 4	mir2A X 4	X

* AAV made and mice injected to assess silencing *in vivo*

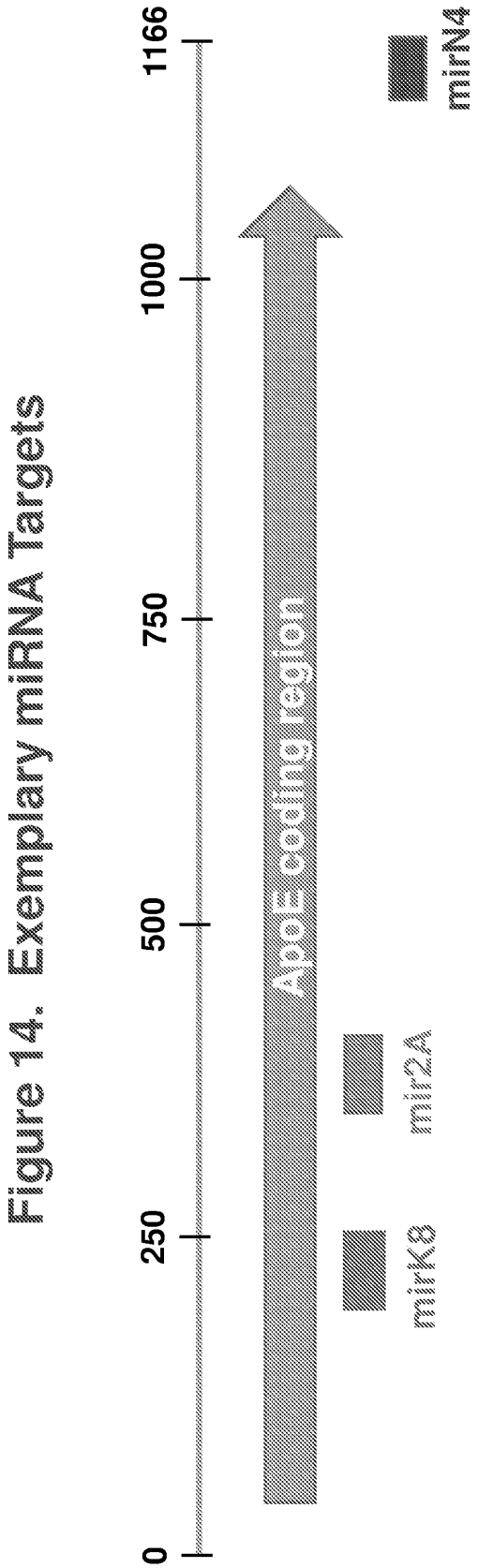


Figure 14. Exemplary miRNA Targets

Figure 15A. Designing an APOE2 Gene to Circumvent Silencing by mirK and mir2A

Example

mirK8 target is eliminated by multiple silent codon changes

CpG dinucleotide is eliminated by silent codon change

```
                AspTyrLeuArgTrpValGlnThrLeuSerGluGlnValGlnGlu
Native          GATTACCTGCGCTGGGTGCAGACGCTGAGCGAGCAGGTGCAGGAG
                |||||||||| | |||||||||| || || || || ||||||||||
Novel           GATTACCTGAGATGGGTGCAAACTCTATCAGAACAAGTGCAGGAG
```

Figure 15B. APOE gene resistant to K3 – altered nucleotides *red*

```
AMINO ACID SEQ:            ALA ALA PRO VAL PRO SER ASP ASN HIS ***
NATIVE APOE:     ACC AGC GCC GCC CCT GTG CCC AGC GAC AAT CAC TGA ACGCCGAA
 SHRNA K3:                              AC GGG TCG CTG TTA GTG ACT T
K3 RESISTANT APOE:                     GTT CCT TCG GAT AAC CAT TAG A
```

APOE GENE RESISTANT TO K3

ATGAAGGTTCTGTGGGCTGCGTTGCTGGTCACATTCCTGGCAGGATGCCAGGCCAAGGTGGAGCAAGCGGTGGAGACAGAGCCGGA
GCCCGAGCTGCGCCAGCAGACCGAGTGGCAGAGCGGCCAGCGCTGGGAACTGGCACTGGGTCGCTTTTGGGATTACCTGCGCTGG
GTGCAGACACTGTCTGAGCAGGTGCAGGAGGAGCTGCTCAGCTCCCAGGTCACCCAGGAACTGAGGGCGCTGATGGACGAGACCAT
GAAGGAGTTGAAGGCCTACAAATCGGAACTGGAGGAACAACTGACCCCGGTGGCGGAGGAGACGCGGGCACGGCTGTCCAAGGA
GCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGACATGGAGGACGTGTGCGGCCGCCTGGTGCAGTACCGCGGCGAGGTGCAGGC
CATGCTCGGCCAGAGCACCGAGGAGCTGCGGGTGCGCCTCGCCTCCCACCTGCGCAAGCTGCGTAAGCGGCTCCTCCGCGATGCCGA
TGACCTGCAGAAGCGCCTGGCAGTGTACCAGGCCGGGGCCCGCGAGGGCGCCGAGCGCGGCCTCAGCGCCATCCGCGAGCGCCTG
GGGCCCCTGGTGGAACAGGGCCGCGTGCGGGCCGCCACTGTGGGCTCCCTGGCCGGCCAGCCGCTACAGGAGCGGGCCCAGGCCT
GGGGCGAGCGGCTGCGCGCGCGGATGGAGGAGATGGGCAGCCGGACCCGCGACCGCCTGGACGAGGTGAAGGAGCAGGTGGCG
GAGGTGCGCGCCAAGCTGGAGGAGCAGGCCCAGCAGATACGCCTGCAGGCCGAGGCCTTCCAGGCCCGCCTCAAGAGCTGGTTCG
AGCCCCTGGTGGAAGACATGCAGCGCCAGTGGGCCGGGCTGGTGGAGAAGGTGCAGGCTGCCGTGGGCACCAGCGCCGCCCCTG
TTCCTTCGATAACCATTAGAC

Figure 15C. APOE gene resistant to K13 – altered nucleotides *red*

```
AMINO ACID SEQ:  GLU GLU GLN ALA GLN GLN ILE ARG LEU GLN ALA GLU
NATIVE APOE:     GAG GAG CAG GCC CAG CAG ATA CGC CTG CAG GCC GAG
SHRNA K13:                TC CGG GTC GTC TAT GCG GAC GT
K13 RESISTANT APOE:      CAA GCG CAA CAA ATC AGA CTC CAA
```

APOE GENE RESISTANT TO K13

ATGAAGGTTCTGTGGGCTGCGTTGCTGGTCACATTCCTGGCAGGATGCCAGGCCAAGGTGGAGCAAGCGGTGGAGACAGAGCCG
GAGCCCGAGCTGCGCCAGCAGACCGAGTGGCAGAGCGGCCAGCGCTGGGAACTGGCACTGGGTCGCTTTTGGGATTACCTGCGC
TGGGTGCAGACACTGTCTGAGCAGGTGCAGGAGGAGCTGCTCAGCTCCCAGGTCACCCAGGAACTGAGGGCGCTGATGGACGAG
ACCATGAAGGAGTTGAAGGCCTACAAATCGGAACTGGAGGAACAACTGACCCCGGTGGCGGAGGAGACGCGGGCACGGCTGTC
CAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGACATGGAGGACGTGTGCGGCCGCCTGGTGCAGTACCGCGGCGAG
GTGCAGGCCATGCTCGGCCAGAGCACCGAGGAGCTGCGGGTGCGCCTCGCCTCCCACCTGCGCAAGCTGCGTAAGCGGCTCCTCC
GCGATGCCGATGACCTGCAGAAGCGCCTGGCAGTGTACCAGGCCGGGGCCCGCGAGGGCGCCGAGCGCGGCCTCAGCGCCATCC
GCGAGCGCCTGGGGCCCCTGGTGGAACAGGGCCGCGTGCGGGCCGCCACTGTGGGCTCCCTGGCCGGCCAGCCGCTACAGGAG
CGGGCCCAGGCCTGGGGCGAGCGGCTGCGCGCGCGGATGGAGGAGATGGGCAGCCGGACCCGCGACCGCCTGGACGAGGTGA
AGGAGCAGGTGGCGGAGGTGCGCGCCAAGCTGGAGGAGCAAGCGCAACAAATCAGACTCCAGGCCGAGGCCTTCCAGGCCCGC
CTCAAGAGCTGGTTCGAGCCCCTGGTGGAAGACATGCAGCGCCAGTGGGCCGGGCTGGTGGAGAAGGTGCAGGCTGCCGTGGG
CACCAGCGCCGCCCCTGTGCCCAGCGACAATCACTGA

Figure 15D. APOE gene resistant to K7 – altered nucleotides red

AMINO ACID SEQ:	ALA TYR LYS SER GLU LEU GLU GLU GLN LEU THR PRO
NATIVE APOE:	GCC TAC AAA TCG GAA CTG GAG GAA CAA CTG ACC CCG
SHRNA K13:	*G TTT AGC CTT GAC CTC CTT GT*
K13 RESISTANT APOE:	T AAG AGT GAG TTA GAA GAG CA

APOE GENE RESISTANT TO K7

ATGAAGGTTCTGTGGGCTGCGTTGCTGGTCACATTCCTGGCAGGATGCCAGGCCAAGGTGGAGCAAGCGGTGGAGACAGA
GCCGGAGCCCGAGCTGCGCCAGCAGACCGAGTGGCAGAGCGGCCAGCGCTGGGAACTGGCACTGGGTCGCTTTTGGGAT
TACCTGCGCTGGGTGCAGACACTGTCTGAGCAGGTGCAGGAGGAGCTGCTCAGCTCCCAGGTCACCCAGGAACTGAGGGC
GCTGATGGACGAGACCATGAAGGAGTTGAAGGCCTATAAGAGTGAGTTAGAAGAGCAACTGACCCCGGTGGCGGAGGA
GACGCGGGCACGGCTGTCCAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGACATGGAGGACGTGTGCGGCCG
CCTGGTGCAGTACCGCGGCGAGGTGCAGGCCATGCTCGGCCAGAGCACCGAGGAGCTGCGGGTGCGCCTCGCCTCCCACC
TGCGCAAGCTGCGTAAGCGGCTCCTCCGCGATGCCGATGACCTGCAGAAGCGCCTGGCAGTGTACCAGGCCGGGGCCCGC
GAGGGCGCCGAGCGCGGCCTCAGCGCCATCCGCGAGCGCCTGGGGCCCCTGGTGGAACAGGGCCGCGTGCGGGCCGCC
ACTGTGGGCTCCCTGGCCGGCCAGCCGCTACAGGAGCGGGCCCAGGCCTGGGGCGAGCGGCTGCGCGCGCGGATGGAG
GAGATGGGCAGCCGGACCCGCGACCGCCTGGACGAGGTGAAGGAGCAGGTGGCGGAGGTGCGCGCCAAGCTGGAGGA
GCAGGCCCAGCAGATACGCCTGCAGGCCGAGGCCTTCCAGGCCCGCCTCAAGAGCTGGTTCGAGCCCCTGGTGGAAGACA
TGCAGCGCCAGTGGGCCGGGCTGGTGGAGAAGGTGCAGGCTGCCGTGGGCACCAGCGCCGCCCCTGTGCCCAGCGACAA
TCACTGA

Figure 16. mRNA Expression from Engineered APOE cDNA

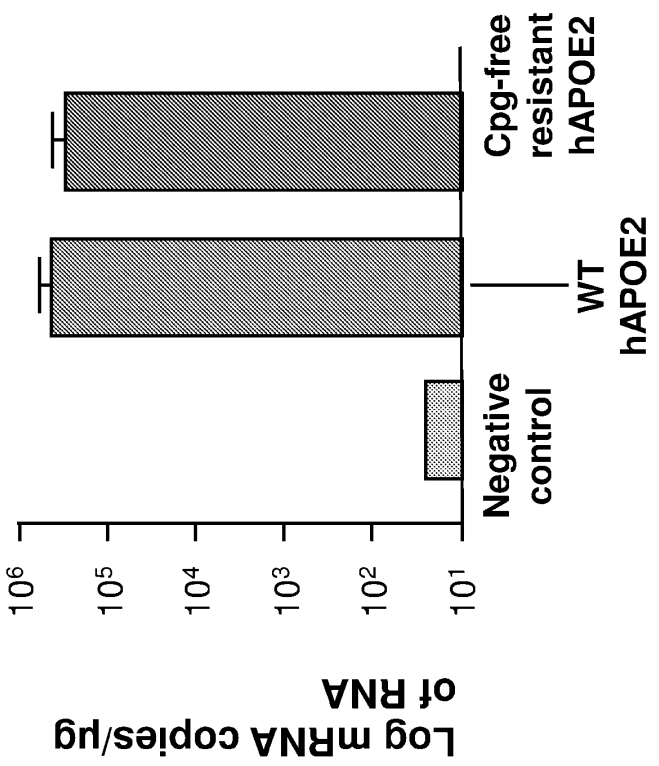
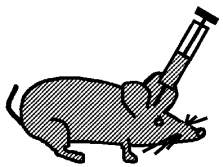


Figure 17. In Vivo Silencing of Human APOE in TRE4 Mouse Brain

- **Intrahippocampal, unilateral right**

  AAVrh.10-mCherry
  AAVS2-mCherry
  AAVrh.10-mCherry-4xmir**N4/2A**
  AAVrh.10-mCherry-4xmir**2A**(polyA)
  AAVS2-mCherry-4xmir**N4/2A**
  AAVS2-mCherry-4xmir**2A**(polyA)
  PBS



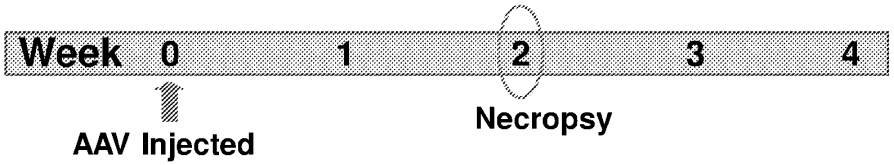
TRE4 mice expressing human APOE4
6-8 wk old
n=5 mice/condition

$2.5x10^{10}$ gc in 2 µl

Weekly health checks

Evaluate

- 4 coronal sections
- hAPOE4 expression (RTqPCR, ELISA)



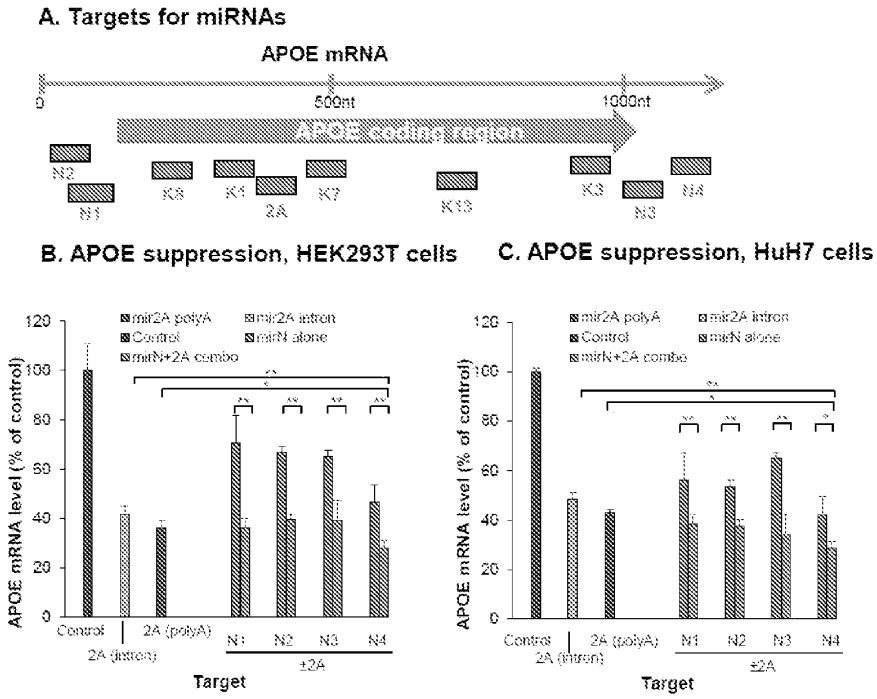

A. Targets for miRNAs
APOE mRNA
0
500nt
1000nt
APOE coding region
N2
N1
K8
K1
2A
K7
K13
K3
N3
N4
B. APOE suppression, HEK293T cells
C. APOE suppression, HuH7 cells
mir2A polyA
mir2A intron
Control
mirN alone
mirN+2A combo
APOE mRNA level (% of control)
Control
2A (polyA)
2A (intron)
N1
N2
N3
N4
±2A
Target

Figure 18

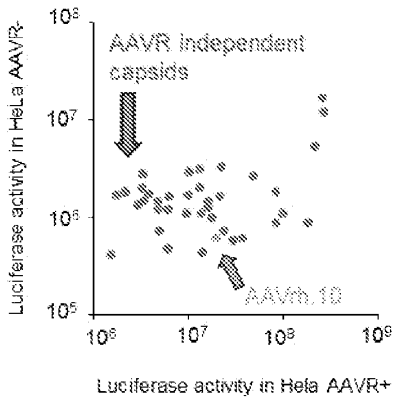

AAVR independent capsids
AAVrh.10
Luciferase activity in HeLa AAVR-
Luciferase activity in Hela AAVR+
10^8
10^7
10^6
10^5
10^6
10^7
10^8
10^9

Figure 18D

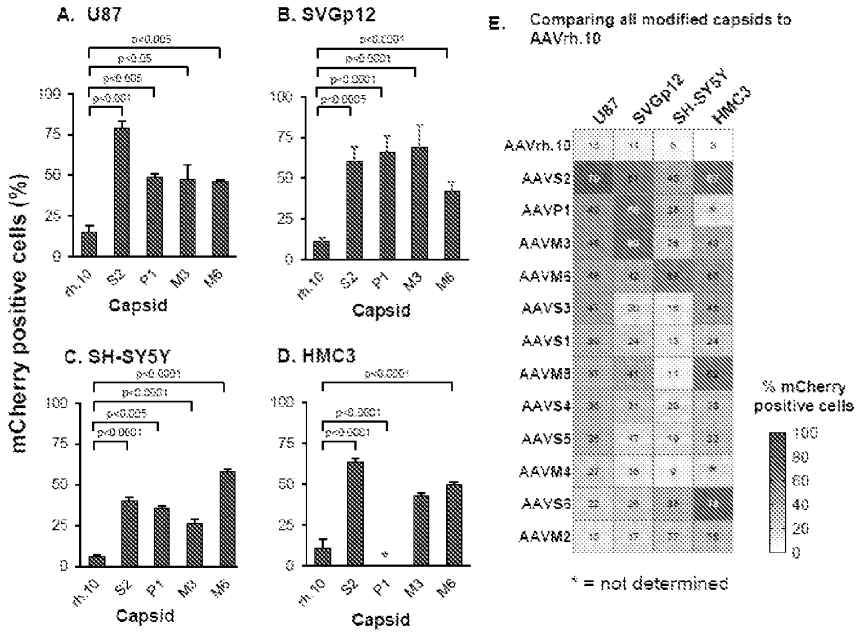

A. U87
B. SVGp12
C. SH-SY5Y
D. HMC3
mCherry positive cells (%)
Capsid
rh.10
S2
P1
M3
M6
E. Comparing all modified capsids to AAVrh.10
U87
SVGp12
SH-SY5Y
HMC3
AAVrh.10
AAVS2
AAVP1
AAVM3
AAVM6
AAVS3
AAVS1
AAVM8
AAVS4
AAVS5
AAVM4
AAVS6
AAVM2
% mCherry positive cells
100
80
60
40
20
0
* = not determined

Figure 19

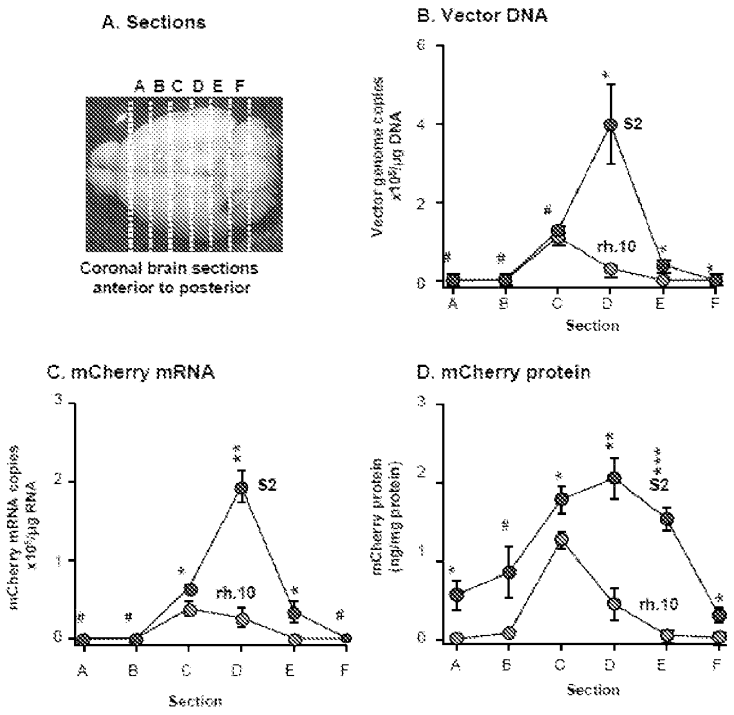

A. Sections
A B C D E F
Coronal brain sections
anterior to posterior
B. Vector DNA
Vector genome copies
x10⁶/µg DNA
S2
rh.10
Section
C. mCherry mRNA
mCherry mRNA copies
x10⁵/µg RNA
S2
rh.10
Section
D. mCherry protein
mCherry protein
(ng/mg protein)
S2
rh.10
Section

Figure 20

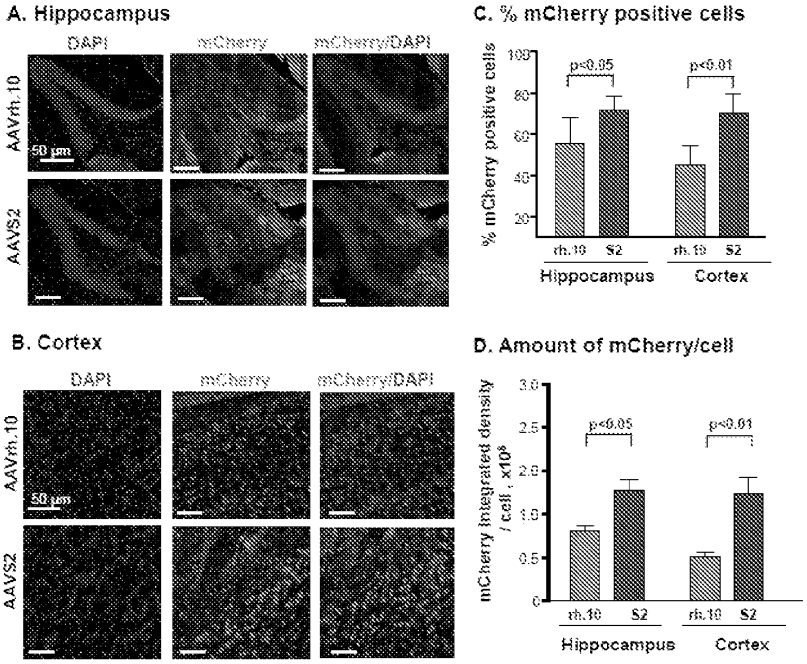

A. Hippocampus
DAPI
mCherry
mCherry/DAPI
AAVrh.10
AAVS2
50 μm
B. Cortex
C. % mCherry positive cells
% mCherry positive cells
p<0.05
p<0.01
rh.10
S2
Hippocampus
Cortex
D. Amount of mCherry/cell
mCherry integrated density / cell , x10⁶

Figure 21

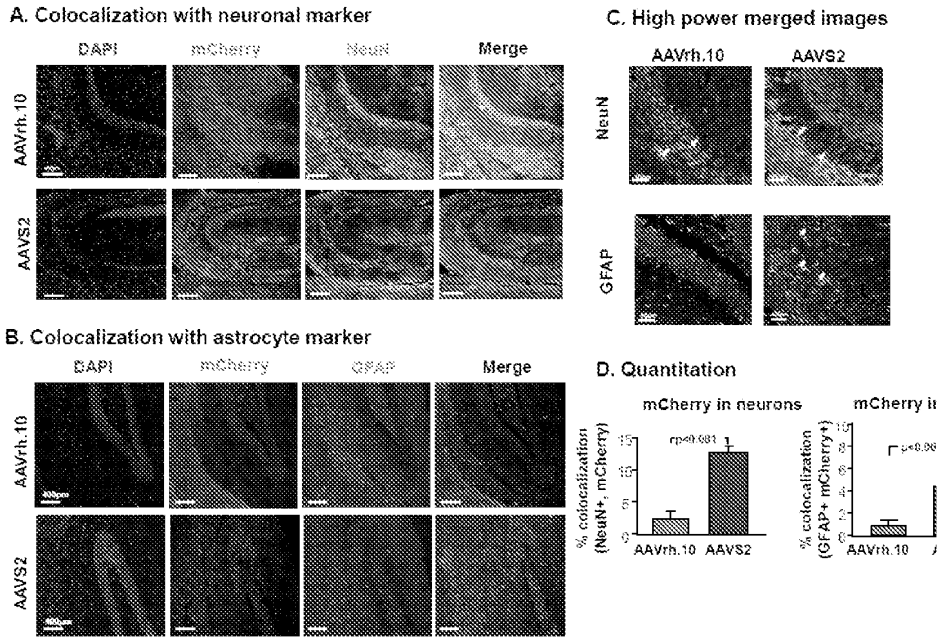

A. Colocalization with neuronal marker
DAPI
mCherry
NeuN
Merge
AAVrh.10
AAVS2
B. Colocalization with astrocyte marker
DAPI
mCherry
GFAP
Merge
AAVrh.10
AAVS2
C. High power merged images
AAVrh.10
AAVS2
NeuN
GFAP
D. Quantitation
mCherry in neurons
% colocalization (NeuN+, mCherry)
AAVrh.10
AAVS2
mCherry in
% colocalization (GFAP+ mCherry+)
AAVrh.10

Figure 22

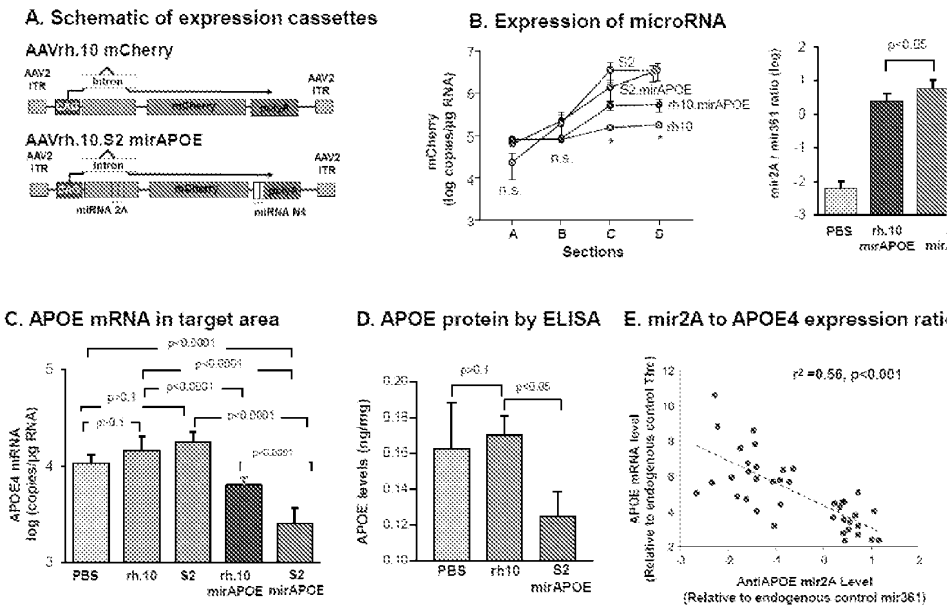

A. Schematic of expression cassettes
AAVrh.10 mCherry
AAV2 ITR
hybrid intron
AAV2 ITR
AAVrh.10.S2 mirAPOE
AAV2 ITR
hybrid intron
AAV2 ITR
miRNA 2A
miRNA N4
B. Expression of microRNA
mCherry (log copies/µg RNA)
S2
S2.mirAPOE
rh10.mirAPOE
rh10
n.s.
n.s.
A
B
C
D
Sections
mir2A / mir361 ratio (log)
PBS
rh.10 mirAPOE
C. APOE mRNA in target area
APOE4 mRNA log (copies/µg RNA)
PBS
rh.10
S2
rh.10 mirAPOE
S2 mirAPOE
D. APOE protein by ELISA
APOE levels (ng/mg)
PBS
rh10
S2 mirAPOE
E. mir2A to APOE4 expression rati
r² =0.56, p<0.001
APOE mRNA level (Relative to endogenous control Thrc)
AntiAPOE mir2A Level (Relative to endogenous control mir361)

Figure 23

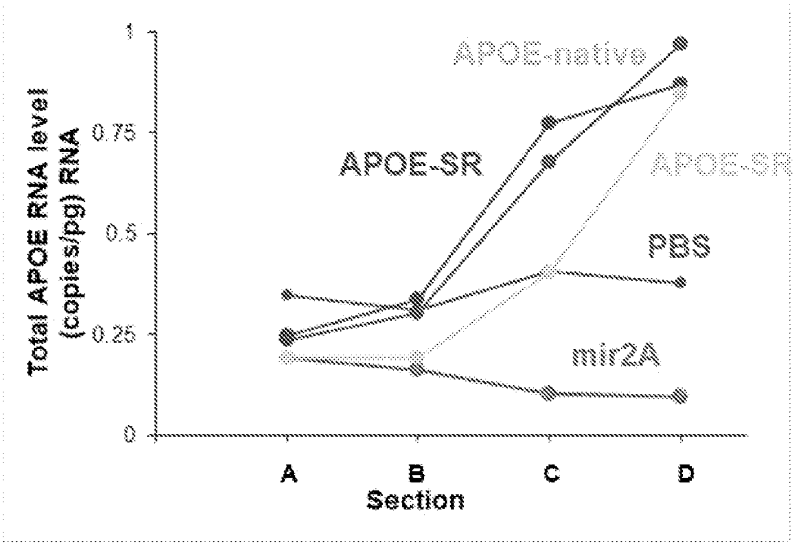

Total APOE RNA level (copies/pg) RNA
1
0.75
0.5
0.25
0
APOE-native
APOE-SR
APOE-SR+Mir2A
PBS
mir2A
A
B
C
D
Section

Figure 24

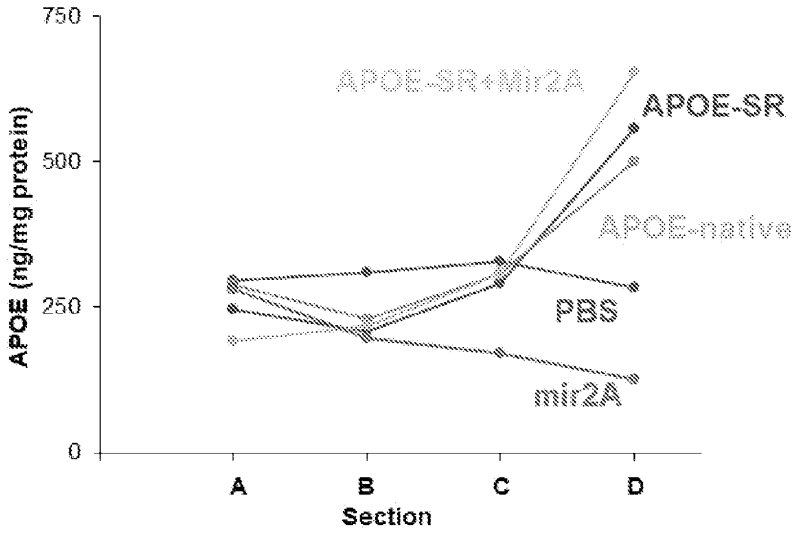

750
500
250
0
APOE (ng/mg protein)
APOE-SR+Mir2A
APOE-SR
APOE-native
PBS
mir2A
A
B
C
D
Section

Figure 25