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Li et al.

(54) **COMPOSITION OF ASYMMETRIC RNA DUPLEX AS MICRORNA MIMETIC OR INHIBITOR**

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§ 371 (c)(1),
(2), (4) Date: **Jul. 26, 2010**

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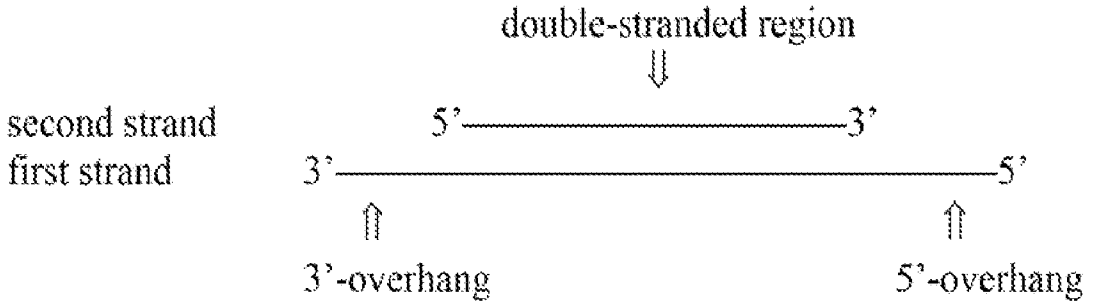
Publication Classification

(51) **Int. Cl.**
C07H 21/02 (2006.01)

(52) **U.S. Cl.** **536/23.1**

(57) **ABSTRACT**

The present invention provides double-stranded RNA molecules that are asymmetrical in strand length. The RNA molecule of the invention, the asymmetric RNA duplex, has one or two overhangs at the end. In one aspect, these novel RNA duplex molecules serve as effective mimetics of miRNA. In another aspect, they are designed to function as effective inhibitors of miRNA. Accordingly, the RNA molecules of the present invention can be used to modulate miRNA pathway activities, with tremendous implications for research, drug discovery and development, and treatment of human diseases.



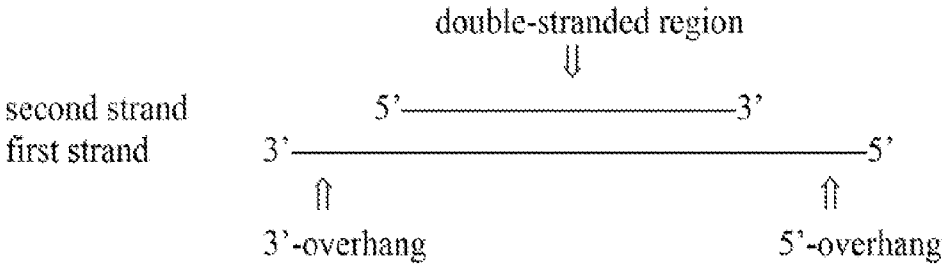


Figure 1A

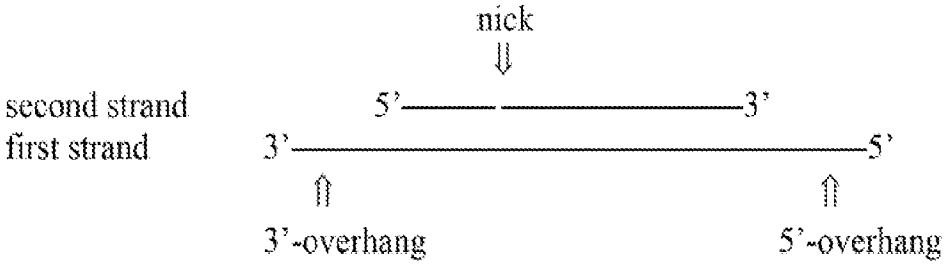


Figure 1B

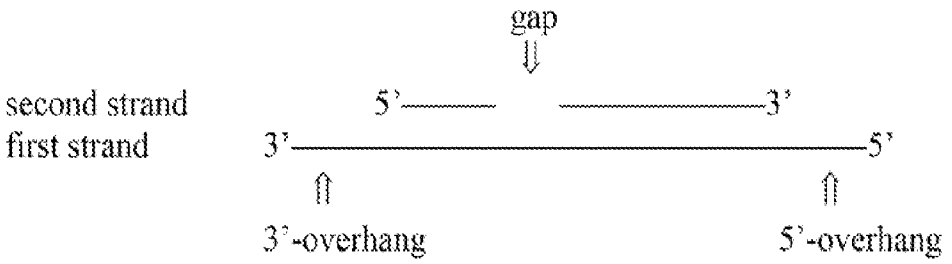


Figure 1C

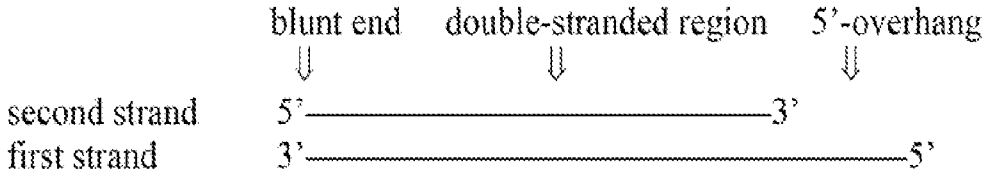


Figure 2A

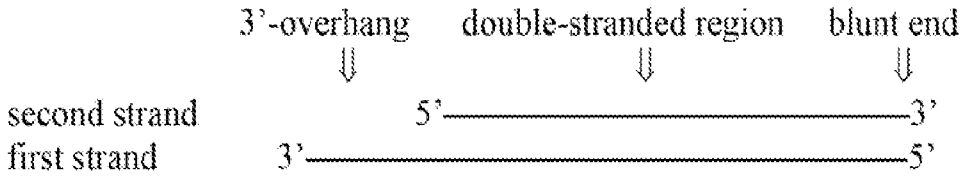


Figure 2B

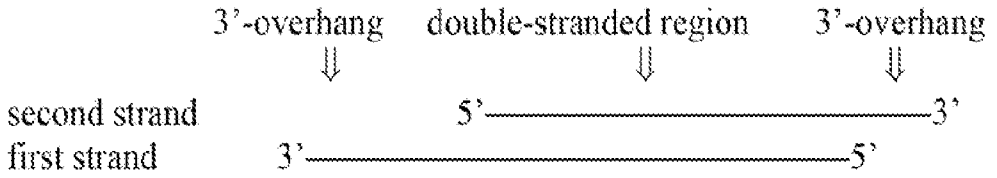


Figure 2C

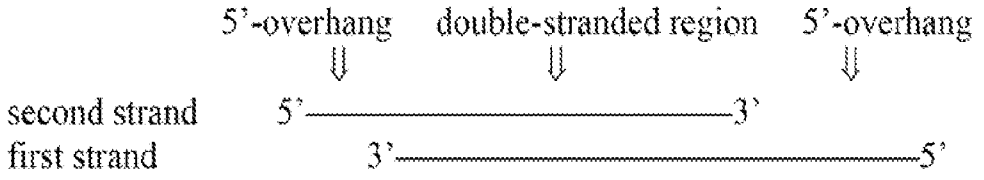


Figure 2D

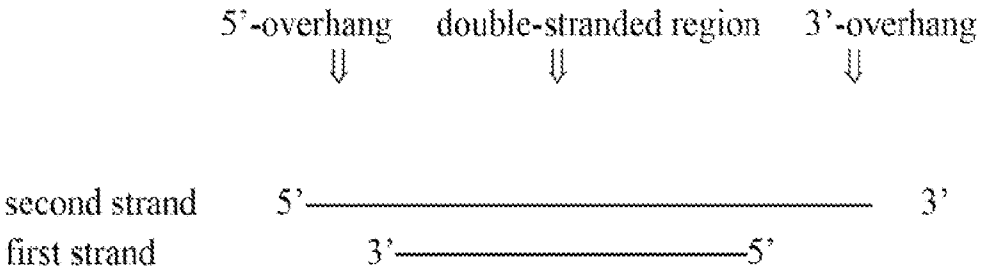


Figure 2E

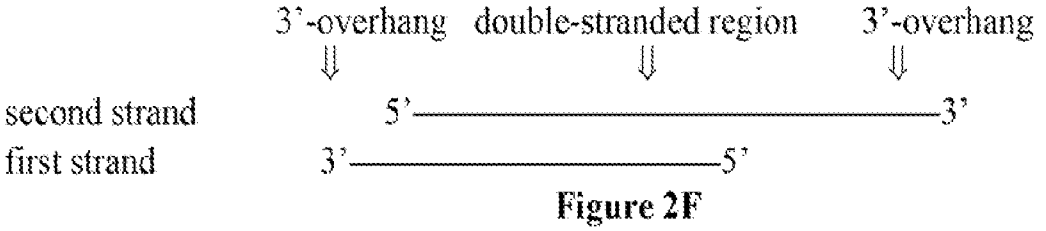


Figure 2F

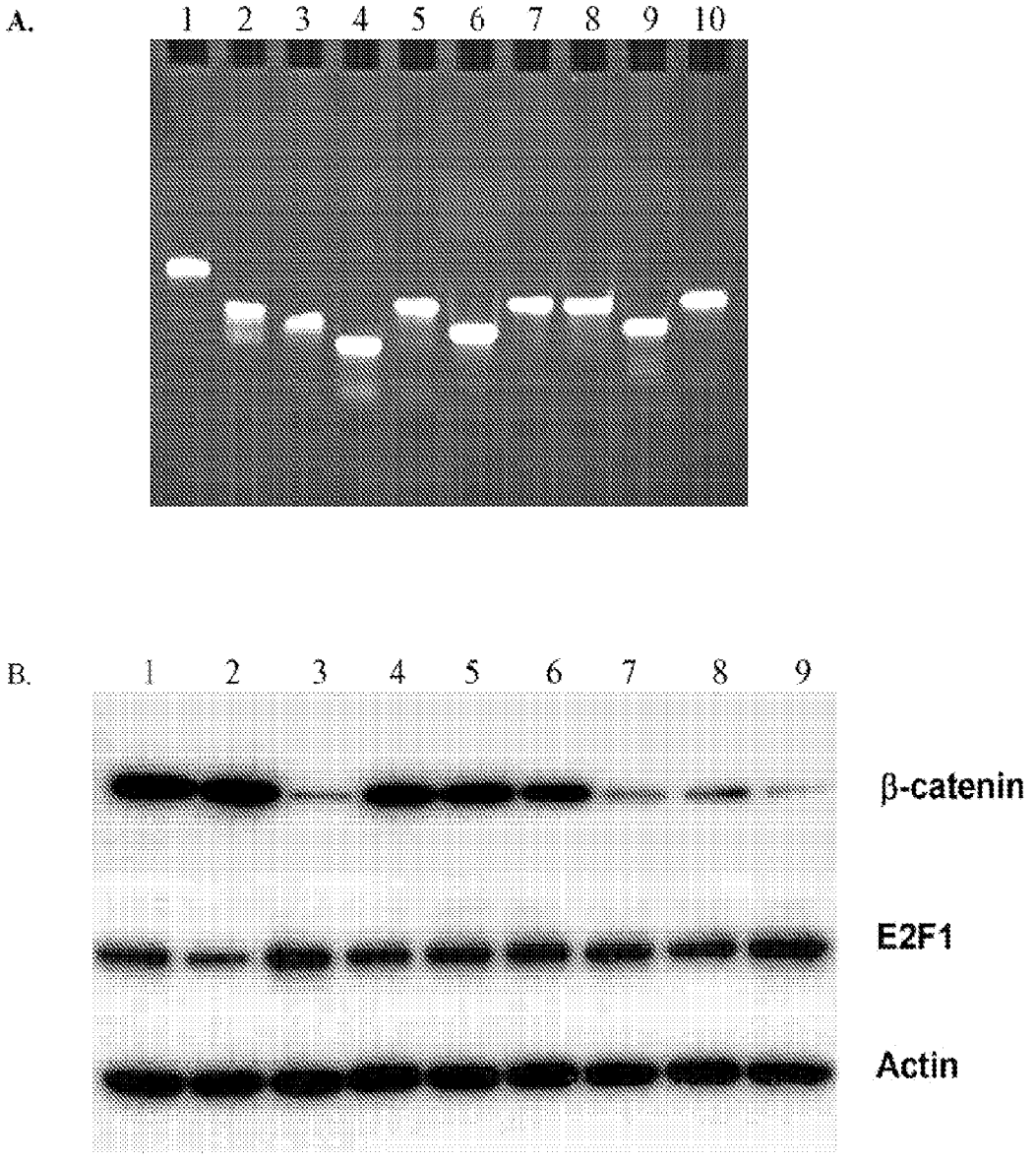


Figure 3

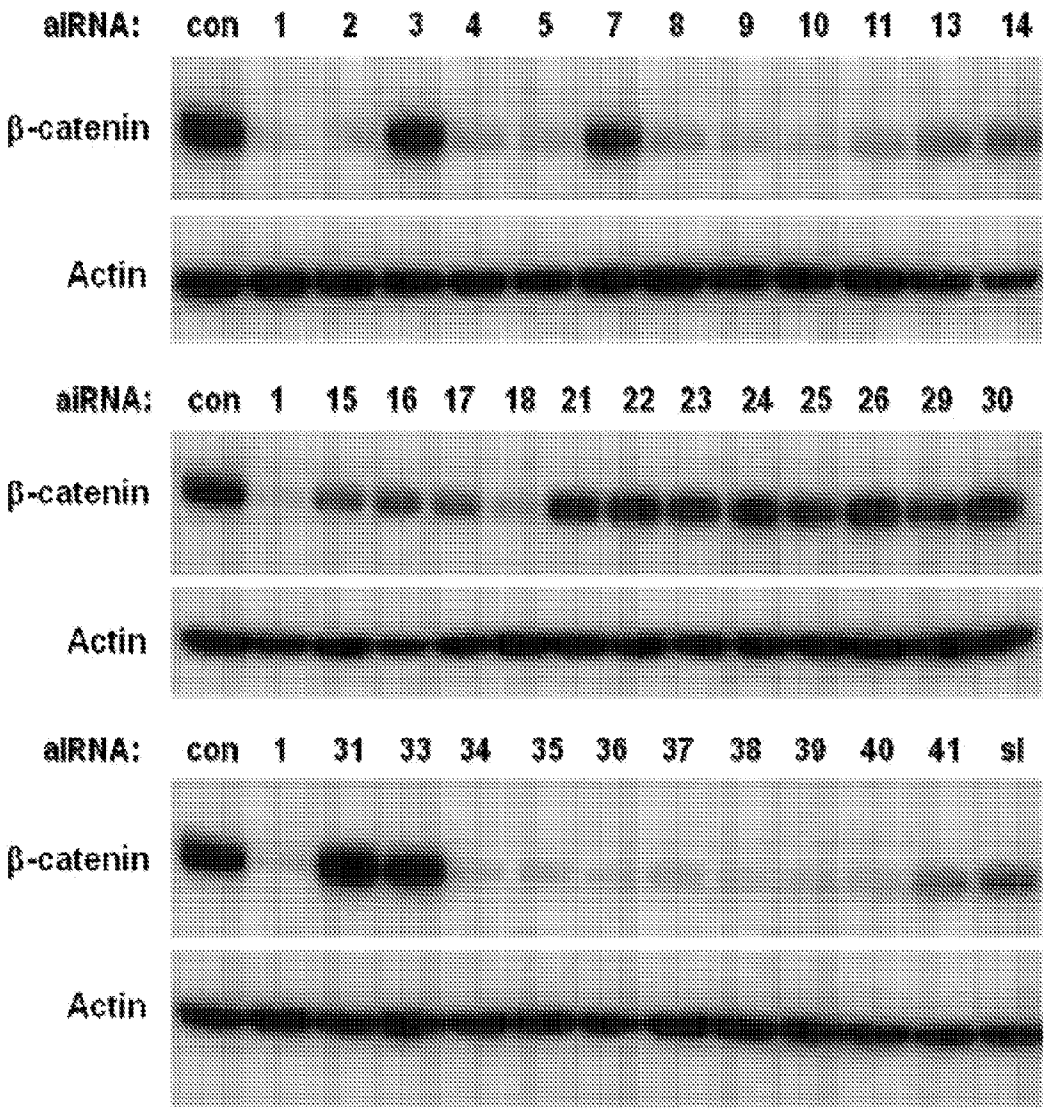
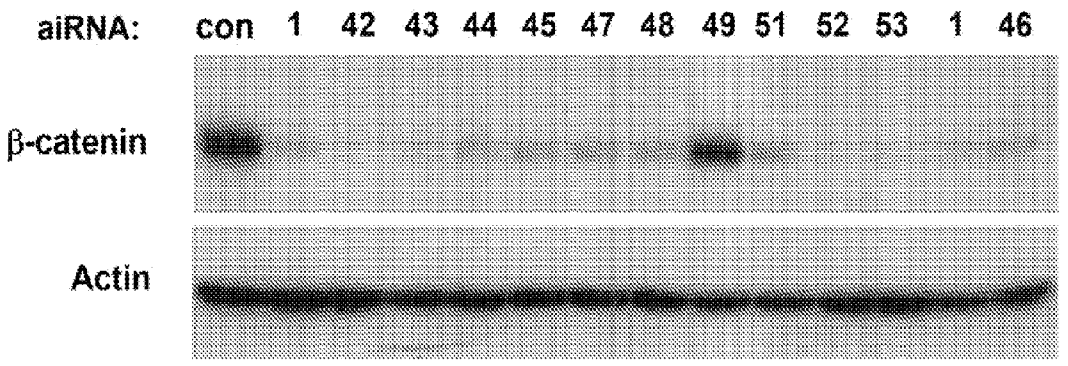

siRNA: con 1 2 3 4 5 7 8 9 10 11 13 14
β-catenin
Actin
siRNA: con 1 15 16 17 18 21 22 23 24 25 26 29 30
β-catenin
Actin
siRNA: con 1 31 33 34 35 36 37 38 39 40 41 sl
β-catenin
Actin

Figure 4


aiRNA:
con 1 42 43 44 45 47 48 49 51 52 53 1 46
β-catenin
Actin

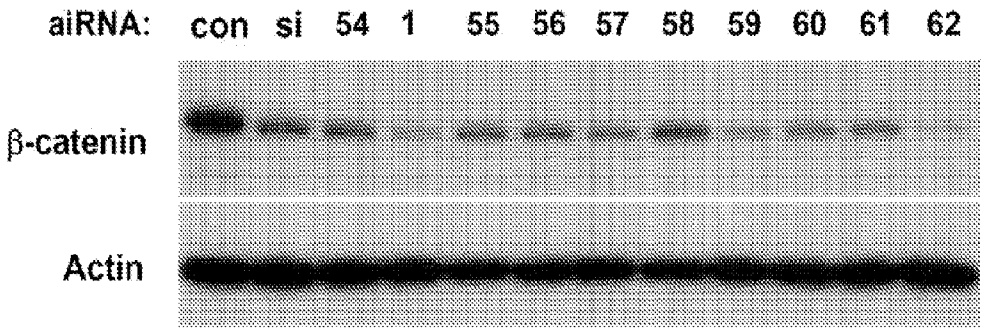
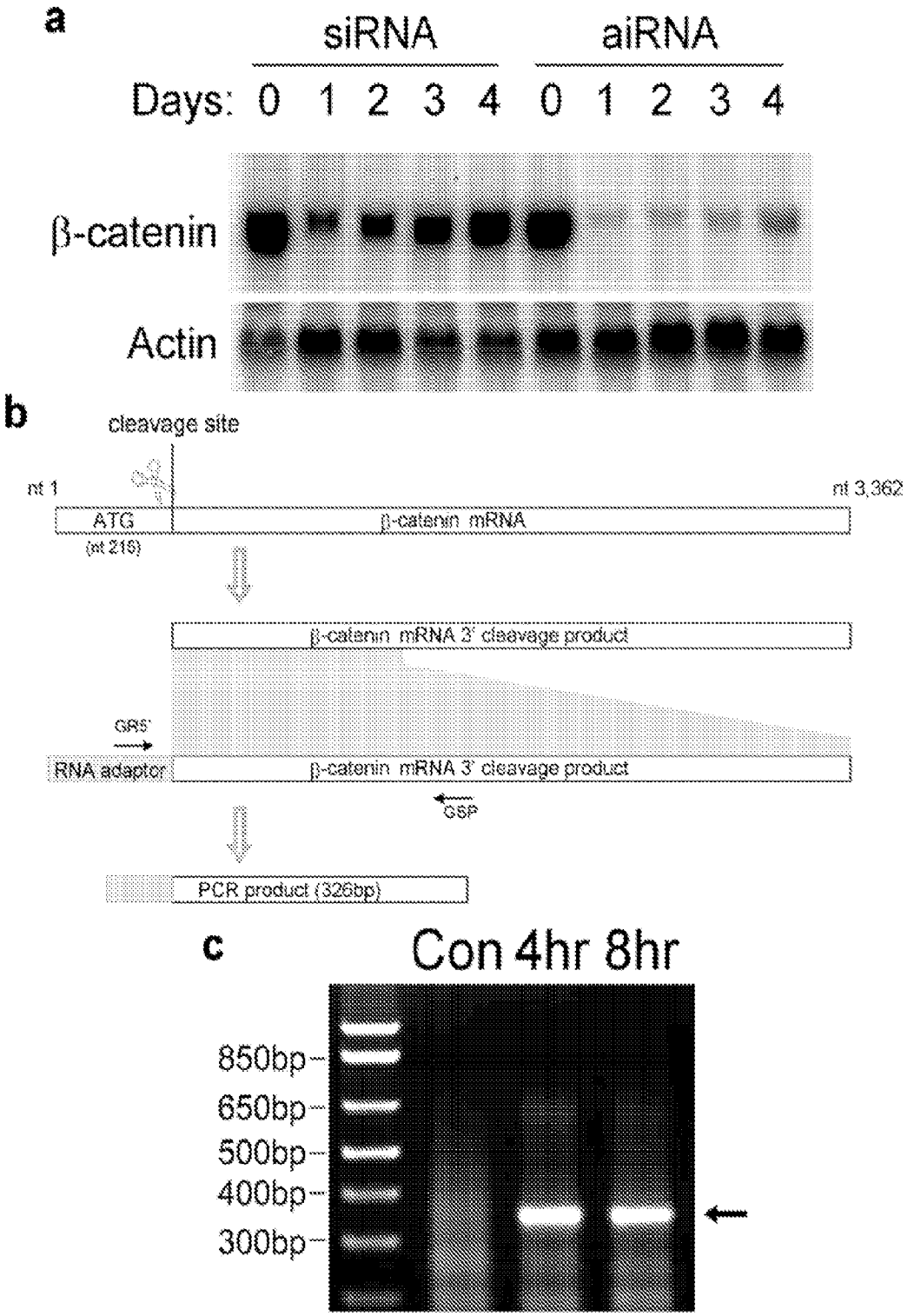
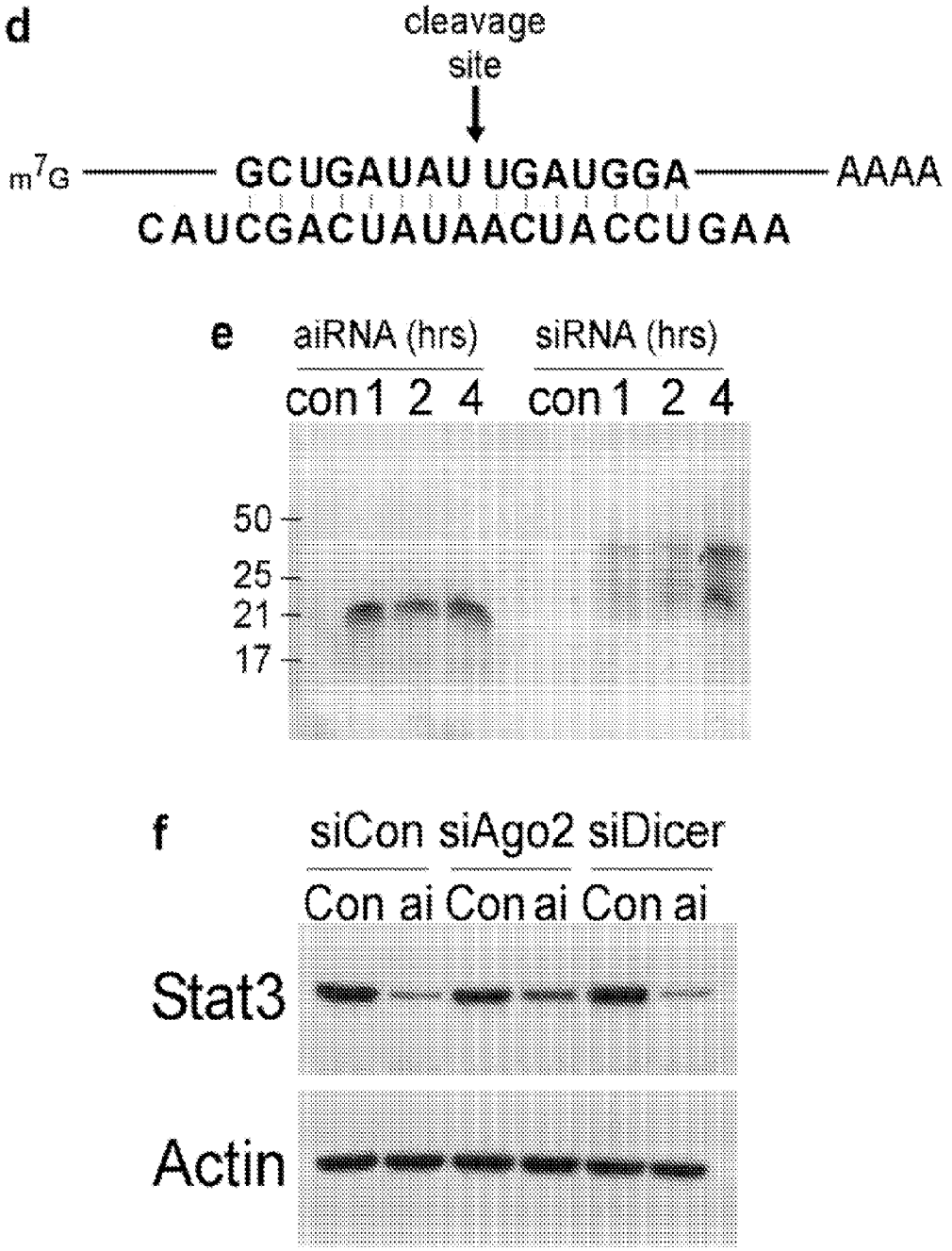

aiRNA:
con si 54 1 55 56 57 58 59 60 61 62
β-catenin
Actin

Figure 5



Figures 6a-6c



Figures 6d-6f

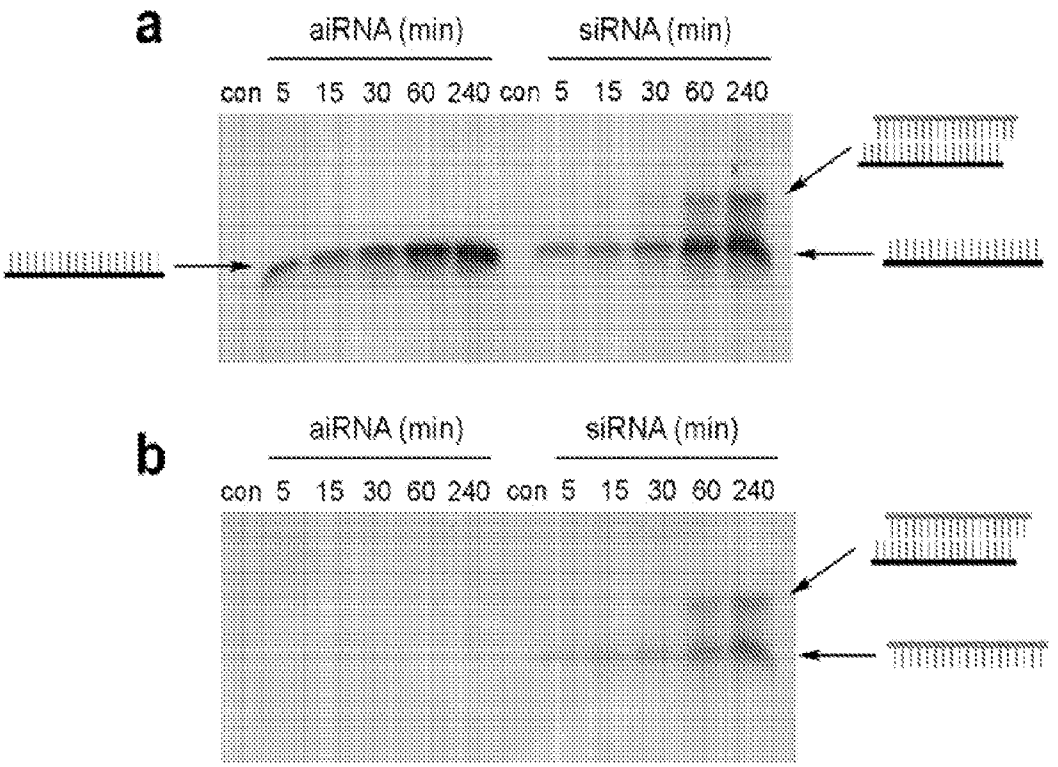

a
aiRNA (min)
siRNA (min)
con 5 15 30 60 240 con 5 15 30 60 240
b
aiRNA (min)
siRNA (min)
con 5 15 30 60 240 con 5 15 30 60 240

Figure 7

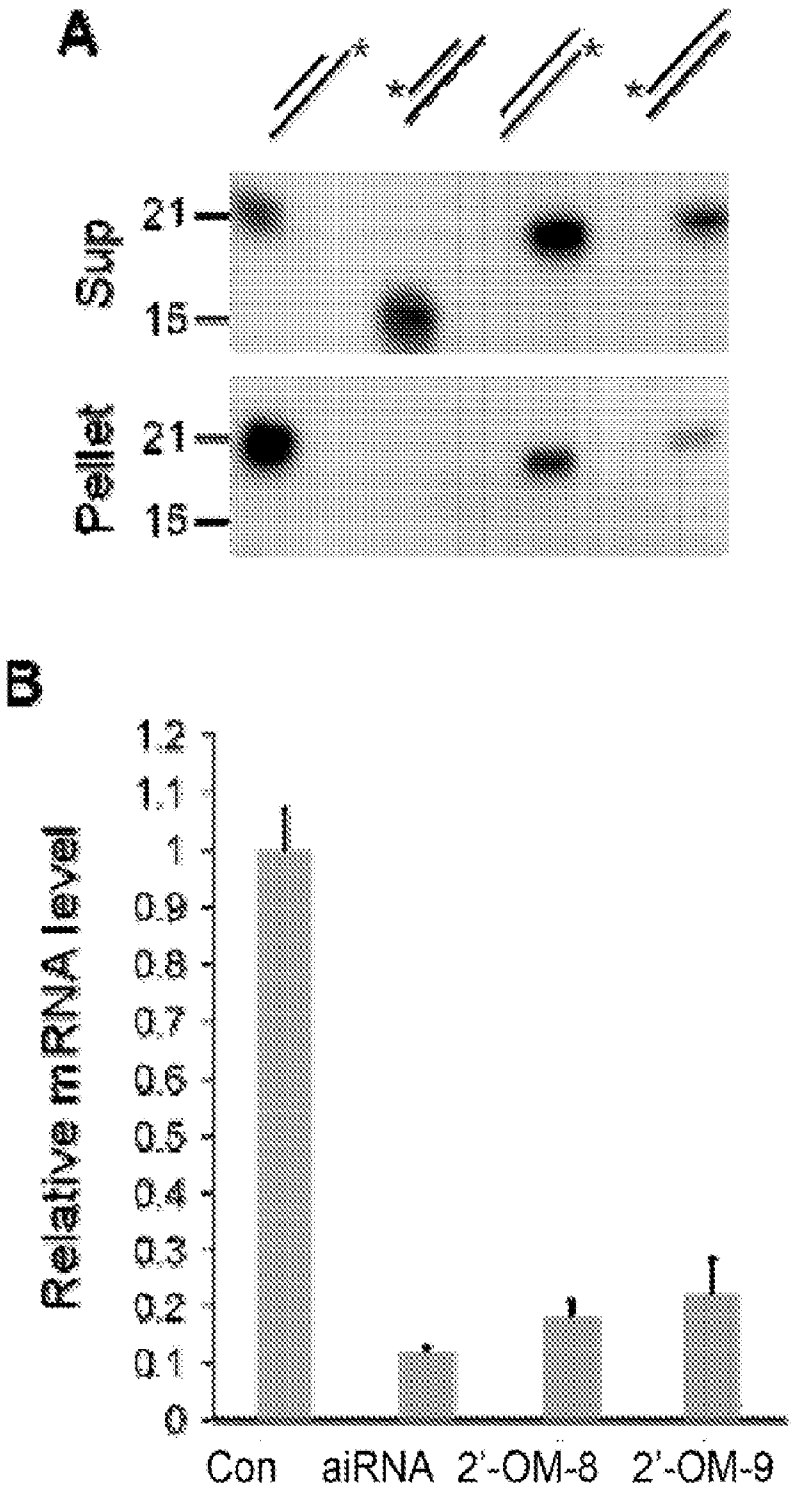

A
Sup
Pellet
21
15
21
15
B
Relative mRNA level
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Con
aiRNA
2'-OM-8
2'-OM-9

Figure 8

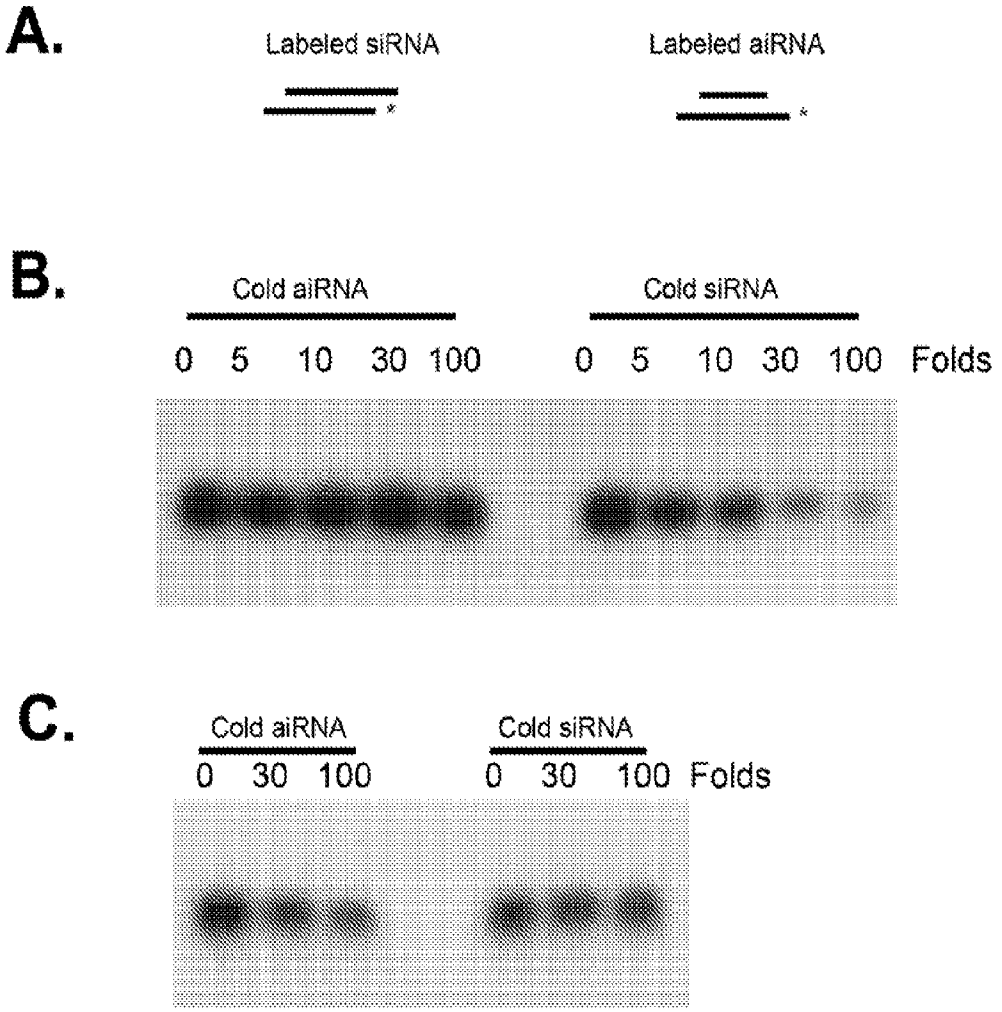

A.
Labeled siRNA
Labeled aiRNA
B.
Cold aiRNA
Cold siRNA
0 5 10 30 100
0 5 10 30 100 Folds
C.
Cold aiRNA
Cold siRNA
0 30 100
0 30 100 Folds

Figure 9

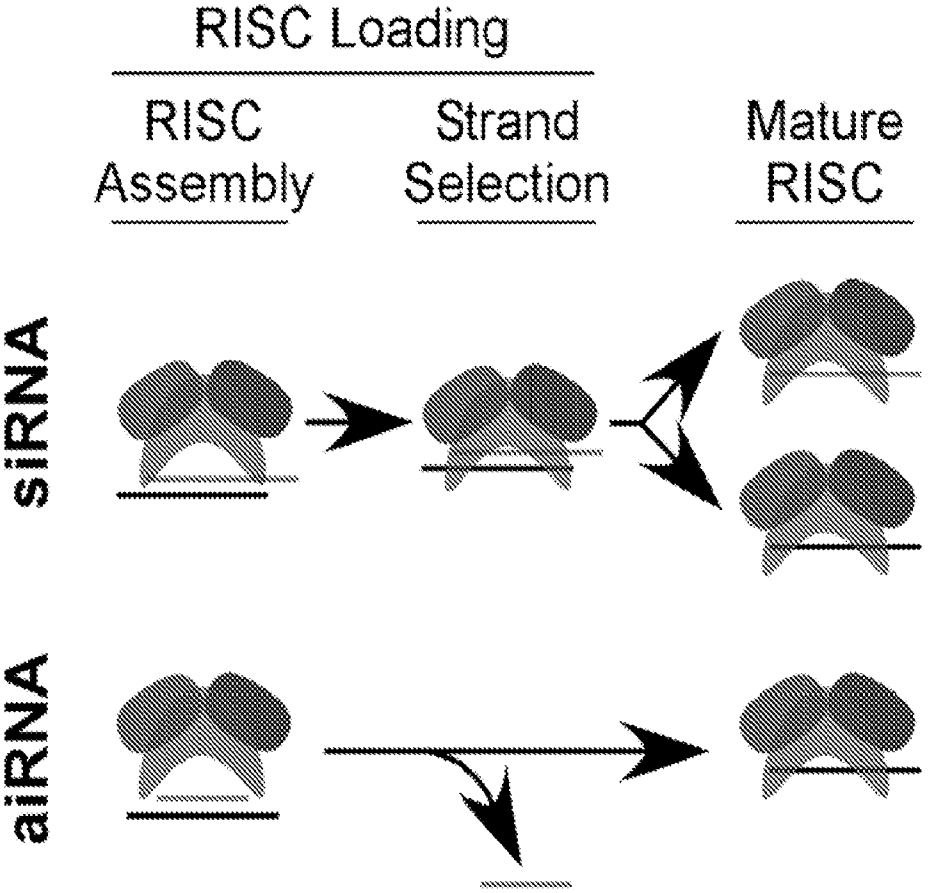
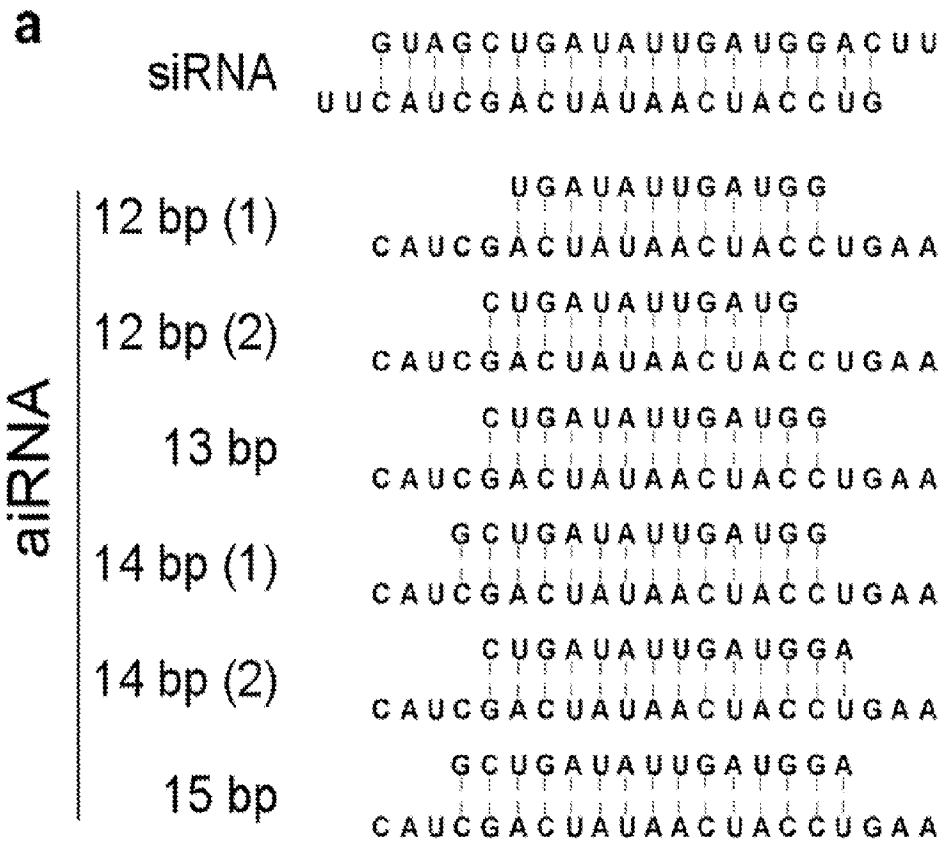
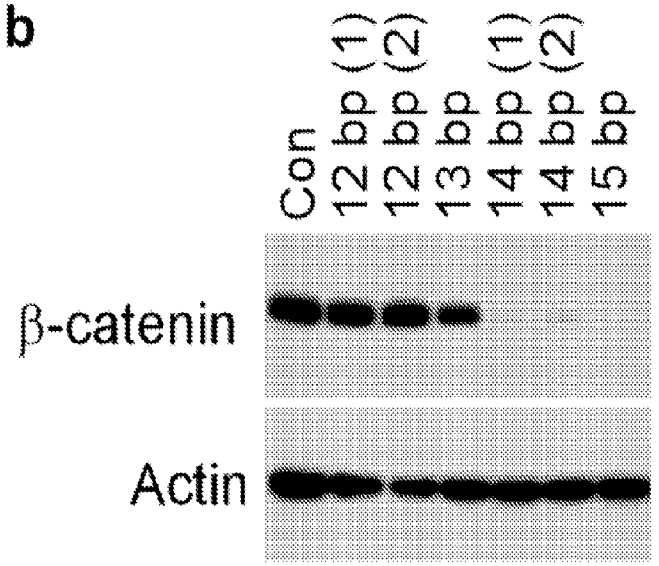
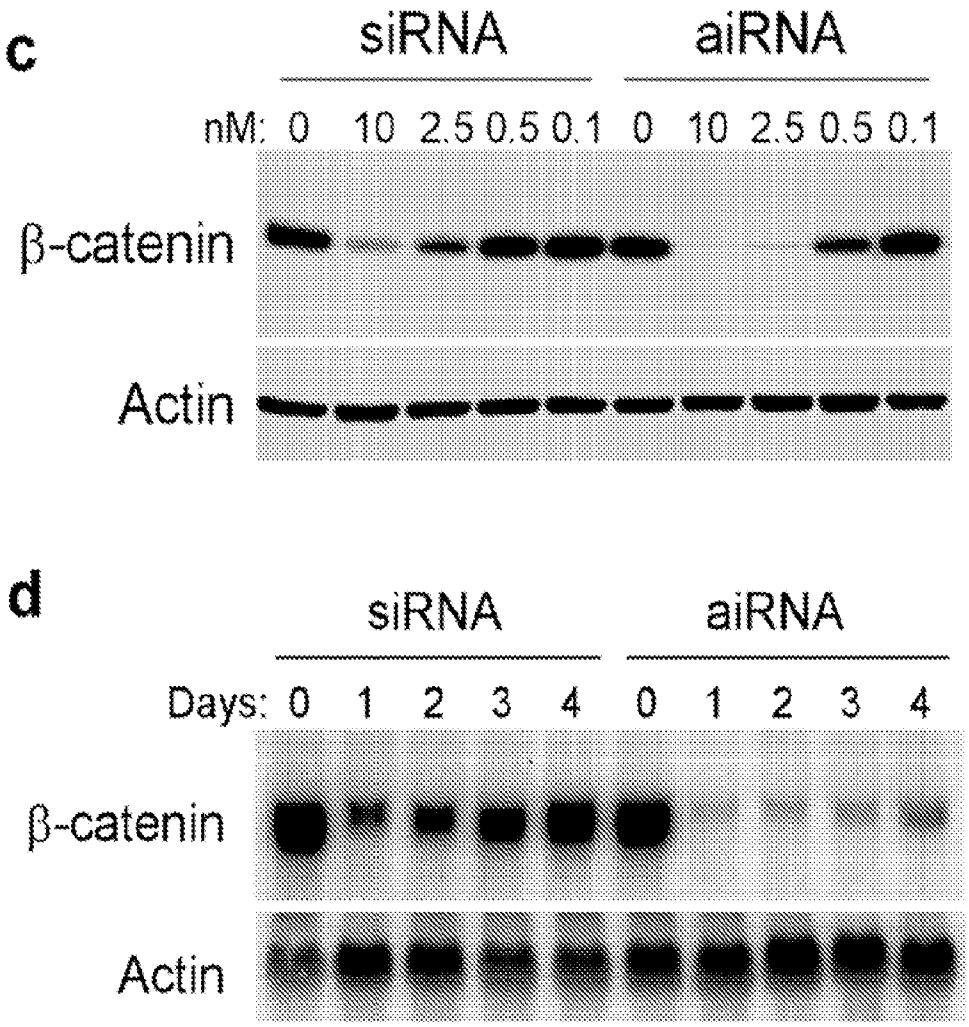

RISC Loading
RISC Assembly
Strand Selection
Mature RISC
siRNA
aiRNA

Figure 10

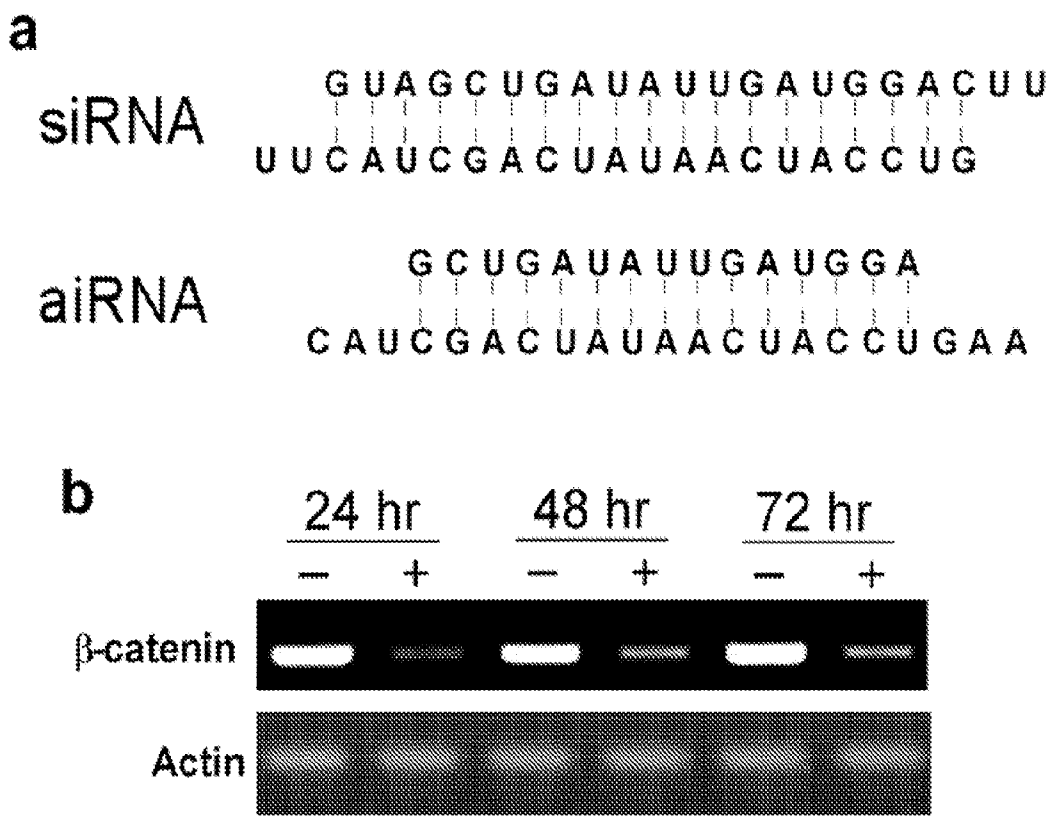




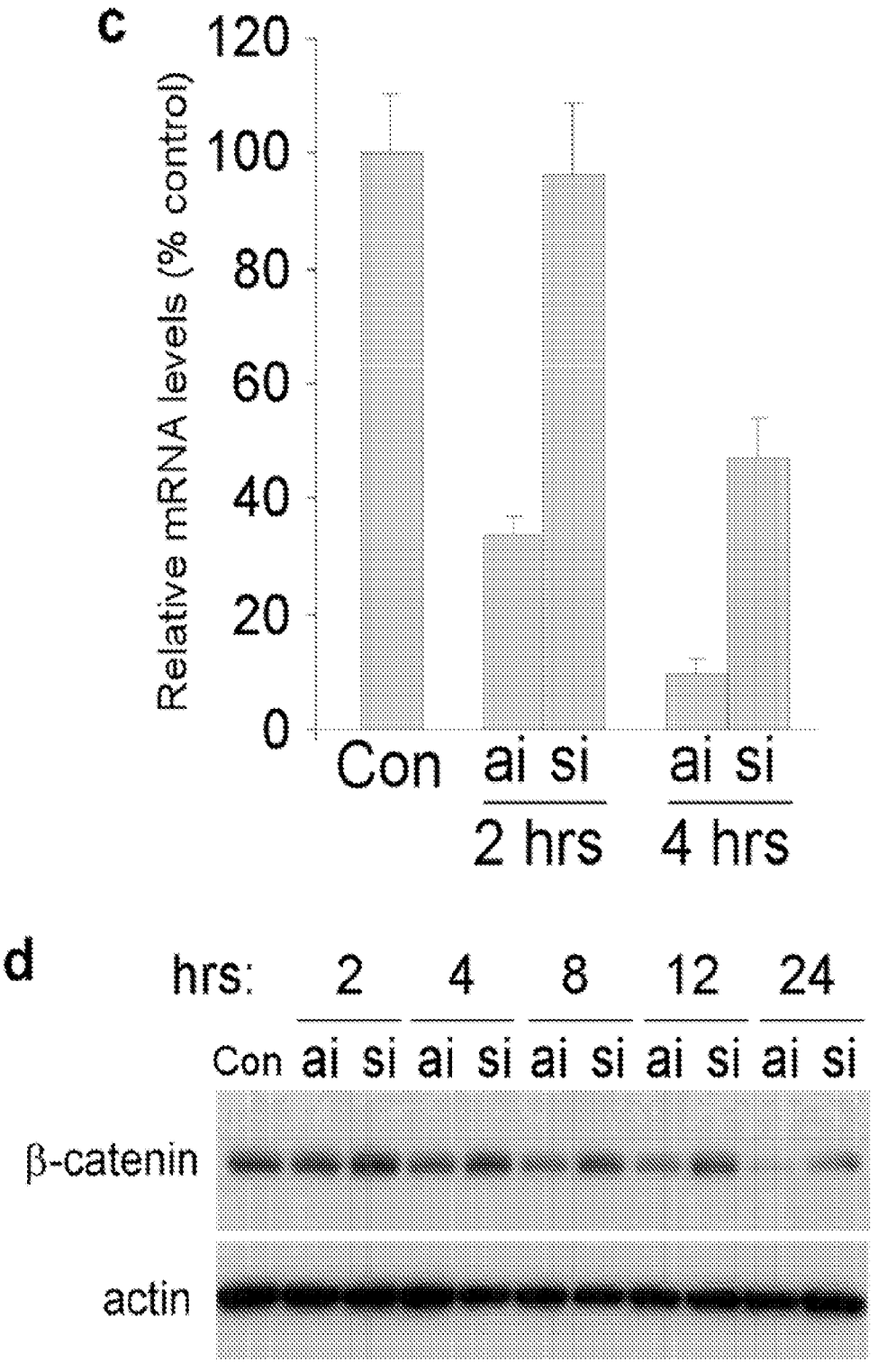
Figures 11A-11B



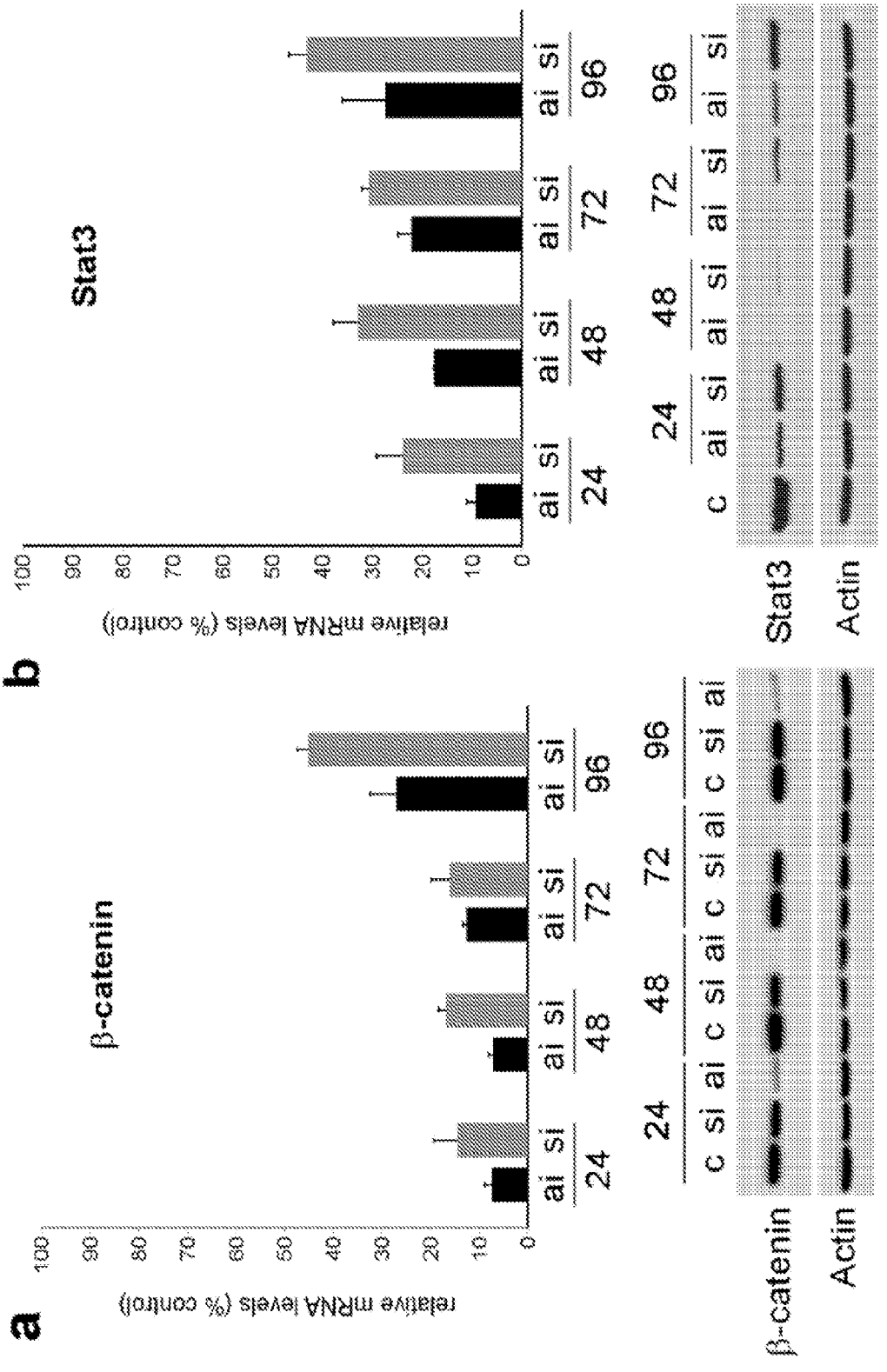
Figures 11C-11D



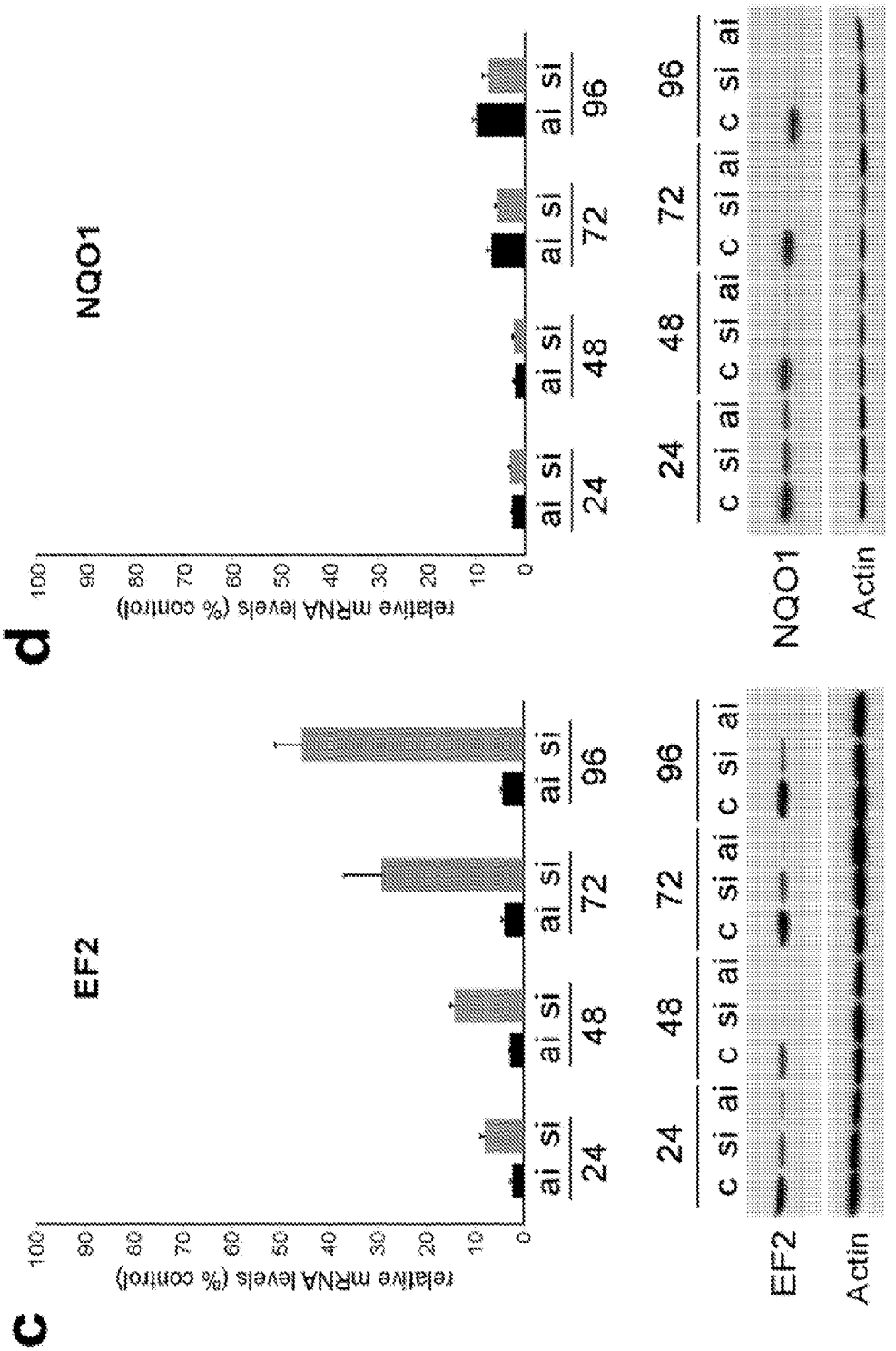
Figures 12A-12B



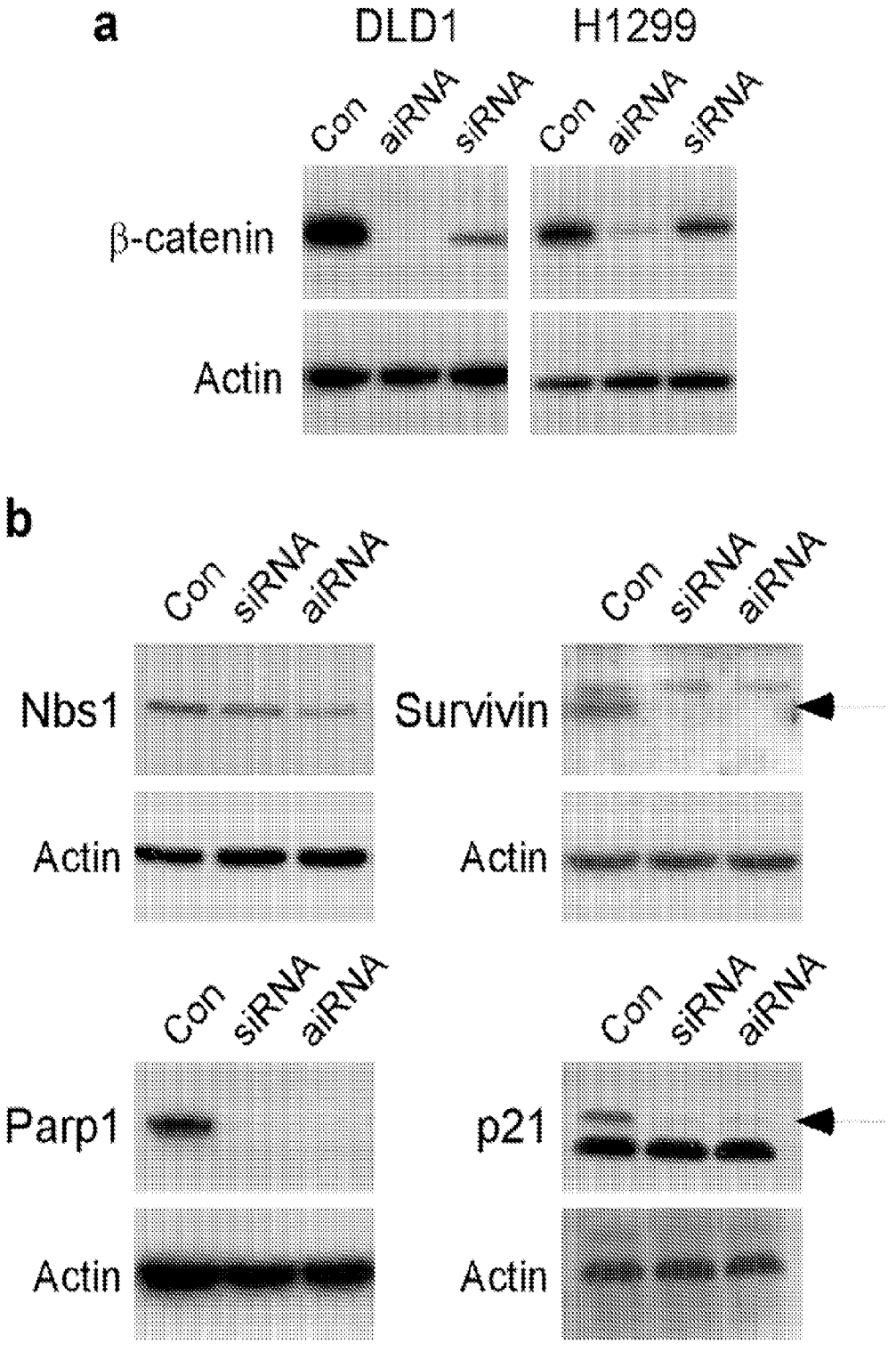
Figures 12C-12D



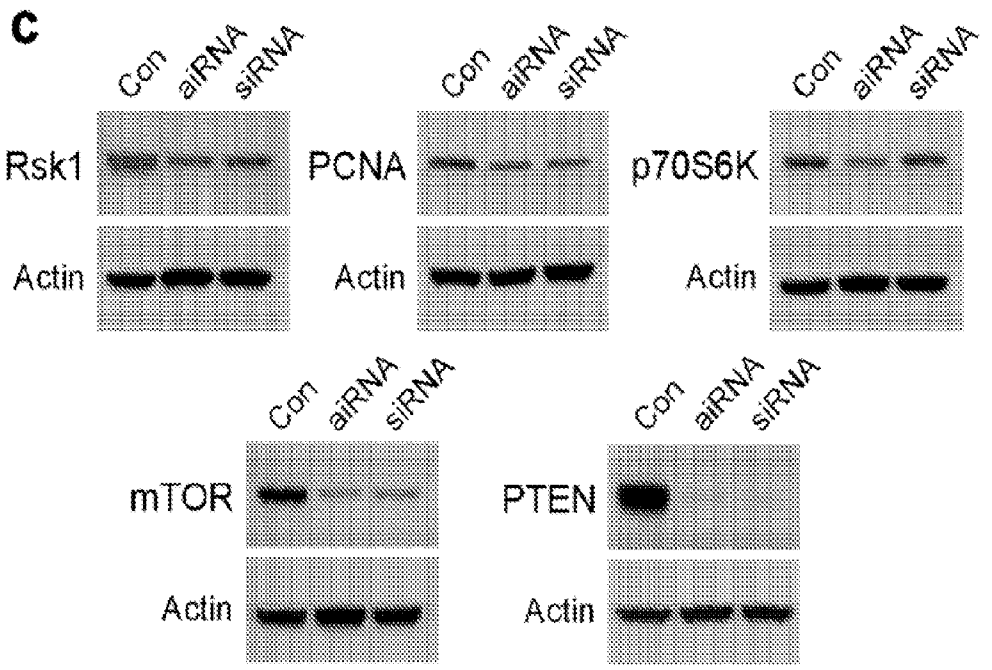
Figures 13a-13b

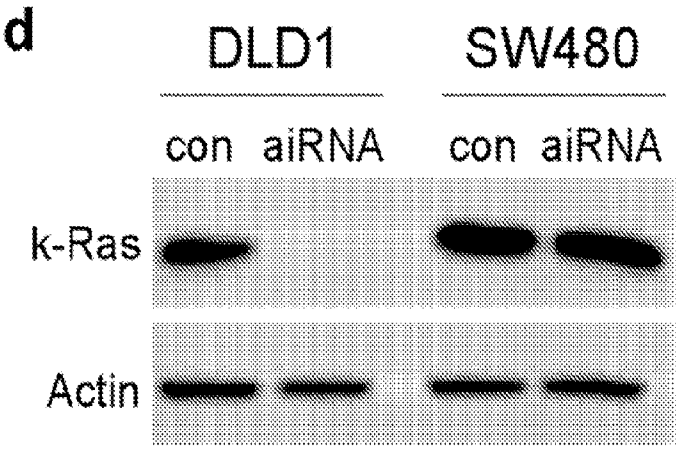


Figures 13c-13d

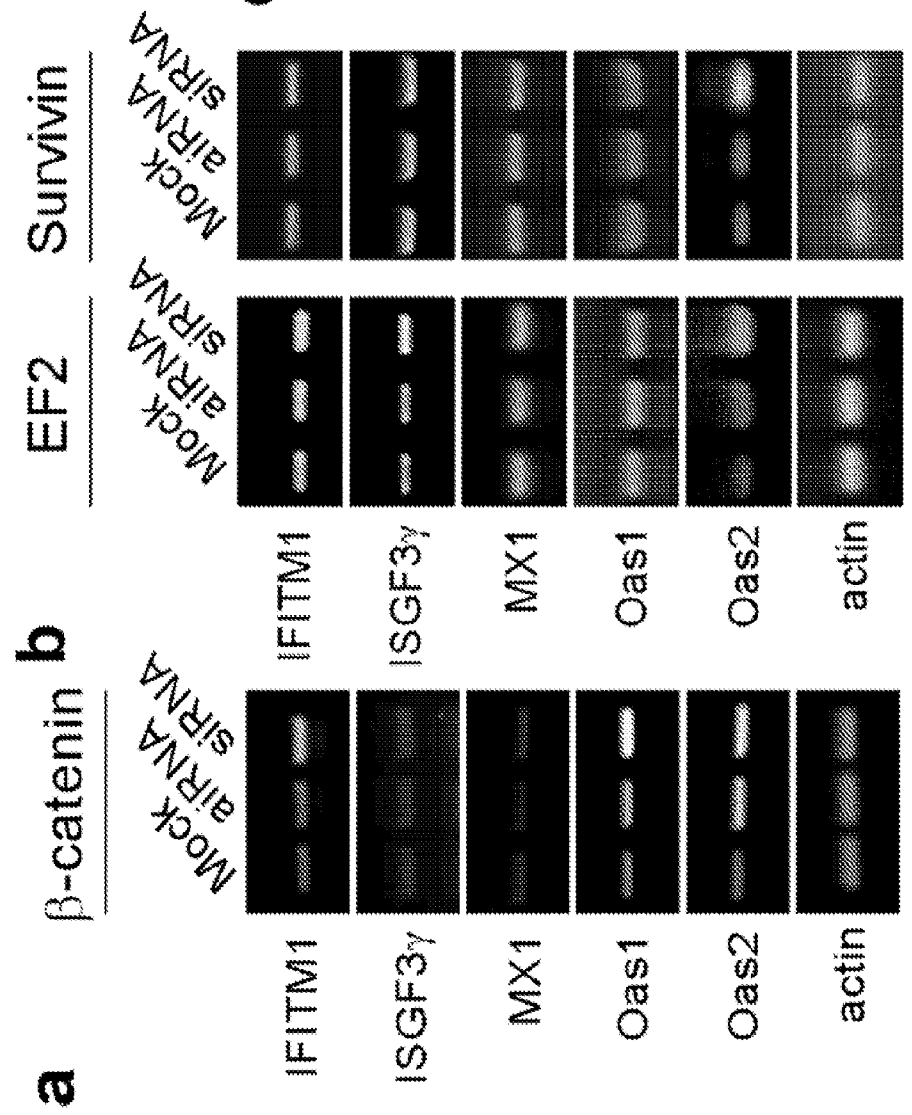


Figures 14a-14b





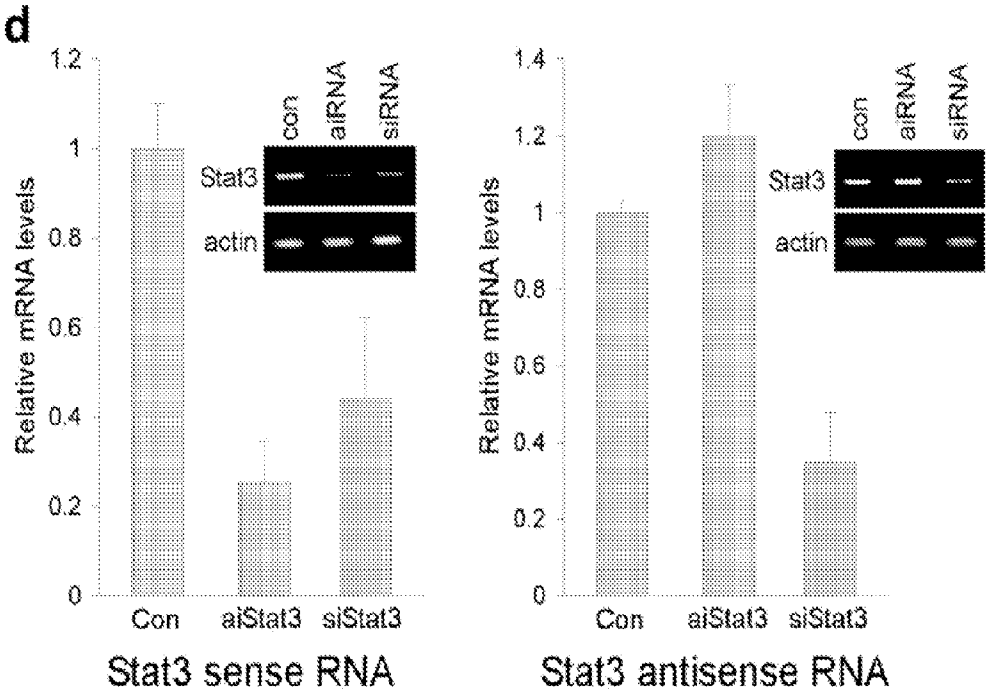
Figures 14c-14d

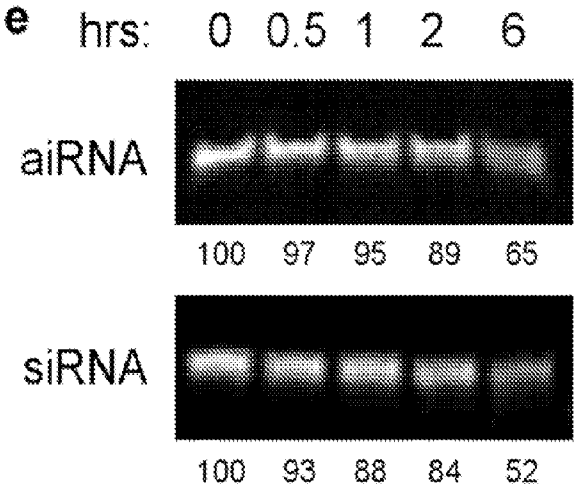


c

	aiRNA	siRNA
ATF3-1	1.74	2
IFIH1	1.32	1.32
RSAD2-1	1.07	4.92
SP110-1	1.07	1.41
ATF3-2	0.93	1.23
SP110-2	0.93	1.14
OAS1	0.87	1.51
IFIT5	0.87	1.23
RSAD2-2	0.81	2.82
OAS3	0.81	1.23
ISGF3γ	0.87	1.14
MX1	0.93	0.75
IFITM1	0.93	1.07

Figures 15a-15c





Figures 15d-15e

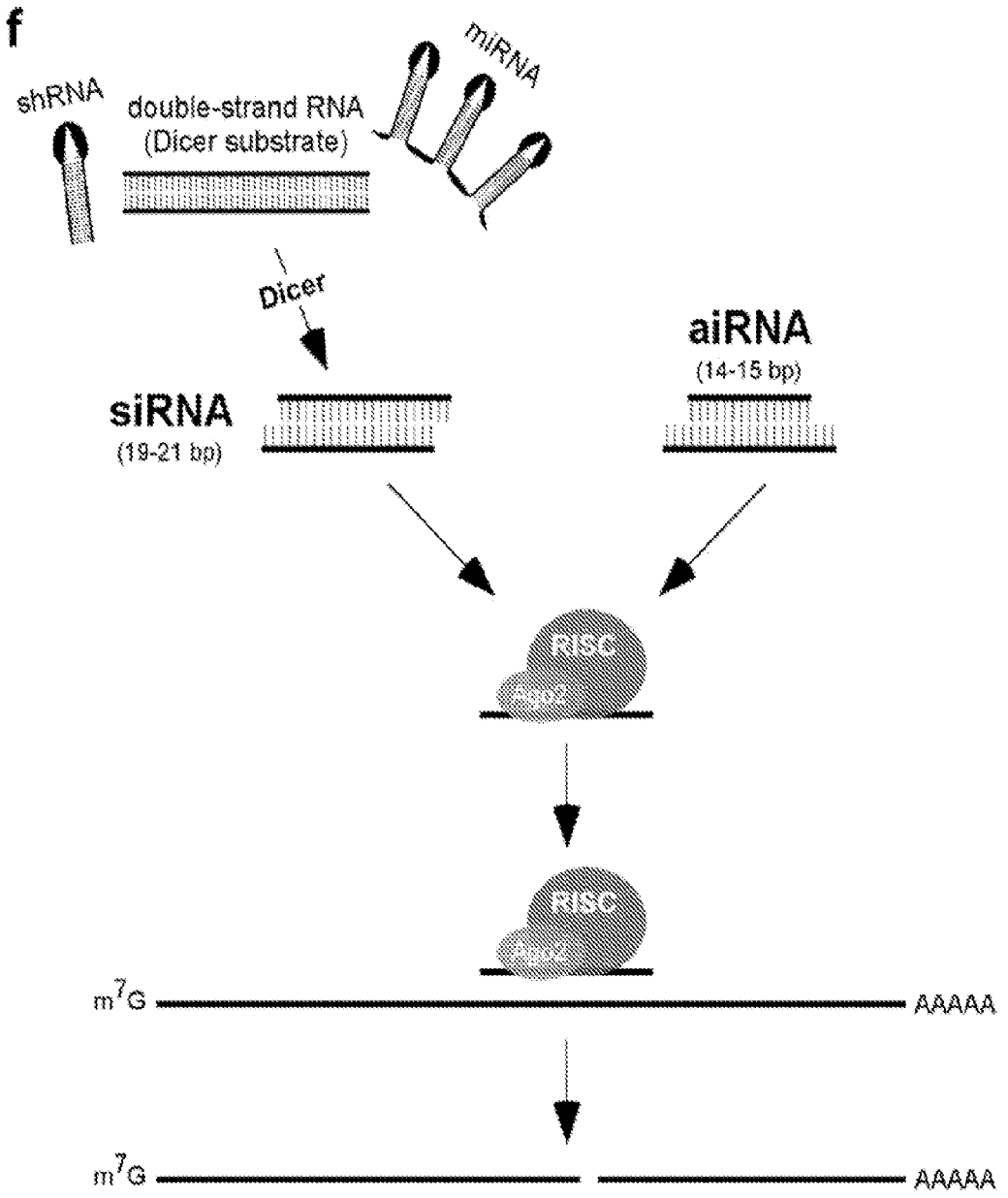

f
shRNA
double-strand RNA
(Dicer substrate)
miRNA
Dicer
siRNA
(19-21 bp)
aiRNA
(14-15 bp)
RISC
Ago2
RISC
Ago2
m7G
AAAAA
m7G
AAAAA

Figure 15f

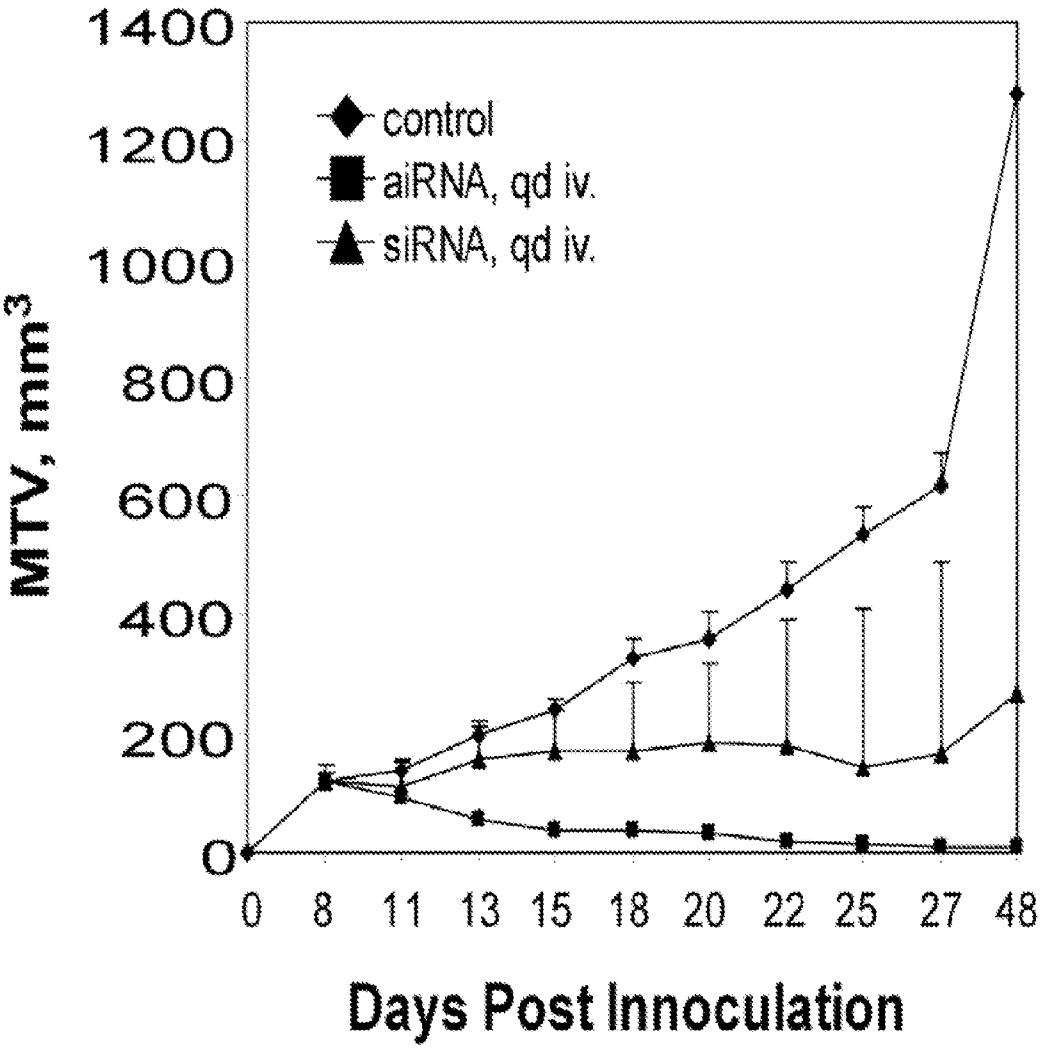

1400
1200
1000
800
600
400
200
0
MTV, mm3
control
aiRNA, qd iv.
siRNA, qd iv.
0 8 11 13 15 18 20 22 25 27 48
Days Post Innoculation

Figure 16

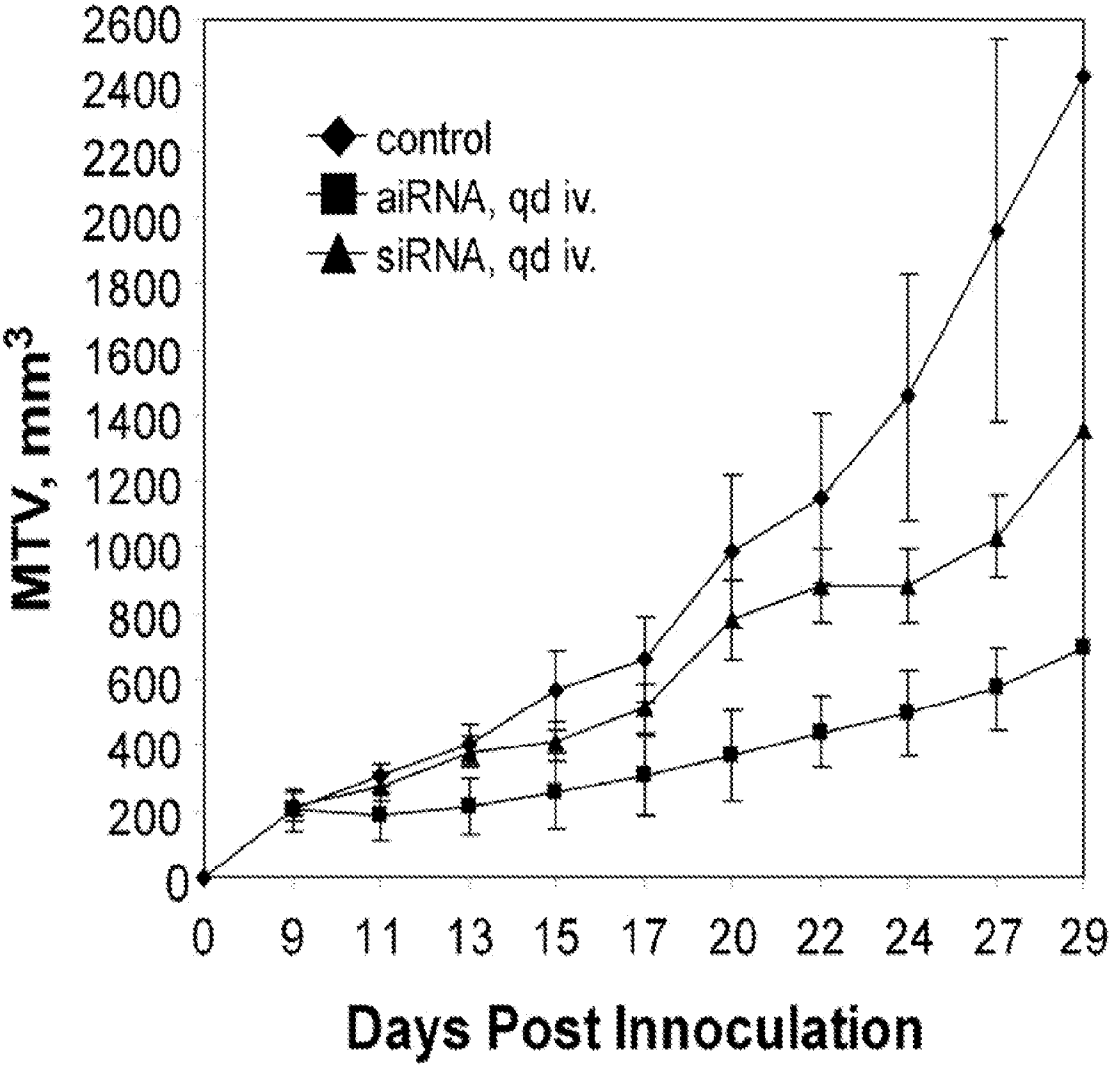

2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
MTV, mm3
control
aiRNA, qd iv.
siRNA, qd iv.
0
9
11
13
15
17
20
22
24
27
29
Days Post Innoculation

Figure 17

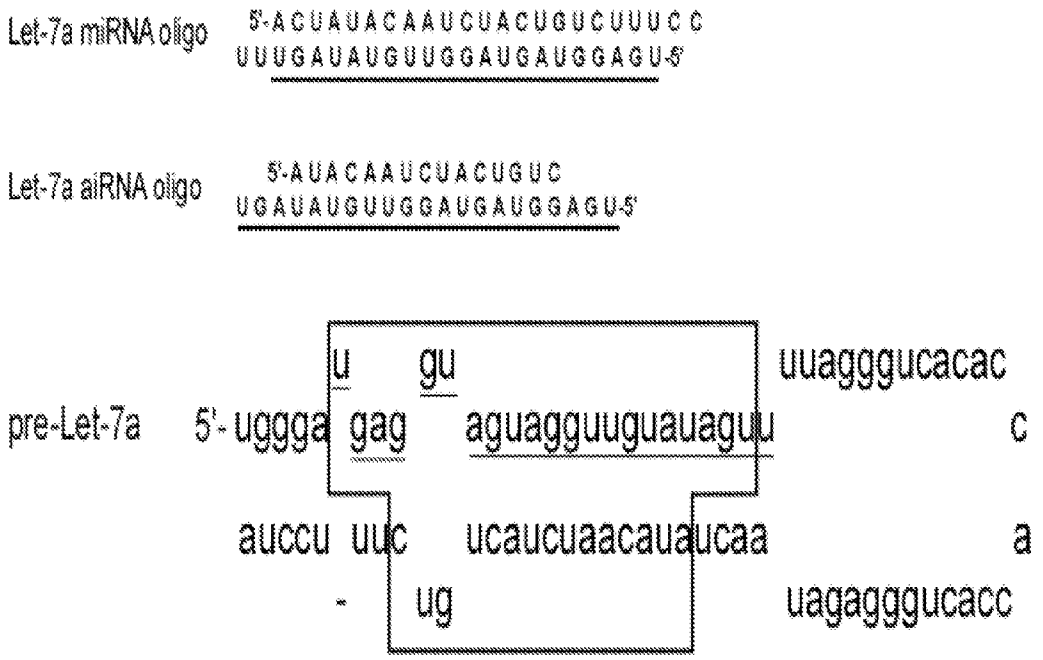


Figure 18A

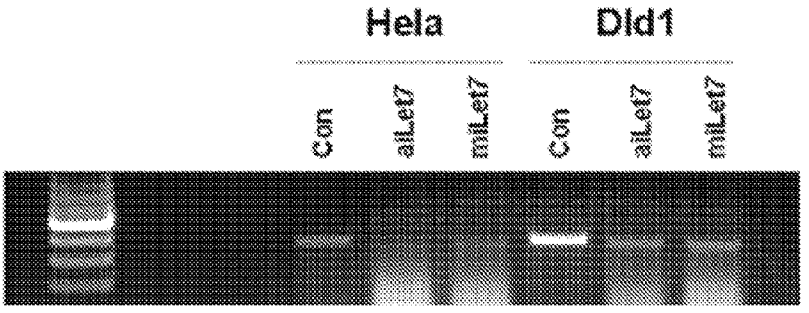


Figure 18B

aiLet-7c mimic:	5'-ACAACCUACUACCUC GUAUGUUGGAUGAUGGAGUAA-5'
Let-7c inhibitor:	5'-GGUAGUAGGUUGUAU ACUCCAUCAUCCAACAUACAA-5'
miR-21 inhibitor:	5'-AUCAGACUGAUGUUG GAAUAGUCUGACUACAACUAA-5'
miR-155 inhibitor:	5'-AUGCUAAUCGUGAUA AAUUACGAUUAGCACUAUCAA-5'

Figure 19A

hsa-Let-7c
Mature miRNA: UGAGGUAGUAGGUUGUAUGGUU

hsa-miR-21
Mature miRNA: UAGCUUAUCAGACUGAUGUUGA

hsa-miR-155
Mature miRNA: UUAAUGCUAAUCGUGAUAGGGGU

Figure 19B

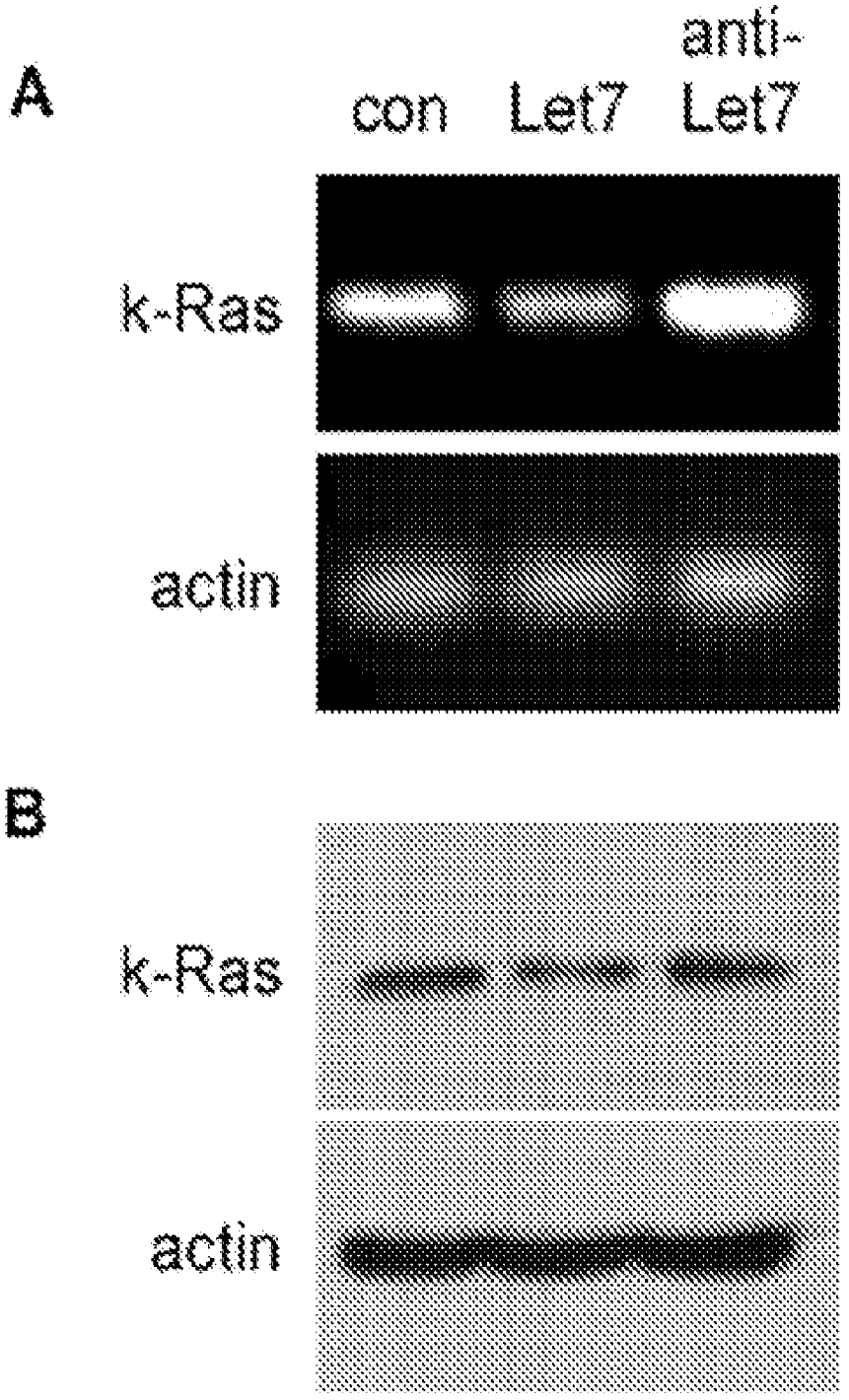
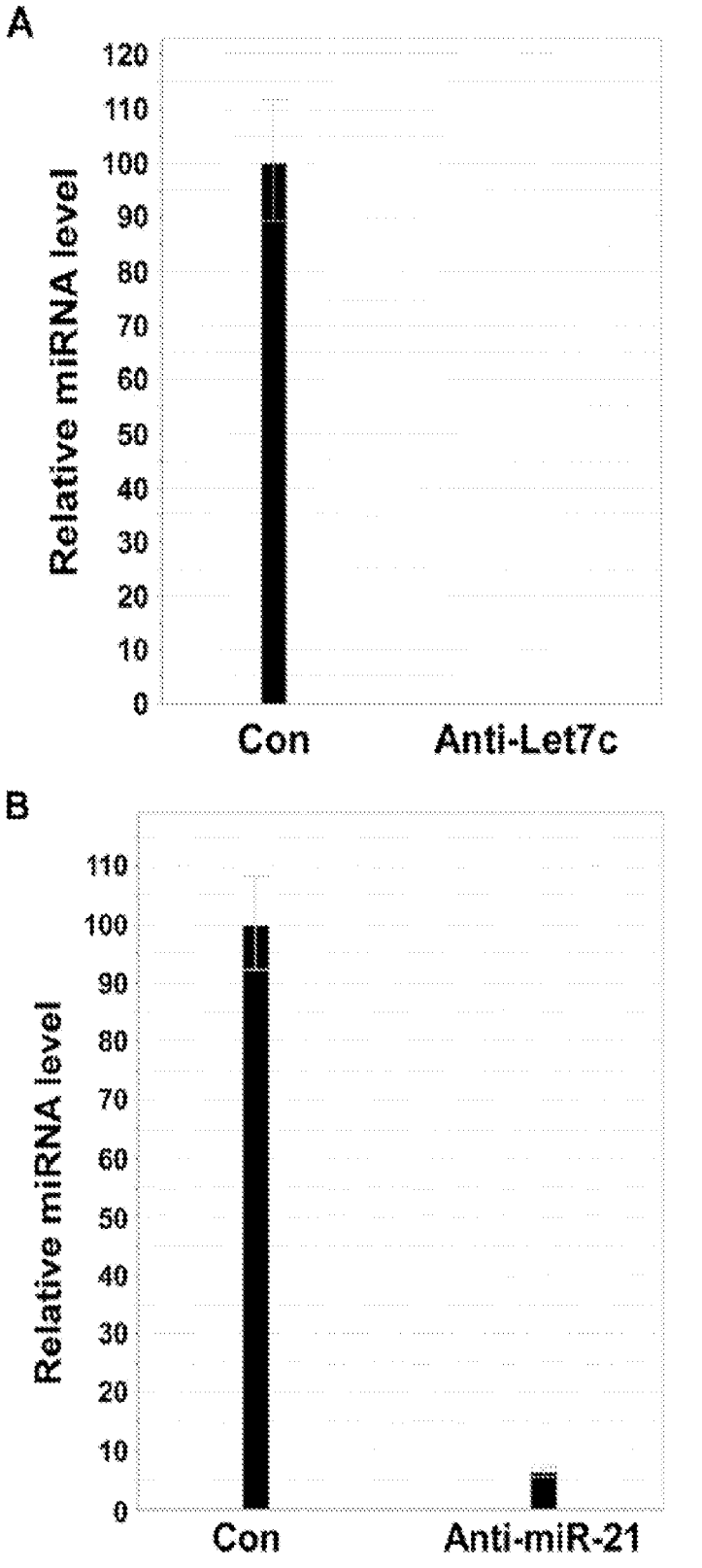


Figure 20



Figures 21A-21B

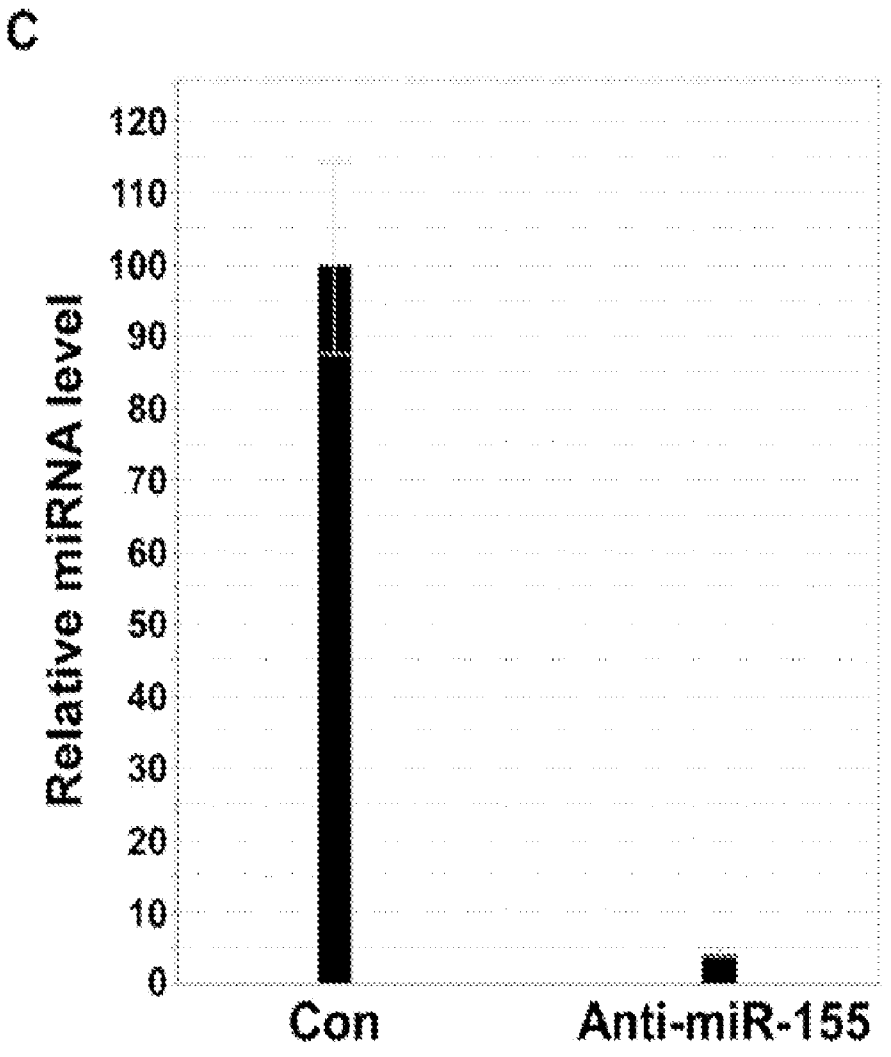

C
Relative miRNA level
120
110
100
90
80
70
60
50
40
30
20
10
0
Con
Anti-miR-155

Figure 21C

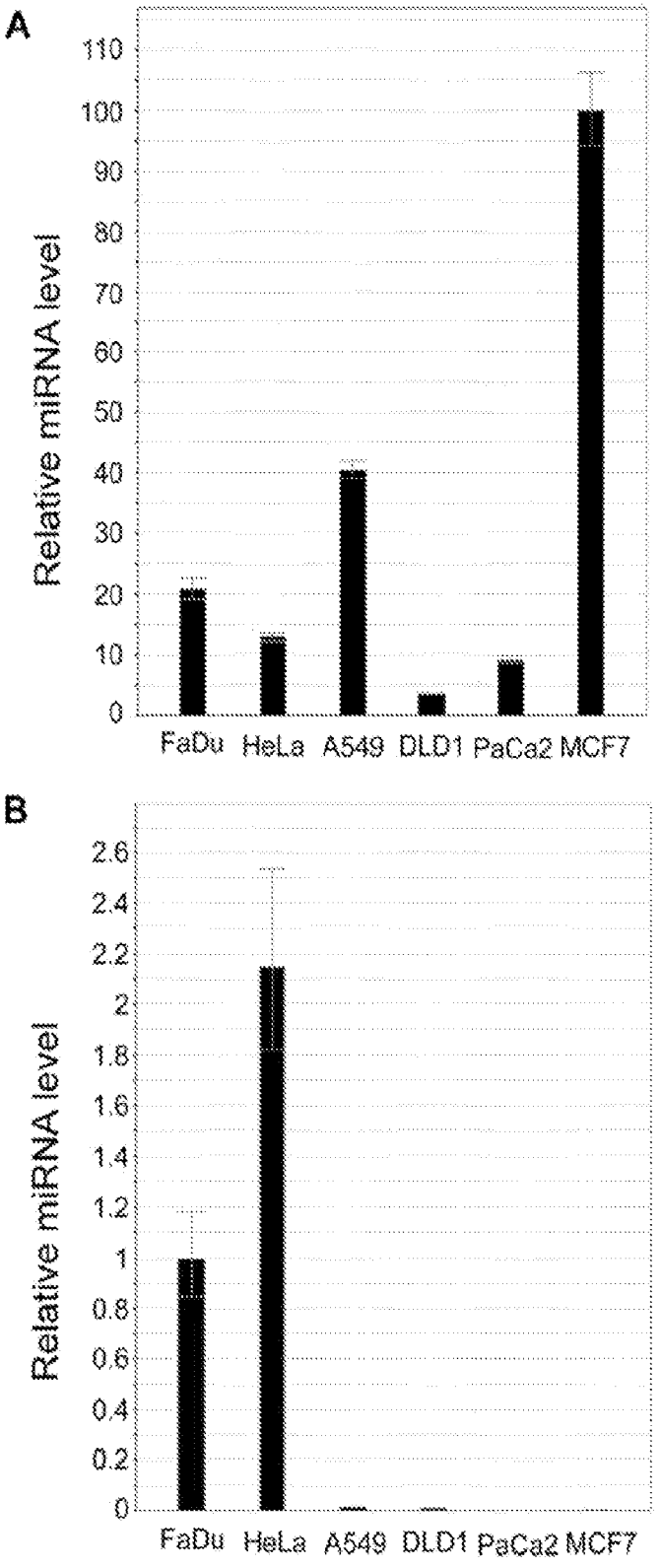

A
Relative miRNA level
110
100
90
80
70
60
50
40
30
20
10
0
FaDu HeLa A549 DLD1 PaCa2 MCF7
B
Relative miRNA level
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
FaDu HeLa A549 DLD1 PaCa2 MCF7

Figure 22

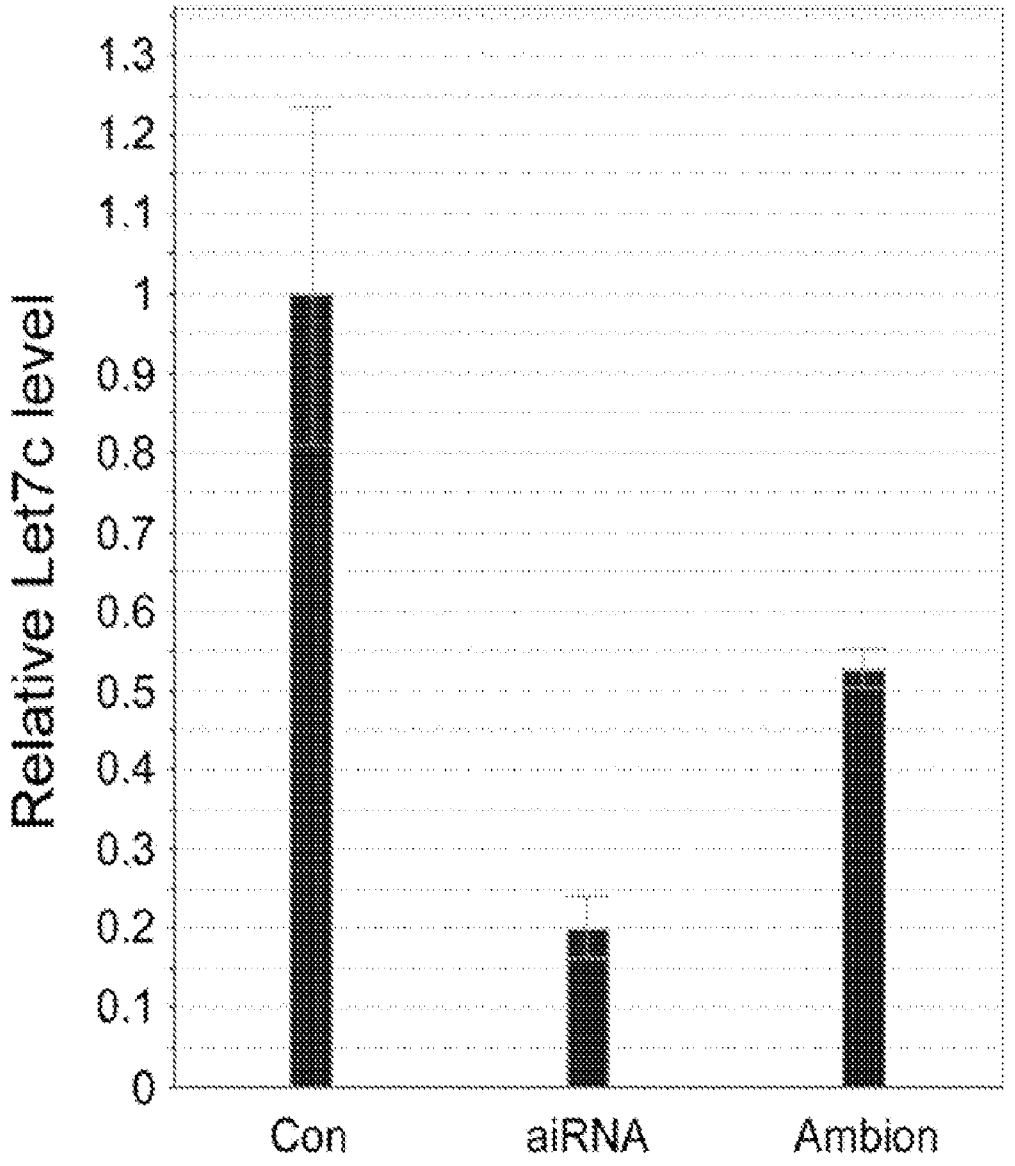

Relative Let7c level
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Con
aiRNA
Ambion

Figure 23

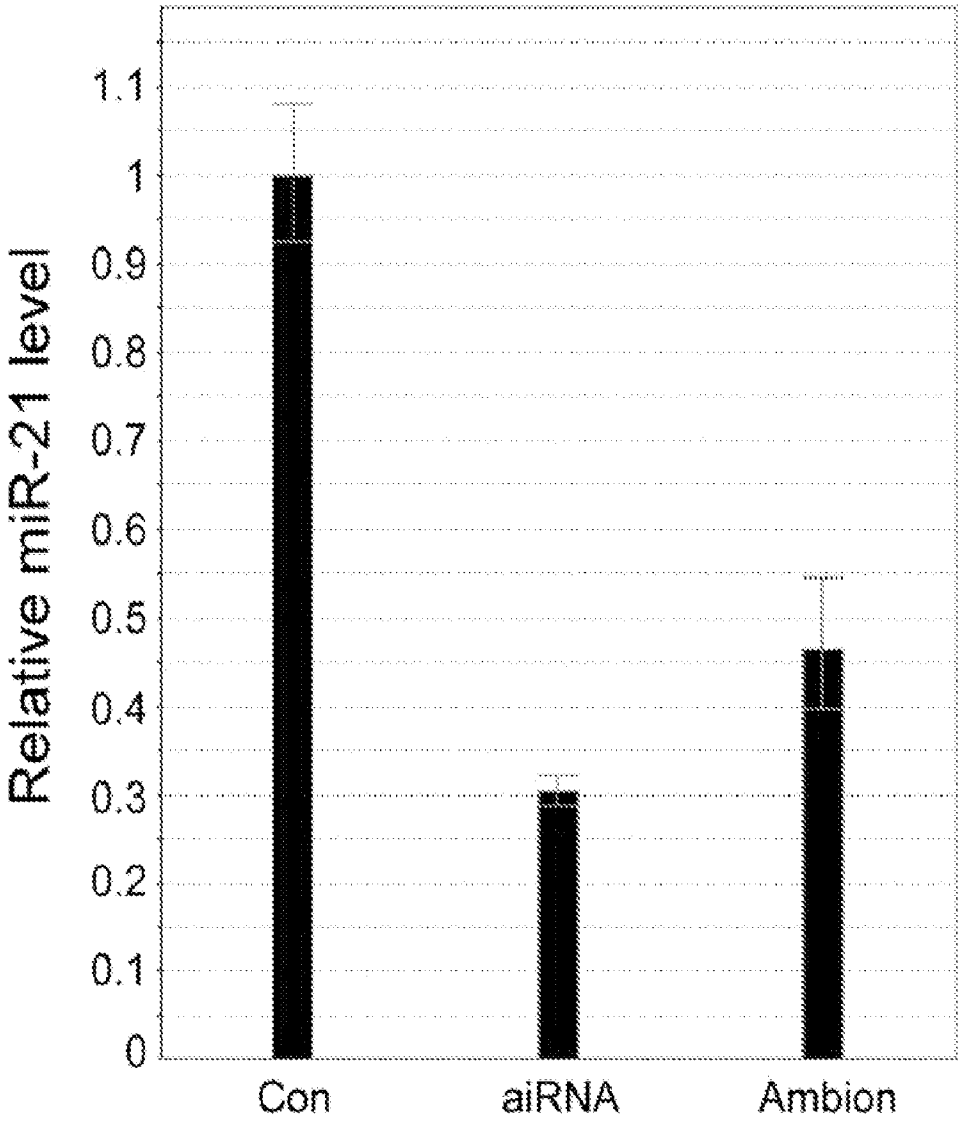

Relative miR-21 level
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Con
aiRNA
Ambion

Figure 24

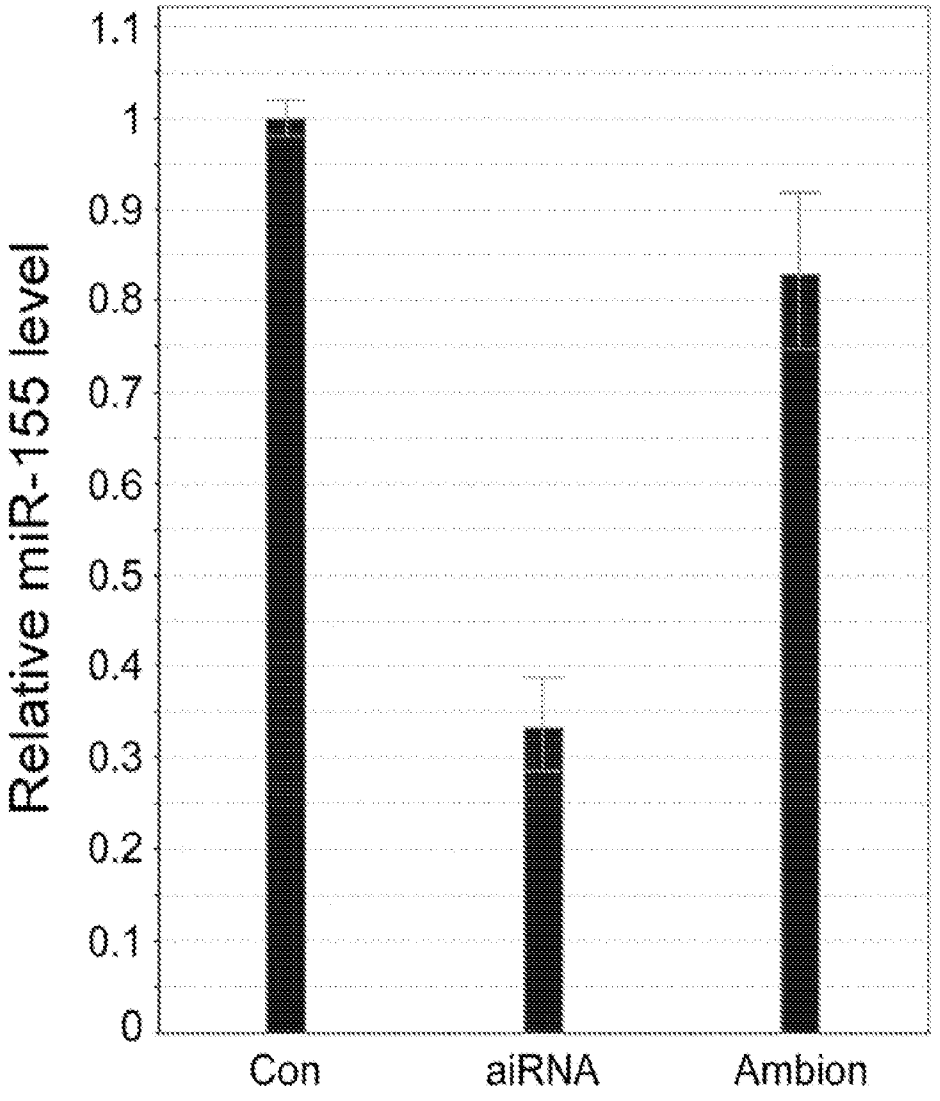

Relative miR-155 level
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Con
aiRNA
Ambion

Figure 25

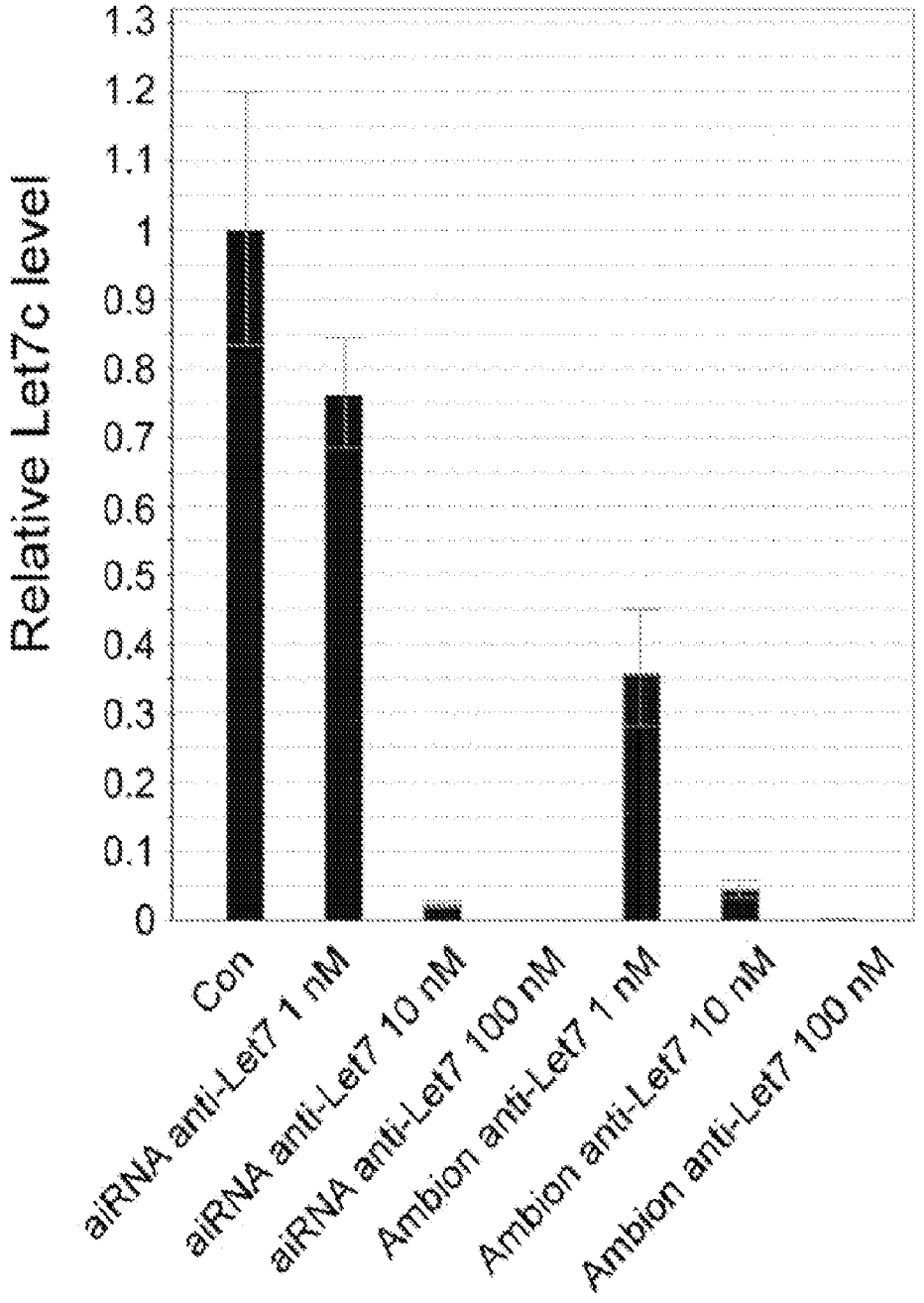

Relative Let7c level
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Con
aiRNA anti-Let7 1 nM
aiRNA anti-Let7 10 nM
aiRNA anti-Let7 100 nM
Ambion anti-Let7 1 nM
Ambion anti-Let7 10 nM
Ambion anti-Let7 100 nM

Figure 26

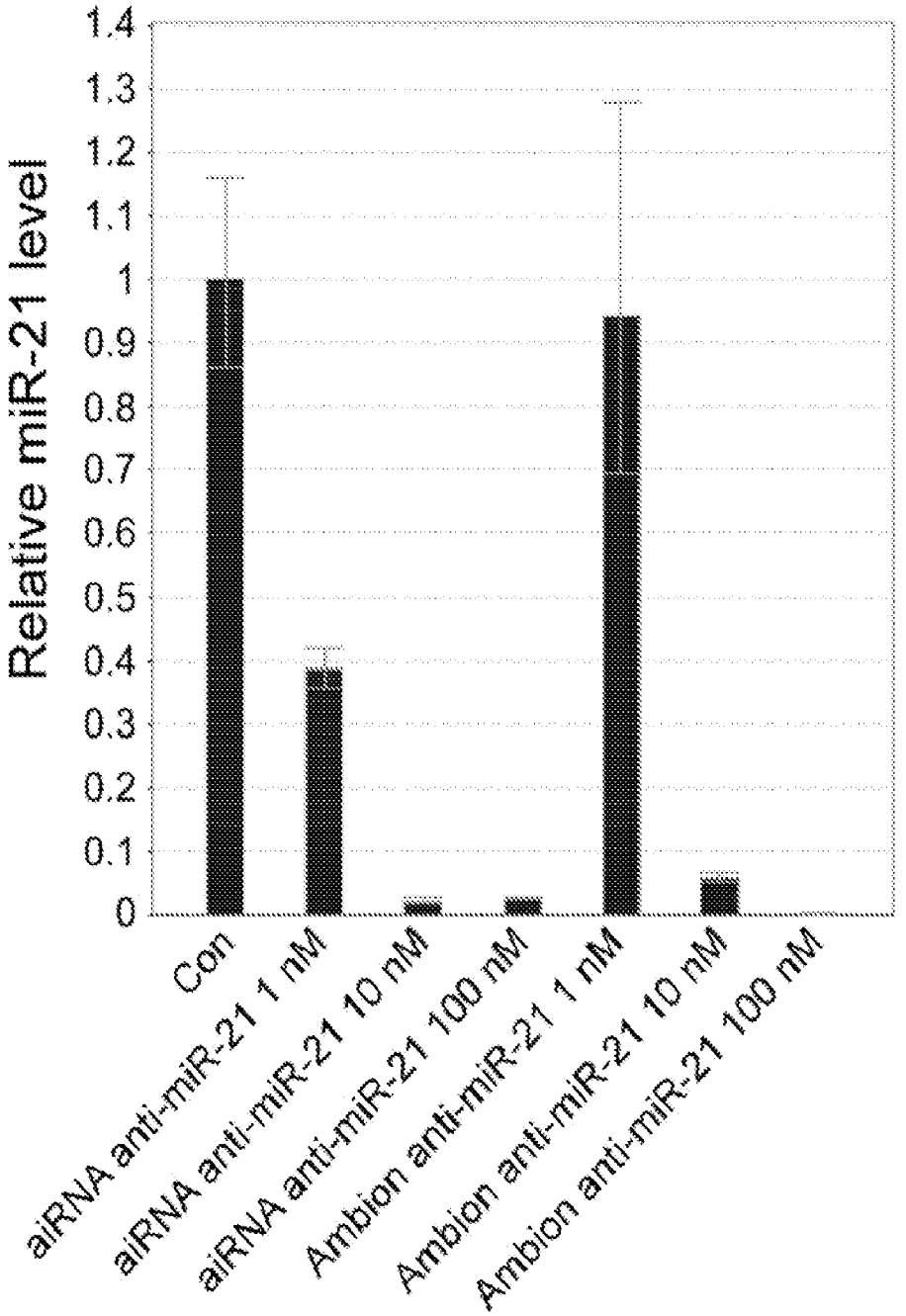

Relative miR-21 level
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Con
aiRNA anti-miR-21 1 nM
aiRNA anti-miR-21 10 nM
aiRNA anti-miR-21 100 nM
Ambion anti-miR-21 1 nM
Ambion anti-miR-21 10 nM
Ambion anti-miR-21 100 nM

Figure 27

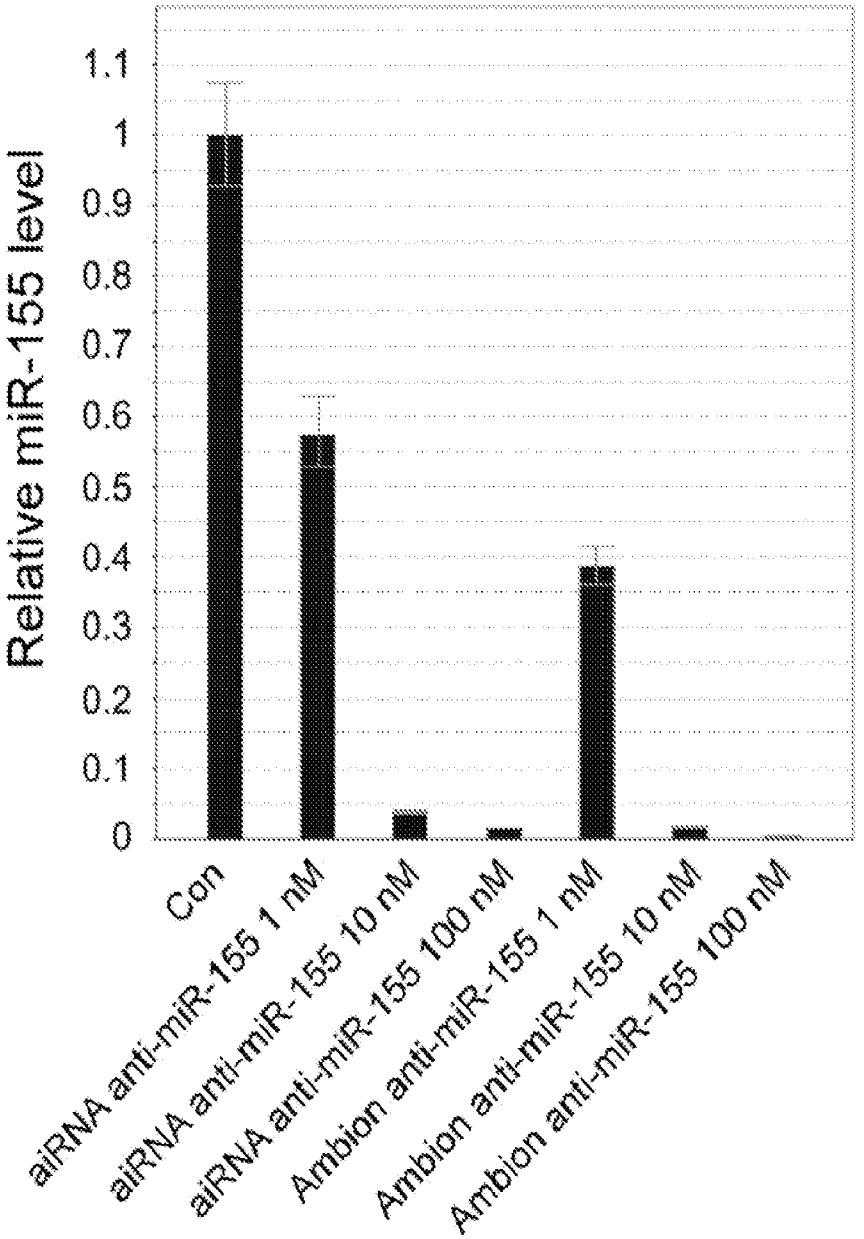

Relative miR-155 level
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Con
aiRNA anti-miR-155 1 nM
aiRNA anti-miR-155 10 nM
aiRNA anti-miR-155 100 nM
Ambion anti-miR-155 1 nM
Ambion anti-miR-155 10 nM
Ambion anti-miR-155 100 nM

Figure 28

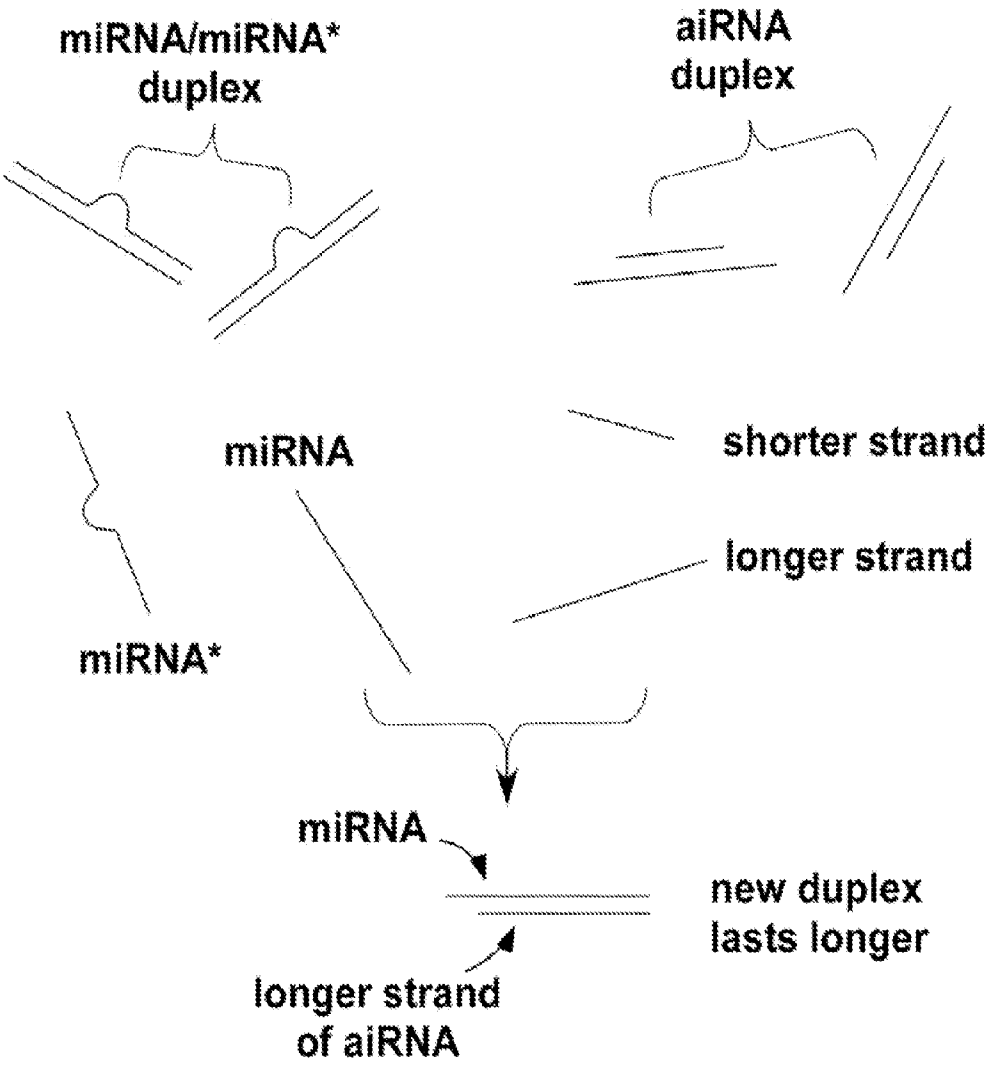

miRNA/miRNA*
duplex
aiRNA
duplex
miRNA
shorter strand
longer strand
miRNA*
miRNA
new duplex
lasts longer
longer strand
of aiRNA

Figure 29

hsa-let-7a-1	hsa-mir-29a	hsa-mir-128-2	hsa-mir-184	hsa-mir-220c	hsa-mir-365-2	hsa-mir-483
hsa-let-7a-2	hsa-mir-29b-1	hsa-mir-129-1	hsa-mir-185	hsa-mir-221	hsa-mir-367	hsa-mir-484
hsa-let-7a-3	hsa-mir-29b-2	hsa-mir-129-2	hsa-mir-186	hsa-mir-222	hsa-mir-369	hsa-mir-485
hsa-let-7b	hsa-mir-29c	hsa-mir-130a	hsa-mir-187	hsa-mir-223	hsa-mir-370	hsa-mir-486
hsa-let-7c	hsa-mir-30a	hsa-mir-130b	hsa-mir-188	hsa-mir-224	hsa-mir-371	hsa-mir-487a
hsa-let-7d	hsa-mir-30b	hsa-mir-132	hsa-mir-190	hsa-mir-296	hsa-mir-372	hsa-mir-487b
hsa-let-7e	hsa-mir-30c-1	hsa-mir-133a-1	hsa-mir-190b	hsa-mir-297	hsa-mir-373	hsa-mir-488
hsa-let-7f-1	hsa-mir-30c-2	hsa-mir-133a-2	hsa-mir-191	hsa-mir-298	hsa-mir-374a	hsa-mir-489
hsa-let-7f-2	hsa-mir-30d	hsa-mir-133b	hsa-mir-192	hsa-mir-299	hsa-mir-374b	hsa-mir-490
hsa-let-7g	hsa-mir-30e	hsa-mir-134	hsa-mir-193a	hsa-mir-300	hsa-mir-375	hsa-mir-491
hsa-let-7i	hsa-mir-31	hsa-mir-135a-1	hsa-mir-193b	hsa-mir-301a	hsa-mir-376a-1	hsa-mir-492
hsa-mir-1-1	hsa-mir-32	hsa-mir-135a-2	hsa-mir-194-1	hsa-mir-301b	hsa-mir-376a-2	hsa-mir-493
hsa-mir-1-2	hsa-mir-33a	hsa-mir-135b	hsa-mir-194-2	hsa-mir-302a	hsa-mir-376b	hsa-mir-494
hsa-mir-7-1	hsa-mir-33b	hsa-mir-136	hsa-mir-195	hsa-mir-302b	hsa-mir-376c	hsa-mir-495
hsa-mir-7-2	hsa-mir-34a	hsa-mir-137	hsa-mir-196a-1	hsa-mir-302c	hsa-mir-377	hsa-mir-496
hsa-mir-7-3	hsa-mir-34b	hsa-mir-138-1	hsa-mir-196a-2	hsa-mir-302d	hsa-mir-378	hsa-mir-497
hsa-mir-9-1	hsa-mir-34c	hsa-mir-138-2	hsa-mir-196b	hsa-mir-302e	hsa-mir-379	hsa-mir-498
hsa-mir-9-2	hsa-mir-92a-1	hsa-mir-139	hsa-mir-197	hsa-mir-302f	hsa-mir-380	hsa-mir-499
hsa-mir-9-3	hsa-mir-92a-2	hsa-mir-140	hsa-mir-198	hsa-mir-320a	hsa-mir-381	hsa-mir-500
hsa-mir-10a	hsa-mir-92b	hsa-mir-141	hsa-mir-199a-1	hsa-mir-320b-1	hsa-mir-382	hsa-mir-501
hsa-mir-10b	hsa-mir-93	hsa-mir-142	hsa-mir-199a-2	hsa-mir-320b-2	hsa-mir-383	hsa-mir-502
hsa-mir-15a	hsa-mir-95	hsa-mir-143	hsa-mir-199b	hsa-mir-320c-1	hsa-mir-384	hsa-mir-503
hsa-mir-15b	hsa-mir-96	hsa-mir-144	hsa-mir-200a	hsa-mir-320c-2	hsa-mir-409	hsa-mir-504
hsa-mir-16-1	hsa-mir-98	hsa-mir-145	hsa-mir-200b	hsa-mir-320d-1	hsa-mir-410	hsa-mir-505
hsa-mir-16-2	hsa-mir-99a	hsa-mir-146a	hsa-mir-200c	hsa-mir-320d-2	hsa-mir-411	hsa-mir-506
hsa-mir-17	hsa-mir-99b	hsa-mir-146b	hsa-mir-202	hsa-mir-323	hsa-mir-412	hsa-mir-507
hsa-mir-18a	hsa-mir-100	hsa-mir-147	hsa-mir-203	hsa-mir-324	hsa-mir-421	hsa-mir-508
hsa-mir-18b	hsa-mir-101-1	hsa-mir-147b	hsa-mir-204	hsa-mir-325	hsa-mir-422a	hsa-mir-509-1
hsa-mir-19a	hsa-mir-101-2	hsa-mir-148a	hsa-mir-205	hsa-mir-326	hsa-mir-423	hsa-mir-509-2
hsa-mir-19b-1	hsa-mir-103-1	hsa-mir-148b	hsa-mir-206	hsa-mir-328	hsa-mir-424	hsa-mir-509-3
hsa-mir-19b-2	hsa-mir-103-2	hsa-mir-149	hsa-mir-208a	hsa-mir-329-1	hsa-mir-425	hsa-mir-510
hsa-mir-20a	hsa-mir-105-1	hsa-mir-150	hsa-mir-208b	hsa-mir-329-2	hsa-mir-429	hsa-mir-511-1
hsa-mir-20b	hsa-mir-105-2	hsa-mir-151	hsa-mir-210	hsa-mir-330	hsa-mir-431	hsa-mir-511-2
hsa-mir-21	hsa-mir-106a	hsa-mir-152	hsa-mir-211	hsa-mir-331	hsa-mir-432	hsa-mir-512-1
hsa-mir-22	hsa-mir-106b	hsa-mir-153-1	hsa-mir-212	hsa-mir-335	hsa-mir-433	hsa-mir-512-2
hsa-mir-23a	hsa-mir-107	hsa-mir-153-2	hsa-mir-214	hsa-mir-337	hsa-mir-448	hsa-mir-513a-1
hsa-mir-23b	hsa-mir-122	hsa-mir-154	hsa-mir-215	hsa-mir-338	hsa-mir-449a	hsa-mir-513a-2
hsa-mir-24-1	hsa-mir-124-1	hsa-mir-155	hsa-mir-216a	hsa-mir-339	hsa-mir-449b	hsa-mir-513b
hsa-mir-24-2	hsa-mir-124-2	hsa-mir-181a-1	hsa-mir-216b	hsa-mir-340	hsa-mir-450a-1	hsa-mir-513c
hsa-mir-25	hsa-mir-124-3	hsa-mir-181a-2	hsa-mir-217	hsa-mir-342	hsa-mir-450a-2	hsa-mir-514-1
hsa-mir-26a-1	hsa-mir-125a	hsa-mir-181b-1	hsa-mir-218-1	hsa-mir-345	hsa-mir-450b	hsa-mir-514-2
hsa-mir-26a-2	hsa-mir-125b-1	hsa-mir-181b-2	hsa-mir-218-2	hsa-mir-346	hsa-mir-451	hsa-mir-514-3
hsa-mir-26b	hsa-mir-125b-2	hsa-mir-181c	hsa-mir-219-1	hsa-mir-361	hsa-mir-452	hsa-mir-515-1
hsa-mir-27a	hsa-mir-126	hsa-mir-181d	hsa-mir-219-2	hsa-mir-362	hsa-mir-453	hsa-mir-515-2
hsa-mir-27b	hsa-mir-127	hsa-mir-182	hsa-mir-220a	hsa-mir-363	hsa-mir-454	hsa-mir-516a-1
hsa-mir-28	hsa-mir-128-1	hsa-mir-183	hsa-mir-220b	hsa-mir-365-1	hsa-mir-455	hsa-mir-516a-2

Figure 30

hsa-mir-516b-1	hsa-mir-548c	hsa-mir-571	hsa-mir-619	hsa-mir-665	hsa-mir-941-4	hsa-mir-1255a	hsa-mir-1296
hsa-mir-516b-2	hsa-mir-548d-1	hsa-mir-572	hsa-mir-620	hsa-mir-668	hsa-mir-942	hsa-mir-1255b-1	hsa-mir-1297
hsa-mir-517a	hsa-mir-548d-2	hsa-mir-573	hsa-mir-621	hsa-mir-671	hsa-mir-943	hsa-mir-1255b-2	hsa-mir-1298
hsa-mir-517b	hsa-mir-548e	hsa-mir-574	hsa-mir-622	hsa-mir-675	hsa-mir-944	hsa-mir-1256	hsa-mir-1299
hsa-mir-517c	hsa-mir-548f-1	hsa-mir-575	hsa-mir-623	hsa-mir-708	hsa-mir-1178	hsa-mir-1257	hsa-mir-1300
hsa-mir-518a-1	hsa-mir-548f-2	hsa-mir-576	hsa-mir-624	hsa-mir-720	hsa-mir-1179	hsa-mir-1258	hsa-mir-1301
hsa-mir-518a-2	hsa-mir-548f-3	hsa-mir-577	hsa-mir-625	hsa-mir-744	hsa-mir-1180	hsa-mir-1259	hsa-mir-1302-1
hsa-mir-518b	hsa-mir-548f-4	hsa-mir-578	hsa-mir-626	hsa-mir-758	hsa-mir-1181	hsa-mir-1260	hsa-mir-1302-2
hsa-mir-518c	hsa-mir-548f-5	hsa-mir-579	hsa-mir-627	hsa-mir-760	hsa-mir-1182	hsa-mir-1261	hsa-mir-1302-3
hsa-mir-518d	hsa-mir-548g	hsa-mir-580	hsa-mir-628	hsa-mir-765	hsa-mir-1183	hsa-mir-1262	hsa-mir-1302-4
hsa-mir-518e	hsa-mir-548h-1	hsa-mir-581	hsa-mir-629	hsa-mir-766	hsa-mir-1184	hsa-mir-1263	hsa-mir-1302-5
hsa-mir-518f	hsa-mir-548h-2	hsa-mir-582	hsa-mir-630	hsa-mir-767	hsa-mir-1185-1	hsa-mir-1264	hsa-mir-1302-6
hsa-mir-519a-1	hsa-mir-548h-3	hsa-mir-583	hsa-mir-631	hsa-mir-768	hsa-mir-1185-2	hsa-mir-1265	hsa-mir-1302-7
hsa-mir-519a-2	hsa-mir-548h-4	hsa-mir-584	hsa-mir-632	hsa-mir-769	hsa-mir-1197	hsa-mir-1266	hsa-mir-1302-8
hsa-mir-519b	hsa-mir-548i-1	hsa-mir-585	hsa-mir-633	hsa-mir-770	hsa-mir-1200	hsa-mir-1267	hsa-mir-1303
hsa-mir-519c	hsa-mir-548i-2	hsa-mir-586	hsa-mir-634	hsa-mir-802	hsa-mir-1201	hsa-mir-1268	hsa-mir-1304
hsa-mir-519d	hsa-mir-548i-3	hsa-mir-587	hsa-mir-635	hsa-mir-873	hsa-mir-1202	hsa-mir-1269	hsa-mir-1305
hsa-mir-519e	hsa-mir-548i-4	hsa-mir-588	hsa-mir-636	hsa-mir-874	hsa-mir-1203	hsa-mir-1270	hsa-mir-1306
hsa-mir-520a	hsa-mir-548j	hsa-mir-589	hsa-mir-637	hsa-mir-875	hsa-mir-1204	hsa-mir-1271	hsa-mir-1307
hsa-mir-520b	hsa-mir-548k	hsa-mir-590	hsa-mir-638	hsa-mir-876	hsa-mir-1205	hsa-mir-1272	hsa-mir-1308
hsa-mir-520c	hsa-mir-548l	hsa-mir-591	hsa-mir-639	hsa-mir-877	hsa-mir-1206	hsa-mir-1273	hsa-mir-1321
hsa-mir-520d	hsa-mir-548m	hsa-mir-592	hsa-mir-640	hsa-mir-885	hsa-mir-1207	hsa-mir-1274a	hsa-mir-1322
hsa-mir-520e	hsa-mir-548n	hsa-mir-593	hsa-mir-641	hsa-mir-886	hsa-mir-1208	hsa-mir-1274b	hsa-mir-1323
hsa-mir-520f	hsa-mir-548o	hsa-mir-595	hsa-mir-642	hsa-mir-887	hsa-mir-1224	hsa-mir-1275	hsa-mir-1324
hsa-mir-520g	hsa-mir-548p	hsa-mir-596	hsa-mir-643	hsa-mir-888	hsa-mir-1225	hsa-mir-1276	hsa-mir-1825
hsa-mir-520h	hsa-mir-549	hsa-mir-597	hsa-mir-644	hsa-mir-889	hsa-mir-1226	hsa-mir-1277	hsa-mir-1826
hsa-mir-521-1	hsa-mir-550-1	hsa-mir-598	hsa-mir-645	hsa-mir-890	hsa-mir-1227	hsa-mir-1278	hsa-mir-1827
hsa-mir-521-2	hsa-mir-550-2	hsa-mir-599	hsa-mir-646	hsa-mir-891a	hsa-mir-1228	hsa-mir-1279	
hsa-mir-522	hsa-mir-551a	hsa-mir-600	hsa-mir-647	hsa-mir-891b	hsa-mir-1229	hsa-mir-1280	
hsa-mir-523	hsa-mir-551b	hsa-mir-601	hsa-mir-648	hsa-mir-892a	hsa-mir-1231	hsa-mir-1281	
hsa-mir-524	hsa-mir-552	hsa-mir-602	hsa-mir-649	hsa-mir-892b	hsa-mir-1233	hsa-mir-1282	
hsa-mir-525	hsa-mir-553	hsa-mir-603	hsa-mir-650	hsa-mir-920	hsa-mir-1234	hsa-mir-1283-1	
hsa-mir-526a-1	hsa-mir-554	hsa-mir-604	hsa-mir-651	hsa-mir-921	hsa-mir-1236	hsa-mir-1283-2	
hsa-mir-526a-2	hsa-mir-555	hsa-mir-605	hsa-mir-652	hsa-mir-922	hsa-mir-1237	hsa-mir-1284	
hsa-mir-526b	hsa-mir-556	hsa-mir-606	hsa-mir-653	hsa-mir-923	hsa-mir-1238	hsa-mir-1285-1	
hsa-mir-527	hsa-mir-557	hsa-mir-607	hsa-mir-654	hsa-mir-924	hsa-mir-1243	hsa-mir-1285-2	
hsa-mir-532	hsa-mir-558	hsa-mir-608	hsa-mir-655	hsa-mir-933	hsa-mir-1244	hsa-mir-1286	
hsa-mir-539	hsa-mir-559	hsa-mir-609	hsa-mir-656	hsa-mir-934	hsa-mir-1245	hsa-mir-1287	
hsa-mir-541	hsa-mir-561	hsa-mir-610	hsa-mir-657	hsa-mir-935	hsa-mir-1246	hsa-mir-1288	
hsa-mir-542	hsa-mir-562	hsa-mir-611	hsa-mir-658	hsa-mir-936	hsa-mir-1247	hsa-mir-1289-1	
hsa-mir-543	hsa-mir-563	hsa-mir-612	hsa-mir-659	hsa-mir-937	hsa-mir-1248	hsa-mir-1289-2	
hsa-mir-544	hsa-mir-564	hsa-mir-613	hsa-mir-660	hsa-mir-938	hsa-mir-1249	hsa-mir-1290	
hsa-mir-545	hsa-mir-566	hsa-mir-614	hsa-mir-661	hsa-mir-939	hsa-mir-1250	hsa-mir-1291	
hsa-mir-548a-1	hsa-mir-567	hsa-mir-615	hsa-mir-662	hsa-mir-940	hsa-mir-1251	hsa-mir-1292	
hsa-mir-548a-2	hsa-mir-568	hsa-mir-616	hsa-mir-663	hsa-mir-941-1	hsa-mir-1252	hsa-mir-1293	
hsa-mir-548a-3	hsa-mir-569	hsa-mir-617	hsa-mir-663b	hsa-mir-941-2	hsa-mir-1253	hsa-mir-1294	
hsa-mir-548b	hsa-mir-570	hsa-mir-618	hsa-mir-664	hsa-mir-941-3	hsa-mir-1254	hsa-mir-1295	

Figure 31

COMPOSITION OF ASYMMETRIC RNA DUPLEX AS MICRORNA MIMETIC OR INHIBITOR

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to and the benefit of U.S. provisional patent applications Ser. Nos. 60/968,257 filed on Aug. 27, 2007, 61/029,753 filed on Feb. 19, 2008, and 61/038,954 filed on Mar. 24, 2008, the entire contents of which applications are incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] MicroRNA (miRNA) is a class of endogenous, small RNA molecules that was first discovered to regulate gene expression at the translation level, and is part of the cell's RNA interference (RNAi) mechanism. First discovered in *Caenorhabditis elegans,* miRNAs have been found in both plants and animals including humans. The sequences of many miRNAs are homologous among different organisms, suggesting that miRNAs represent a relativley old and important regulatory pathway (Grosshans et al. *J Cell Biol* 156: 17-21 (2002)). Encoded by genes transcribed from DNA but not translated into protein (non-protein-coding RNA), miRNAs regulate as much as 30% of mamalian genes. (Czech, *NEJM* 354:1194-1195 (2006); Mack, *Nature Biotech.* 25:631-638 (2007); Eulalio, et al., *Cell* 132:9-14 (2008)). Recent researches have found that miRNA represses protein production by blocking translation or causing transcript degradation, thereby regulating gene expression. A single miRNA may target 250-500 different mRNAs, proving this class of RNA to be an extremely important mediator of a wide range of cellular functions. Many of the genes regulated by miRNAs are disease-causing genes, therefore, any mechanism that modulates miRNA functionality has great thearapeutic potentials.

[0003] In animals, miRNAs are first expressed from the genome as RNA transcripts called primary miRNAs (pri-miRNAs). They are transcribed by RNA Polymerase II, and likely form hairpin structures. In the nucleaus, the dsRNA-specific ribonuclease Drosha processes the pri-miRNAs into shorter, about 70- to 100-nucleotide long stem-loop structures known as pre-miRNAs, which are then exported out into the cytoplasm, likely by Exportin-5 (Exp5). (Yi, et al. *Genes Dev.* 17: 3011-3016 (2003)). In the cytoplasm, Dicer, a member of the RNase III ribonuclease family, cleaves the pre-miRNA into a double-stranded guide/passenger (miRNA/miRNA*) duplex with 3' overhangs at both ends. The two strands of the miRNA duplex often have mismatches from imperfect complementarity in their sequences and when they separate, a mature miRNA, in many cases, between about 19 and 23 nucelotides long, is bound by the RNA-induced silencing complex (RISC) or a similar protein complex. RISC is also the protein complex that effects target-specific mRNA degradation mediated by small or short interfereing RNAs (siRNAs).

[0004] While it is not entirely clear at this point how the miRNA duplex or the single-stranded mature miRNA interacts with RISC, it is believed that once it is selected by a catalytic component of RISC, argonaute, as the guide strand, the mature miRNA is integrated into the complex, and binds to a messenger RNA (mRNA) molecule that has a significantly, though often not perfectly, complementary sequence. The passenger strand, miRNA*, is likely degraded. Translation of the mRNA bound by the miRNA-RISC complex is then repressed, resulting in reduced expression of the corresponding gene. In some cases, the bound mRNA is cleaved or deadenylated and degraded.

[0005] References cited herein are not admitted to be prior art to the claimed invention.

SUMMARY OF THE INVENTION

[0006] The present invention is about the discovery that a novel class of duplex RNAs can effectively modulate miRNA activities in mammalian cells, which is termed here "asymmetrical interfering RNAs" (aiRNAs). The hallmark of this novel class of RNAs is the length asymmetry between the two RNA strands. The present invention provides evidence that double-stranded RNAs asymmetric in strand-length can be constructed to either mimic or inhibit miRNAs in cells, and modulate miRNA pathway activities in both directions, i.e., up and down.

[0007] In one aspect, the present invention provides a mimetic of a microRNA (miRNA), comprising: a double stranded RNA molecule comprising a first strand of a first length and a second strand of a second and shorter length, said first strand having a sequence substantially the same as at least a portion of said miRNA, said first and second strands being substantially complementary to each other such that they form at least one double stranded region, wherein said RNA molecule further comprises a terminal overhang of 1-10 nucleotides; and wherein said mimetic is adapted to mimic said miRNA in modulating expression of at least one gene.

[0008] In a preferred aspect, the present invention provides a mimetic of a mature microRNA (miRNA), and the mimetic comprises: a double stranded RNA molecule comprising a first strand of 15-28 nucleotides and a second shorter strand of 12-26 nucleotides, said first strand having a sequence substantially the same as at least a portion of said mature miRNA, said first and second strands being substantially complementary to each other such that they form at least one double stranded region, wherein said first strand further comprises both a 3' overhang of 1-8 nucleotides and a 5' overhang of 1-8 nucleotides, wherein said mimetic is adapted to mimic said mature miRNA in modulating expression of at least one gene.

[0009] In one feature, the miRNA being mimicked by the mimetics of the invention is a guide strand or a mature miRNA. In an embodiment, the miRNA is an endogenous miRNA duplex comprising a mature miRNA and a substantially complementary passenger strand; said second strand of said mimetic has a sequence substantially the same as at least a portion of said passenger strand.

[0010] In a further aspect, the present invention provides an inhibitor of a microRNA (miRNA), comprising: a double stranded RNA molecule comprising a first strand of a first length and a second strand of a second and shorter length, said first strand having a sequence substantially complementary to at least a portion of a target miRNA, said first and second strands being substantially complementary to each other such that they form at least one double stranded region, wherein said RNA molecule further comprises a terminal overhang of 1-10 nucleotides, wherein said inhibitor is adapted to inhibit said target miRNA.

[0011] And in a preferred aspect, the present invention provides an inhibitor of a mature microRNA (miRNA), comprising: a double stranded RNA molecule comprising a first strand of 15-28 nucleotides and a second, shorter strand of

12-26 nucleotides, said first strand having a sequence substantially complementary to at least a portion of said mature miRNA, said first and second strands being substantially complementary to each other such that they form at least one double stranded region, wherein said first strand further comprises both a 3' overhang of 1-8 nucleotides and a 5' terminal overhang of 1-8 nucleotides, wherein said inhibitor is adapted to inhibit said target miRNA.

[0012] In one feature, the miRNA targeted by the inhibitors of the invention is a guide strand, or a mature strand. In some embodiments, the inhibitors of the invention is capable of decreasing the amount of mature miRNA by about at least 30%, 50%, 70%, 80% or 90%.

[0013] In one feature, the mimetics and inhibitors of the invention further includes at least one mismatched or unmatched nucleotide in sequence between said first and second strands. In one embodiment, the at least one mismatched or unmatched nucleotide forms a loop. In an alternative embodiment, the first and second strands in the mimetics and inhibitors are perfectly complementary to each other in said double stranded region.

[0014] The terminal overhang of the mimetics and inhibitors of the invention, in some embodiments, is of 1-8 nucleotides, and 1-3 nucleotides, in some other embodiments. The terminal overhang can be a 3' overhang, and in a preferred embodiment, on the first strand, which is the longer strand. In another embodiment, the terminal overhang is a 5' overhang, and in a preferred embodiment, on the first strand. In some embodiments, the mimetics and inhibitors have both a 3' overhang and a 5' overhang on said first strand, and preferably, both said 3' and 5' overhangs are of 1-3 nucleotides. In another embodiment, the mimetics and inhibitors of the invention has one terminal overhang on one end and a blunt end on the other end.

[0015] In various embodiments of the mimetics: said first strand (the longer strand) has a length of 13-100 nucleotides, and said second strand has a length of 5-30 nucleotides; said first strand has a length of 15-30 nucleotides, and said second strand has a length of 12-29 nucleotides; said first strand has a length of 15-28 nucleotides and said second strand has a length from 12-26 nucleotides; said first strand has a length of 19-25 nucleotides and said second strand has a length of 12-24 nucleotides; said first strand has a length of 19-23 nucleotides and said second strand has a length of 14-20 nucleotides.

[0016] In various embodiments of the inhibitors: said first strand (the longer strand) has a length of 10-100 nucleotides, and said second strand has a length of 5-30 nucleotides; said first strand has a length of 15-60 nucleotides, and said second strand has a length of 5-28 nucleotides; said first strand has a length of 15-28 nucleotides and said second strand has a length from 12-26 nucleotides; said first strand has a length of 19-25 nucleotides and said second strand has a length of 12-20 nucleotides.

[0017] In one feature, in the mimetics and inhibitors of the invention, said first strand is longer than said second strand by a length selected from the group consisting of 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 nucleotides.

[0018] In one feature, in the mimetics and inhibitors of the invention, said terminal overhang is stabilized against degradation.

[0019] In one feature, the mimetics and inhibitors further have at least one nick in at least one of said first and second strands. In another feature, the double-stranded region of the mimetics or inhibitors further comprises a gap of one or more unpaired nucleotides.

[0020] In one feature, the mimetics and inhibitors include a modified nucleotide or a nucleotide analogue. In another feature, they include at least one deoxynucleotide, which can be in one or more regions selected from the group consisting of 3'-overhang, 5'-overhang, and double-stranded region.

[0021] In one feature, in the mimetics of the invention, said first strand has a sequence that is at least 60 percent, or in some embodiments, at least 70 percent, the same as at least said portion of said miRNA. In one embodiment, said first strand of the mimetics shares the same seed region as said miRNA.

[0022] In one feature, in the inhibitors of the invention, said first strand has a sequence that is at least 60 percent, or in some embodiments, at least 70 percent, complementary to at least said portion of its target miRNA.

[0023] In an embodiment, the GC content of the double stranded region in the mimetics and inhibitors of the invention is about 20-60%, or preferably, about 30-50%.

[0024] In an embodiment of the mimetics and inhibitors, said first strand comprises a sequence motif with at least one nucleotide selected from the group consisting of A, U, and dT. In one further embodiment, the 5' overhang has a sequence motif "AA," "UU," or "dTd." In one feature, a mimetic and inhibitor of the invention is further conjugated to an entity selected from the group consisting of peptide, antibody, polymer, lipid, oligonucleotide, cholesterol and aptamer.

[0025] In one feature of the mimetics and inhibitors of the invention, the double stranded RNA molecule is synthetic or isolated. In an embodiment, the double stranded RNA molecule of the invention, whether a mimetic or inhibitor, is transcribed from a recombinant vector or its progeny.

[0026] In one feature, the mimetics of the invention are adapted to modulate at least 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% the expression of said at least one gene. In one embodiment, the mimetics of the invention mimic an miRNA of the Let? family.

[0027] In an embodiment, the mimetic of the invention comprises one of the following duplex sequence:

```
Sense: 5'-AUACAAUCUACUGUC
Antisense: 5'-UGAGGUAGUAGGUUGUAUAGU,
and

Sense: 5'-ACAACCUACUACCUC
Antisense: 5'-AAUGAGGUAGUAGGUUGUAUG.
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[0028] In another embodiment, the inhibitor of the invention comprises one of the following duplex sequence:

```
Sense: 5'-GGUAGUAGGUUGUAU
Antisense: 5'-AACAUACAACCUACUACCUCA,

Sense: 5'-AUCAGACUGAUGUUG
Antisense: 5'-AAUCAACAUCAGUCUGAUAAG,
and

Sense: 5'-AUGCUAAUCGUGAUA
Antisense: 5'-AACUAUCACGAUUAGCAUUAA.
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[0029] In one aspect, the present invention provides an expression vector comprising a DNA sequence encoding at least the first strand of the double stranded RNA molecule of

the mimetics and inhibitors of the invention, said sequence operably linked to an expression control sequence, e.g., a promoter.

[0030] In an embodiment, the expression vector further comprises a second DNA sequence encoding at least the second strand of the double stranded RNA molecule of the mimetics and inhibitors of the invention, said sequence operably linked to a second promoter. The vector may be selected from a group consisting of viral, eukaryotic and bacterial expression vectors.

[0031] In one aspect, the present invention provides a cell comprising the above expression vector. In another aspect, the present invention provides a cell that comprises the double-strand RNA molecule of the invention.

[0032] In a further aspect, the present invention provides a method of making a mimetic of a microRNA (miRNA), said method comprising the steps of: selecting a miRNA sequence; synthesizing a first RNA strand having a region substantially the same as at least a portion of contiguous nucleotides in said miRNA; synthesizing a second and shorter RNA strand, and combining the synthesized strands under suitable conditions to form a double stranded RNA molecule with at least one terminal overhang such that said RNA molecule is capable of mimicking said miRNA in modulating expression of at least one gene.

[0033] In another aspect, the present invention provides a method of making an inhibitor of a target microRNA (miRNA), said method comprising the steps of: selecting a target miRNA sequence; synthesizing a first RNA strand having a region substantially complementary to at least a portion of contiguous nucleotides in said target mRNA; synthesizing a second and shorter RNA strand, and combining the synthesized strands under suitable conditions to form a double stranded RNA molecule with at least one terminal overhang such that said RNA molecule is capable of inhibiting said target miRNA.

[0034] In one feature, the methods of making a mimetic or inhibitor according to the invention further includes one or more of the following steps: chemically modifying said at least one terminal overhang against degradation; introducing at least one deoxynucleotide into said double stranded RNA molecule; introducing at least one modified nucleotide or a nucleotide analogue into said double stranded RNA molecule; introducing at least one mismatch, nick or gap in said double stranded region; conjugating at least one of said first and second strands with an entity selected from the group consisting of peptide, antibody, polymer, lipid, oligonucleotide, cholesterol, and aptamer; modifying at least one base in one of the strands. In an embodiment, the methods of making a mimetic or inhibitor further includes a step of introducing at least one modified nucleotide analogue into the duplex RNA molecule during the synthesizing step, after the synthesizing and before the combining step, or after the combining step.

[0035] In one feature, in the methods of making a mimetic or inhibitor, at least one of the RNA strands is enzymatically or biologically synthesized. In some embodiments, the first strand and the second strand are synthesized separately or simultaneously.

[0036] In another aspect, the present invention provides a method of modulating an miRNA pathway in a cell or organism, said method comprising the steps of contacting said cell or organism with a mimetic of the invention, under conditions where said mimicking of said miRNA can occur; and modulating the expression of at least one gene using said mimetic, thereby modulating an endogenous miRNA pathway. In an embodiment, the first strand in the double stranded RNA molecule of the mimetic mimics said endogenous miRNA in its interaction with RISC.

[0037] In another aspect, the present invention provides a method of modulating an miRNA pathway in a cell, said method comprising the steps of: contacting said cell or organism with an inhibitor of the invention, under conditions where said inhibition of said target miRNA can occur; and reducing the amount of target miRNA available with said inhibitor, thereby modulating an endogenous miRNA pathway.

[0038] In one feature, the modulation methods of the invention, whether using a mimetic or an inhibitor, further include the step of introducing said duplex RNA molecule into a target cell in culture or in an organism in which the modulation of gene expression can occur.

[0039] In an embodiment, the introducing step is selected from the group consisting of transfection, lipofection, electroporation, infection, injection, oral administration, inhalation, topic and regional administration. Further, the introducing step may use a pharmaceutically acceptable excipient, carrier, or diluent selected from the group consisting of a pharmaceutical carrier, a positive-charge carrier, a liposome, a protein carrier, a polymer, a nanoparticle, a nanoemulsion, a lipid, and a lipoid,

[0040] In another aspect, the present invention provides use of the modulation methods of the invention for various purposes including: determining the function or utility of a gene in a cell or an organism, for modulating the expression of at least one gene in a cell or an organism. In an embodiment, the gene is associated with a disease, a pathological condition, or an undesirable condition. In anther embodiment, the gene is associated with a human or animal diseases. The gene may be a gene of a pathogenic microorganism, a viral gene, a tumor-associated gene, and so on. In an embodiment, the gene is associated with a disease selected from the group consisting of autoimmune disease, inflammatory diseases, degenerative diseases, infectious diseases, proliferative diseases, metabolic diseases, immune-mediated disorders, allergic diseases, dermatological diseases, malignant diseases, gastrointestinal disorders, respiratory disorders, cardiovascular disorders, renal disorders, rheumatoid disorders, neurological disorders, endocrine disorders, and aging. In one feature, the methods of the invention are used for studying drug target in vitro or in vivo. In a further feature, the methods of the invention are used for treating or preventing a disease or an undesirable condition.

[0041] In another aspect, the present invention provides a pharmaceutical composition comprising as an active agent at least one mimetic or inhibitor of the invention, and a pharmaceutically acceptable excipient, carrier, or diluent. The carrier may be selected from the group consisting of a pharmaceutical carrier, a positive-charge carrier, a liposome, a protein carrier, a polymer, a nanoparticle, a nanoemulsion, a lipid, and a lipoid.

[0042] In another aspect, the present invention provides a treatment method comprising administering an effective amount of the pharmaceutical composition of the invention to a subject in need. In various embodiments, the pharmaceutical composition is administered via a route selected from the group consisting of intravascular (iv), subcutaneous (se), topic, po, inhalation, intramuscular, intra-peritoneal (ip) and regional routes. In various embodiments, the effective amount of the pharmaceutical composition is about 1 ng to 1

g per day, 100 ng to 1 g per day, or 1 μg to 500 mg per day. In an embodiment, the method is used in treating cancer.

[0043] In a further aspect, the present invention provides a research reagent comprising the mimetic of the invention, or the inhibitor of the invention. The present invention also provides a kit comprising said research reagent.

[0044] In yet another aspect, the present invention provides a method of diagnosing a patient of a disease or condition, comprising contacting cells of the patient with the mimetic or inhibitor of the invention; and looking for at least one change indicating said disease or condition.

[0045] Other features and advantages of the present invention are apparent from the additional descriptions provided herein including the different examples. The provided examples illustrate different components and methodology useful in practicing the present invention. The examples do not limit the claimed invention. Based on the present disclosure the skilled artisan can identify and employ other components and methodology useful for practicing the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0046] FIG. **1**A shows the structure of a duplex RNA molecule that has both a 3'-overhang and a 5'-overhang.

[0047] FIG. **1**B shows the duplex RNA molecule of FIG. **1**A with a nick in one of the strands.

[0048] FIG. **1**C shows the duplex RNA molecule of FIG. **1**A with a gap in one of the strands.

[0049] FIG. **2**A shows the structure of a duplex RNA molecule that has a blunt end, and a 5'-overhang.

[0050] FIG. **2**B shows the structure of a duplex RNA molecule that has a blunt end, and a 3'-overhang.

[0051] FIG. **2**C shows the structure of a duplex RNA molecule that has a 3'-overhang on both ends.

[0052] FIG. **2**D shows the structure of a duplex RNA molecule that has a 5'-overhang on both ends.

[0053] FIG. **2**E shows an alternative structure of a duplex RNA molecule that has both a 3% overhang and a 5'-overhang.

[0054] FIG. **2**F shows an alternative structure of a duplex RNA molecule that has a 3'-overhang on both ends.

[0055] FIG. **3** shows the induction of gene silencing of β-catenin by aiRNA (asymmetric interfering RNAs). FIG. **3**A shows the confirmation of the oligos. After annealing, the oligos were confirmed by 20% polyacrylamide gel. Lane 1, 21 nt/21 nt; lane 2, 12 nt (a)/21 nt; lane 3, 12 nt (b)/21 nt; lane 4, 13 nt/13 nt; lane 5, 13 nt/21 nt; lane 6, 14 nt/14 nt; lane 7, 14 nt(a)/21nt; lane 8, 14 nt(b)/21 nt; lane 9, 15 nt/15 nt; lane 10, 15 nt/21 nt.

[0056] FIG. **3**B shows the effects of the oligos in gene silencing. HeLa cells were plated at 200,000 cells/well into a 6 well culture plate. 24 hours later they were transfected with scramble siRNA (lane 1), 21-bp siRNA targeted E2F1 (lane 2, as a control for specificity) or 21-bp siRNA targeted beta-catenin (lane 3, as a positive control), or the same concentration of aiRNA of different length mix: 12 nt(a)/21 nt (lane 4); 12 nt (b)/21 nt (lane 5); 13 nt/21 nt (lane 6); 14nt (a)/21 nt (lane 7); 14 nt (b)/21 nt (lane 8); 15 nt/21 nt (lane 9). Cells were harvested 48 hours after transfection. Expression of β-catenin was determined by Western blot. E2F1 and actin were used as controls.

[0057] FIGS. **4** and **5** show the structure-activity relationship of aiRNA oligos, with or without base substitutions, in mediating gene silencing. Hela cells were transfected with the indicated aiRNA. Cells were harvested and lysates generated at 48 hours post transfection. Western blots were performed to detect levels of β-catenin and actin. si stands for β-catenin siRNA oligonucleotide. The numerical labeling above each lane corresponds to the aiRNA oligos in Table 3.

[0058] FIG. **6** shows the analysis of the mechanism of gene silencing triggered by aiRNA.

[0059] FIG. **6***a* shows the northern blot analysis of β-catenin mRNA levels in cells transfected with aiRNA or siRNA for the indicated number of days.

[0060] FIG. **6***b* shows the schematic of 5'-RACE-PCR for β-catenin showing cleavage of mRNA and expected PCR product.

[0061] FIG. **6***c* shows β-catenin cleavage products mediated by aiRNA were amplified by 5'-RACE-PCR from cells transfected with aiRNA for 4 or 8 hours.

[0062] FIG. **6***d* shows the schematic of β-catenin mRNA cleavage site confirmed by sequencing the 5'-RACE-PCR fragment.

[0063] FIG. **6***e* shows differential RISC loading efficiency of aiRNA and siRNA. aiRNA or siRNA duplexes were transfected into Hela cells 48 hours after transfection with pCMV-Ago2. Ago2 was immunoprecipitated at the indicated time points following aiRNA or siRNA transfection, and northern blot analysis was performed to determine levels of Ago2/RISC associated small RNAs. Levels of Ago2 (shown below) were determined by western blot following IP.

[0064] FIG. **6***f* shows the effects of knocking down Ago2 or Dicer on gene siliencing activity of aiRNA and siRNA. Cells were transfected with scramble siRNA (siCon), or siRNA targeting Ago2 (siAgo2), or Dicer (siDicer) 24 hours prior to transfection with scramble aiRNA (Con) or aiRNA targeting Stat3 (ai). Cells were harvested and western blot analysis was performed at 48 hours following aiStat3 transfection.

[0065] FIG. **7** shows the advantages of incorporation of aiRNA into RISC compared to siRNA.

[0066] FIG. **7**A shows that aiRNA enters RISC with better efficiency than siRNA. Cells transfected with Ago2 expression plasmid were transfected with aiRNA or siRNA for the indicated times. Following cell lysis, Ago2 was immunoprecipitaed, RNA extracted from the immunoprecipitate, and separated on a 15% acrylamide gel. Following transfer, the membrane was hybridized to a probe to detect the 21 mer antisense strand of the aiRNA or siRNA. IgG control lane shows lack of signal compared to Ago2 immunoprecipitate.

[0067] FIG. **7**B shows that the sense strand of aiRNA does not stay in RISC. Membrane from (A) was stripped and re-probed to detect the sense strand of the transfected oligo. Cartoons in (A) and (B) illustrate the position of the sense strand (upper strand), the antisense strand (lower strand), or the duplex on the membrane.

[0068] FIG. **8** shows that the mechanism of RISC loading by aiRNA.

[0069] FIG. **8**A shows the immunoprecipitation analysis of the interaction between different strands of aiRNA or siRNA and Ago2. Hela S-10 lysate containing overexpressed Ago2 was incubated with the indicated aiRNA or siRNA duplex containing ^{32}P end labeled sense or antisense strands. The (*) marks the location of the label. Following Ago2 immunoprecipitation, the RNA was isolated and separated on a 15% acrylamide gel and exposed to film. The Ago2-associated RNAs are shown in the pellet fraction, while the non-Ago2 bound RNAs remain in the supernatant (Sup).

[0070] FIG. **8**B shows the role of sense strand cleavage in aiRNA activity. Cells were transfected with aiRNA or aiRNA with sense strand 2'-O-methyl at position 8 (predicted Ago2 cleavage site) or position 9 as a control. RNA was collected at 4 hours post transfection and qRT-PCR performed to determine relative levels of β-catenin mRNA remaining.

[0071] FIG. **9** shows the aiRNA and siRNA competition analysis.

[0072] FIG. **9**A illustrates the siRNA and aiRNA duplex containing ^{32}P end labeled antisense strands. The (*) marks the location of the label.

[0073] FIG. **9**B shows that the cold aiRNA does not compete with labeled siRNA for Ago2. Hela S-10 lysate containing overexpressed Ago2 was incubated with the ^{32}P end labeled siRNA and cold aiRNA or siRNA duplex prior to Ago2 immunoprecipitation. RNA was then isolated and analyzed on 15% acrylamide gel.

[0074] FIG. **9**C shows that the cold siRNA does not compete with labeled aiRNA for Ago2. The same S-10 lysate used in B was incubated with the ^{32}P end labeled aiRNA and cold aiRNA or siRNA duplex prior to Ago2 immunoprecipitation. RNA was then isolated and analyzed on 15% acrylamide gel.

[0075] FIG. **10** illustrates the model of aiRNA and siRNA showing observed differences in RISC loading and generation of mature RISC.

[0076] FIG. **11** shows asymmetric RNA duplexes of 14-15 by with antisense overhangs (aiRNA) induced potent, efficacious, rapid, and durable gene silencing.

[0077] FIG. **11**A shows the Diagram showing sequence and design of siRNA and aiRNA targeting β-catenin.

[0078] FIG. **11**B shows the induction of gene silencing by aiRNA of various lengths. β-catenin protein levels were analysed by western blot in cells transfected with indicated aiRNA for 48 hours.

[0079] FIG. **11**C shows that aiRNA is more potent and efficacious than siRNA in inducing β-catenin protein depletion. Hela cells were transfected with aiRNA or siRNA targeting β-catenin at the indicated concentrations. At 48 hours post-transfection, cell lysates were made and western blot analysis was done.

[0080] FIG. **11**D shows that the aiRNA is more efficacious, rapid, and durable than siRNA in reducing β-catenin RNA levels. Cells were transfected with 10 nM 15 by aiRNA or 21-mer siRNA for the indicated number of days before northern blot analysis.

[0081] FIG. **12** shows that aiRNA mediates rapid and potent silencing.

[0082] FIG. **12**A shows the sequence and structure of aiRNA and siRNA used to target β-catenin.

[0083] FIG. **12**B shows RT-PCR of β-catenin mRNA levels from cells transfected with control aiRNA or aiRNA targeting β-catenin. RNA was collected at the indicated times post transfection.

[0084] FIG. **12**C shows the quantitative real-time RT-PCR of β-catenin mRNA levels in cells transfected with control, aiRNA, or siRNA for the indicated number of hours.

[0085] FIG. **12**D shows the western blot analysis of β-catenin protein levels in cells transfected with control, aiRNA, or siRNA for the indicated times.

[0086] FIG. **13** shows the comparison of aiRNA with siRNA in gene silencing efficacy and durability against multiple targets. Hela cells were transfected with scramble siRNA (c), aiRNA (ai), or siRNA (si) targeting (a) β-catenin at 10 nM, (b) Stat3, (c) EF2, or (d) NQO1 at 20 nM. RNA and protein was purified at the indicated time points and analyzed for mRNA levels by quantitative real time polymerase chain reaction (qRT-PCR) and protein levels by western blot. qRT-PCR data is normalized to siCon transfected cells.

[0087] FIG. **14** shows aiRNA mediated gene silencing is effective against various genes in multiple cell lines.

[0088] FIG. **14***a* shows aiRNA duplex is more efficacious than siRNA in targeting β-catenin in different mammalian cell lines.

[0089] FIG. **14***b* shows the western blot analysis of Nbs1, Survivin, Parp1, p21 from cells transfected with 20 nM of the indicated aiRNA or siRNA for 48 hours.

[0090] FIG. **14***c* shows the western blot analysis of Rsk1, PCNA, p70S6K, mTOR, and PTEN from cells transfected with 20 nM of the indicated aiRNA or siRNA for 48 hours.

[0091] FIG. **14***d* shows the allele-specific gene silencing of k-Ras by aiRNA. aiRNA targeting wildtype k-Ras was tested for silencing of k-Ras in both k-Ras wildtype (DLD1) and k-Ras mutant (SW480) cell lines by western blot analysis.

[0092] FIG. **15** show the lack of off-target gene silencing by sense-strand, immuno stimulation, and serum stability of aiRNAs.

[0093] FIG. **15***a* shows RT-PCR analysis of the expression of interferon inducible genes in PBMC mock treated or incubated with β-catenin siRNA or aiRNA duplex for 16 hours.

[0094] FIG. **15***b* shows RT-PCR analysis of the expression of interferon inducible genes in Hela cells mock transfected or transfected with EF2 or Survivin aiRNA or siRNA for 24 hours.

[0095] FIG. **15***c* shows the microarray analysis for changes in the expression of known interferon response related genes. Total RNA isolated from aiRNA and siRNA transfected Hela cells was analyzed by microarray.

[0096] FIG. **15***d* shows that no sense-strand mediated off-target gene silencing is detected for aiRNA. Cells were co-transfected with aiRNA or siRNA and either a plasmid expressing Stat3 (sense RNA) or a plasmid expressing antisense Stat3 (antisense RNA). Cells were harvested and RNA collected at 24 hours post transfection and relative levels of Stat3 sense or antisense RNA were determined by quantitative real time PCR or RT-PCR (inserts).

[0097] FIG. **15***e* shows the Stability of aiRNA and siRNA duplexes in human serum. aiRNA and siRNA duplexes were incubated in 10% human serum at 37° C. for the indicated amount of time prior to gel electrophoresis. Duplex remaining (% of control) is indicated.

[0098] FIG. **15***f* illustrates the proposed model for gene specific silencing mediated by the aiRNA duplex.

[0099] FIG. **16** shows the potent Anti-Tumor Activity of aiRNA against β-catenin in SW480 human colon xenografted mouse model. Immunosurpressed mice with established subcutaneous SW480 human colon cancer were given intravenously (iv) with 0.6 nmol PEI-complexed β-catenin siRNAs, PEI-complexed β-catenin aiRNAs or a PEI-complexed unrelated siRNA as negative control daily. Tumor size was evaluated periodically during treatment. Each point represents the mean±SEM of six tumors.

[0100] FIG. **17** shows the potent Anti-Tumor Activity of aiRNA against β-catenin in HT29 human colon xenografted mouse model. Immunosurpressed mice with established subcutaneous HT29 human colon cancer were given intravenously (iv) with 0.6 nmol PEI-complexed β-catenin siRNAs, PEI-complexed β-catenin aiRNAs or a PEI-complexed unrelated siRNA as negative control every other day. Tumor size

was evaluated periodically during treatment. Each point represents the mean±SEM of five tumors.

[0101] FIG. **18** shows that Let-7a mimetic aiRNA can function as Let-7a with equal or better efficiency.

[0102] FIG. **18**A shows the sequence of the Let-7a and Let-7a mimetic aiRNA.

[0103] FIG. **18**B shows that Let-7a mimetic aiRNA can down regulate the mRNA level of Let-7a target k-Ras.

[0104] FIG. **19**A shows the sequences and structure of the indicated aiRNA mimetic and inhibitors.

[0105] FIG. **19**B shows the sequences of the indicated mature miRNAs.

[0106] FIG. **20**A shows the effect of Let-7c mimetic aiRNA and aiRNA Let-7c inhibitor on mRNA level of k-Ras.

[0107] FIG. **20**B shows the effect of Let-7c mimetic aiRNA and aiRNA Let-7c inhibitor on protein level of k-Ras.

[0108] FIG. **21** shows that designed aiRNAs can inhibit miRNAs.

[0109] FIG. **21**A shows that anti-Let7c aiRNA potently inhibit the expression of Let-7c.

[0110] FIG. **21**B shows that anti-miR21 aiRNA potently inhibit the expression of miR21.

[0111] FIG. **21**C shows that anti-miR155 aiRNA potently inhibit the expression of miR155.

[0112] FIG. **22**A shows that MCF7 cells express high level of miR-21.

[0113] FIG. **22**B shows that FaDu cells express high level of miR-155.

[0114] FIG. **23** shows that the anti-Let7c aiRNA is more efficacious than the commercially available miRNA inhibitor.

[0115] FIG. **24** shows that the anti-miR21 aiRNA is more efficacious than the commercially available miRNA inhibitor.

[0116] FIG. **25** shows that the anti-miR155 aiRNA is more efficacious than the commercially available miRNA inhibitor.

[0117] FIG. **26** compares the potency of the anti-Let7c aiRNA and the commercially available miRNA inhibitor.

[0118] FIG. **27** compares the potency of the anti-miR21 aiRNA and the commercially available miRNA inhibitor.

[0119] FIG. **28** compares the potency of the anti-miR155 aiRNA and the commercially available miRNA inhibitor.

[0120] FIG. **29** theorizes one possible mechanism for inhibition of miRNA by the aiRNA.

[0121] FIGS. **30** and **31** list the known human miRNAs.

DETAILED DESCRIPTION OF THE INVENTION

[0122] As used in the specification and claims, the singular form "a", "an", and "the" include plural references unless the context clearly dictate otherwise. For example, the term "a cell" includes a plurality of cells including mixtures thereof

[0123] As used herein, a "double stranded RNA," a "duplex RNA," or a "RNA duplex" refers to an RNA of two strands and with at least one double-stranded region, and includes RNA molecules that have at least one gap, nick, bulge, loop, and/or bubble either within a double-stranded region or between two neighboring double-stranded regions. If one strand has a gap or a single-stranded region of unmatched nucleotides between two double-stranded regions, that strand is considered as having multiple fragments. A double-stranded RNA as used here can have terminal overhangs on either end or both ends. In some embodiments, the two strands of the duplex RNA can be linked through certain chemical linker.

[0124] As used herein, an "antisense strand" refers to an RNA strand that has substantial sequence complementarity against a target messenger RNA. An antisense strand can be part of an siRNA molecule, part of a miRNA/miRNA* duplex, or a single-strand mature miRNA.

[0125] The term "isolated" or "purified" as used herein refer to a material that is substantially or essentially free from components that normally accompany it in its native state. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography.

[0126] As used herein, "modulating" and its grammatical equivalents refer to either increasing or decreasing (e.g., silencing), in other words, either up-regulating or down-regulating.

[0127] As used herein, the term "subject" refers to any animal (e.g., a mammal), including, but not limited to humans, non-human primates, rodents, and the like, which is to be the recipient of a particular treatment. Typically, the terms "subject" and "patient" are used interchangeably herein in reference to a human subject.

[0128] Terms such as "treating," "treatment," "to treat," "alleviating" and "to alleviate" as used herein refer to both (1) therapeutic measures that cure, slow down, lessen symptoms of and/or halt progression of a diagnosed pathologic condition or disorder and (2) prophylactic or preventative measures that prevent or slow the development of a targeted pathologic condition or disorder. Thus those in need of treatment include those already with the disorder; those prone to have the disorder; and those in whom the disorder is to be prevented. A subject is successfully "treated" according to the methods of the present invention if the patient shows one or more of the following: a reduction in the number of or complete absence of cancer cells; a reduction in the tumor size; inhibition of or an absence of cancer cell infiltration into peripheral organs including the spread of cancer into soft tissue and bone; inhibition of or an absence of tumor metastasis; inhibition or an absence of tumor growth; relief of one or more symptoms associated with the specific cancer; reduced morbidity and mortality; and improvement in quality of life.

[0129] As used herein, the terms "inhibiting", "to inhibit" and their grammatical equivalents, when used in the context of a bioactivity, refer to a down-regulation of the bioactivity, which may reduce or eliminate the targeted function, such as the production of a protein or the phosphorylation of a molecule. In particular embodiments, inhibition may refers to a reduction of about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% of the targeted activity. When used in the context of a disorder or disease, the terms refer to success at preventing the onset of symptoms, alleviating symptoms, or eliminating the disease, condition or disorder.

[0130] As used herein, the term "substantially complementary" refers to complementarity in a base-paired, double-stranded region between two nucleic acids and not any single-stranded region such as a terminal overhang or a gap region between two double-stranded regions. The complementarity does not need to be perfect; there may be any number of base pair mismatches, for example, between the two nucleic acids. However, if the number of mismatches is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a substantially complementary sequence. When two sequences are referred to as "substantially complementary" herein, it is meant that the sequences are sufficiently complementary to each other to hybridize under the selected reaction conditions. The relationship of nucleic acid complementarity and stringency of

hybridization sufficient to achieve specificity is well known in the art. Two substantially complementary strands can be, for example, perfectly complementary or can contain from 1 to many mismatches so long as the hybridization conditions are sufficient to allow, for example discrimination between a pairing sequence and a non-pairing sequence. Accordingly, substantially complementary sequences can refer to sequences with base-pair complementarity of 100, 95, 90, 80, 75, 70, 60, 50 percent or less, or any number in between, in a double-stranded region.

[0131] As used herein, antagomirs are miRNA inhibitors, and can be used in the silencing of endogenous miRNAs.

[0132] As used herein, mimetics or mimics are miRNA agonists, and can be used to replace endogenous miRNAs as functional equivalents and thereby up-regulating pathways affected by such endogenous miRNAs.

[0133] As a natural form of post-transcriptional gene silencing and each with a wide range of targets, miRNAs present great opportunities in disease treatment and prevention. As one can imagine, depending on the regulatory targets of a particular miRNA, it might be desirable to either up-regulate or down-regulate that miRNA's level in cells. For example, if its mRNA targets correspond to one or more tumor suppressor genes, one might want to down-regulate the miRNA, e.g., miR-21. On the other hand, one might want to up-regulate an miRNA if its targets include the mRNA of an oncogene, e.g., let-7 miRNAs which target the mRNA of the RAS family of oncogenes. In situations where multiple miRNAs modulate a single gene target or a network of related gene targets, it may be desirable to up-regulate certain miRNA while simultaneously down-regulate others.

[0134] Currently, effective tools devised to regulate RNAs include single-strand RNAs (e.g., antisense) and double-stranded RNAs where the two strands are very much the same length (e.g., siRNA consisting of 21 nucleotide dsRNA with symmetric 2-nt 3' overhangs). Among these, siRNA evokes RNAi in eukaryotes via an exogenous mechanism (typically through viral or artificial introduction) separate from miRNA, although in the end it utilizes the same RISC as miRNA does to effect more specific gene silencing. Single-strand antisense oligonucleotides are not stable in cell, and although various chemical modifications have been attempted on them, they remain largely ineffective as miRNA inhibitors (Vermeulen A. et al. *RNA* 13: 726-730(2007)). In sum, while miRNA has been discovered for over a decade, little progress has been made in devising effective modulators, whether mimetics or inhibitors, of miRNA (Mack G. *Nat Biotech* 25(6): 631-638 (2007)).

[0135] The present invention provides a novel structural scaffold called asymmetric interfering RNA (aiRNA) that can be used to effect siRNA-like results (described in detail in co-owned PCT and U.S. applications filed on the same day as the present application under the title "Composition of asymmetric interfering RNA and uses thereof," the entire content of which is incorporated herein by reference) and also to modulate miRNA pathway activities.

[0136] The novel structural design of aiRNA is not only functionally potent in effecting gene regulation, but also offers several advantages over the current state-of-art, RNAi regulators (mainly antisense, siRNA). Among the advantages, aiRNA can have RNA duplex structure of much shorter length than the current siRNA constructs, which should reduce the cost of synthesis and abrogate or reduce length-dependent triggering of nonspecific interferon-like immune responses from host cells. The shorter length of the passenger strand in aiRNA should also eliminate or reduce the passenger strand's unintended incorporation in RISC, and in turn, reduce off-target effect observed in miRNA-mediated gene silencing. aiRNA can be used in all areas that current miRNA-based technologies are being applied or contemplated to be applied, including biology research, R&D in biotechnology and pharmaceutical industries, and miRNA-based diagnostics and therapies.

1.0. The aiRNA Structural Scaffold

[0137] The present invention is pertinent to asymmetrical double stranded RNA molecules that are capable of modulating miRNA-mediated gene silencing. In an embodiment, a RNA molecule of the present invention comprises a first strand and a second strand, wherein the second strand is substantially complementary to the first strand, and the first strand and the second strand form at least one double-stranded region, wherein the first strand is longer than the second strand (length asymmetry). The RNA molecule of the present invention has at least one double-stranded region, and two ends independently selected from the group consisting of 5'-overhang, 3'-overhang, and blunt end (e.g., see FIGS. **1**A, 2A-2D). In another embodiment, the first strand is shorter than the second strand. The two form at least one double-stranded region, and can have two ends independently selected from the group consisting of 5'-overhang, 3'-overhang, and blunt end (e.g., see FIGS. **2**E-**2**F).

[0138] In the field of making small RNA regulators where changes, addition and deletion of a single nucleotide can critically affect the functionality of the molecule (Elbashir, et al, *The EMBO Journal* 20:6877-6888 (2001)), the aiRNA scaffold provides a structural platform distinct from the classic siRNA structure of 21-nt double-strand RNA which is symmetric in each strand and their respective 3' overhangs. Further, the aiRNA of the present invention provides a much-needed new approach in designing a new class of small molecule regulators that, as shown by data included in the examples below, can overcome obstacles currently encountered in RNAi-based researches and drug development. For example, data from aiRNAs that structurally mimic siRNAs show that aiRNAs are more efficacious, potent, rapid-onset, durable, and specific than siRNAs in inducing gene silencing. Further evidence is provided below that aiRNAs can be designed to regulate miRNA pathways, either as a mimic or inhibitor.

[0139] Any single-stranded region of the RNA molecule of the invention, including any terminal overhangs and gaps in between two double-stranded regions, can be stabilized against degradation, either through chemical modification or secondary structure. The RNA strands can have unmatched or imperfectly matched nucleotides. Each strand may have one or more nicks (a cut in the nucleic acid backbone, e.g., see FIG. **1**B), gaps (a fragmented strand with one or more missing nucleotides, e.g, see FIG. **1**C), and modified nucleotides or nucleotide analogues. Not only can any and all of the nucleotides in the RNA molecule chemically modified, each strand may be conjugated with one or more moieties to enhance its functionality, for example, with moieties such as one or more peptides, antibodies, antibody fragments, aptamers, polymers, lipids, oligonucleotides and so on. In some embodiments, the moieties are added to enhance delivery. In some other embodiments, the moieties are added to enhance one or more pharmacological properties, e.g., drug absorption.

[0140] In an embodiment, the first strand is at least 1 nt longer than the second strand. In a further embodiment, the first strand is at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nt longer than the second strand. In another embodiment, the first strand is 20-100 nt longer than the second strand. In a further embodiment, the first strand is 2-12 nt longer than the second strand. In an even further embodiment, the first strand is 3-10 nt longer than the second strand.

[0141] In an embodiment, the first strand, or the long strand, has a length of 5-100 nt, or preferably 10-30 or 12-30 nt, or more preferably 15-28 nt. In one embodiment, the first strand is 21 nucleotides in length. In an embodiment, the second strand, or the short strand, has a length of 3-30 nt, or preferably 3-29 nt or 10-26 nt, or more preferably 12-26 nt. In an embodiment, the second strand has a length of 15 nucelotides.

[0142] In an embodiment, the double-stranded region has a length of 3-98 bp. In a further embodiment, the double-stranded region has a length of 5-28 bp. In an even further embodiment, the double-stranded region has a length of 10-19 bp. The length of the double-stranded region can be 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 bp.

[0143] In an embodiment, the double-stranded region of the RNA molecule does not contain any mismatch or bulge, and the two strands are perfectly complementary to each other in the double-stranded region. In another embodiment, the double-stranded region of the RNA molecule contains mismatch and/or bulge.

[0144] In an embodiment, the terminal overhang is 1-10 nucleotides. In a further embodiment, the terminal overhang is 1-8 nucleotides. In another embodiment, the terminal overhang is 3 nt.

1.1. The Duplex RNA Molecule with Both a 5'-Overhang and a 3'-Overhang

[0145] Referring to FIG. **1**A, in one embodiment of the prsent invention, the double stranded RNA molecule has both a 5'-overhang and a 3'-overhang on the first strand. The RNA molecule comprises a first strand and a second strand; the first strand and the second strand form at least one double-stranded region with substantially complementary sequences, wherein the first strand is longer than the second strand. On the first strand, flanking the double-stranded region, there is an unmatched overhang on both the 5' and 3' termini.

[0146] In an embodiment, the first strand is at least 2 nt longer than the second strand. In a further embodiment, the first strand is at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nt longer than the second strand. In another embodiment, the first strand is 20-100 nt longer than the second strand. In a further embodiment, the first strand is 2-12 nt longer than the second strand. In an even further embodiment, the first strand is 3-10 nt longer than the second strand.

[0147] In an embodiment, the first strand has a length of 5-100 nt. In a further embodiment, the first strand has a length of 5-100 nt, and the second strand has a length from 3-30 nucleotides. In an even further embodiment, the first strand has a length of 5-100 nt, and the second strand has a length from 3-18 nucleotides.

[0148] In an embodiment, the first strand has a length from 10-30 nucleotides. In a further embodiment, the first strand has a length from 10-30 nucleotides, and the second strand has a length from 3-28 nucleotides. In an even further embodiment, the first strand has a length from 10-30 nucleotides, and the second strand has a length from 3-19 nucleotides.

[0149] In an embodiment, the first strand has a length from 12-26 nucleotides. In a further embodiment, the first strand has a length from 12-26 nucleotides, and the second strand has a length from 10-24 nucleotides. In an even further embodiment, the first strand has a length from 12-26 nucleotides, and the second strand has a length from 10-19 nucleotides.

[0150] In an embodiment, the first strand has a length of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nt. In another embodiment, the second strand has a length of 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, or 28 nt.

[0151] In an embodiment, the first strand has a length of 21 nt, and the second strand has a length of 15 nt. This particular embodiment is sometimes referred to as the "15/21" configuration herein below. In some of the "15/21" configurations, the longer strand has a 3-nt overhang in both the 3' end and the the 5' end.

[0152] In an embodiment, the 3'-overhang has a length of 1-10 nt. In a further embodiment, the 3'-overhang has a length of 1-8 nt. In an even further embodiment, the 3'-overhang has a length of 2-6 nt. In one embodiment, the 3'-overhang has a length of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nt.

[0153] In an embodiment, the 5'-overhang has a length of 1-10 nt. In a further embodiment, the 5'-overhang has a length of 1-6 nt. In an even further embodiment, the 5'-overhang has a length of 2-4 nt. In one embodiment, the 5'-overhang has a length of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nt.

[0154] In an embodiment, the length of the 3'-overhang is equal to that of the 5'-overhang. In another embodiment, the 3'-overhang is longer than the 5'-overhang. In an alternative embodiment, the 3'-overhang is shorter than the 5'-overhang.

[0155] In an embodiment, the duplex RNA molecule comprises a double-stranded region of substantially complementary sequences of about 15 nt, a 3-nt 3'-overhang, and a 3-nt 5'-overhang. The first strand is 21 nt and the second strand is 15 nt. In one feature, the double-stranded region of various embodiments consists of perfectly complementary sequences. In an alternative feature, the double strand region includes at least one nick (FIG. **1**B), gap (FIG. **1**C), and/or mismatch (bulge or loop).

[0156] In an embodiment, the double-stranded region has a length of 3-98 bp. In a further embodiment, the double-stranded region has a length of 5-28 bp. In an even further embodiment, the double-stranded region has a length of 10-19 bp. The length of the double-stranded region can be 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 bp. There can be more than one double-stranded region.

[0157] In an embodiment, the first strand is the guide strand, which is capable of targeting a substantially complementary gene transcript such as a messenger RNA (mRNA) for gene silencing either by cleavage or by translation repression.

[0158] The same principles and features discussed above also apply to the embodiment where the second strand is longer than the first strand (FIG. **2**E).

1.2. The Duplex RNA Molecule with a Blunt End and a 5'-Overhang or a 3'-Overhang

[0159] In one embodiment, the duplex RNA molecule comprises a double-stranded region, a blunt end, and a 5'-overhang or a 3'-overhang (see, e.g., FIGS. **2**A and **2**B). The RNA molecule comprises a first strand and a second strand, wherein the first strand and the second strand form a double-stranded region, wherein the first strand is longer than the second strand.

[0160] In an embodiment, the double-stranded region has a length of 3-98 bp. In a further embodiment, the double-stranded region has a length of 5-28 bp. In an even further embodiment, the double-stranded region has a length of 10-18 bp. The length of the double-stranded region can be 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 bp. The double-stranded region can have features similar to those described with regard to other embodiments and are not necessarily repeated here. For example, the double-stranded region can consist of perfectly complementary sequences or include at least one nick, gap, and/or mismatch (bulge or loop).

[0161] In an embodiment, the first strand is at least 1 nt longer than the second strand. In a further embodiment, the first strand is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nt longer than the second strand. In another embodiment, the first strand is 20-100 nt longer than the second strand. In a further embodiment, the first strand is 2-12 nt longer than the second strand. In an even further embodiment, the first strand is 4-10 nt longer than the second strand.

[0162] In an embodiment, the first strand has a length of 5-100 nt. In a further embodiment, the first strand has a length of 5-100 nt, and the second strand has a length from 3-30 nucleotides. In an even further embodiment, the first strand has a length of 10-30 nt, and the second strand has a length from 3-19 nucleotides. In another embodiment, the first strand has a length from 12-26 nucleotides, and the second strand has a length from 10-19 nucleotides.

[0163] In an embodiment, the duplex RNA molecule comprises a double-stranded region, a blunt end, and a 3'-overhang (see, e.g., FIG. **2**B).

[0164] In an embodiment, the 3'-overhang has a length of 1-10 nt. In a further embodiment, the 3'-overhang has a length of 1-8 nt. In an even further embodiment, the 3'-overhang has a length of 2-6 nt. In one embodiment, the 3'-overhang has a length of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nt.

[0165] In an alternative embodiment, the duplex RNA molecule comprises a double-stranded region, a blunt end, and a 5'-overhang (see, e.g., FIG. **2**A).

[0166] In an embodiment, the 5'-overhang has a length of 1-10 nt. In a further embodiment, the 5'-overhang has a length of 1-6 nt. In an even further embodiment, the 5'-overhang has a length of 2-4 nt. In one embodiment, the 5'-overhang has a length of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nt.

1.3. The Duplex RNA Molecule with Two 5'-Overhangs or Two 3'-Overhangs

[0167] In one embodiment, the duplex RNA molecule comprises a double-stranded region, and two 3'-overhangs or two 5'-overhangs (see, e.g., FIGS. **2**C and **2**D). The RNA molecule comprises a first strand and a second strand, wherein the first strand and the second strand form a double-stranded region, wherein the first strand is longer than the second strand.

[0168] In an embodiment, the double-stranded region has a length of 3-98 bp. In a further embodiment, the double-stranded region has a length of 5-28 bp. In an even further embodiment, the double-stranded region has a length of 10-18 bp. The length of the double-stranded region can be 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 bp.

[0169] In an embodiment, the first strand is at least 1 nt longer than the second strand. In a further embodiment, the first strand is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nt longer than the second strand. In another embodiment, the first strand is 20-100 nt longer than the second strand. In a further embodiment, the first strand is 2-12 nt longer than the second strand. In an even further embodiment, the first strand is 4-10 nt longer than the second strand.

[0170] In an embodiment, the first strand has a length of 5-100 nt. In a further embodiment, the first strand has a length of 5-100 nt, and the second strand has a length from 3-30 nucleotides. In an even further embodiment, the first strand has a length of 10-30 nt, and the second strand has a length from 3-18 nucleotides. In another embodiment, the first strand has a length from 12-26 nucleotides, and the second strand has a length from 10-16 nucleotides.

[0171] In an alternative embodiment, the duplex RNA molecule comprises a double-stranded region, and two 3'-overhangs (see, e.g., FIG. **2**C). The double-stranded region shares similar features as described with regard to other embodiments.

[0172] In an embodiment, the 3'-overhang has a length of 1-10 nt. In a further embodiment, the 3'-overhang has a length of 1-6 nt. In an even further embodiment, the 3'-overhang has a length of 2-4 nt. In one embodiment, the 3'-overhang has a length of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nt.

[0173] In an embodiment, the duplex RNA molecule comprises a double-stranded region, and two 5'-overhangs (see, e.g., FIG. **2**D).

[0174] In an embodiment, the 5'-overhang has a length of 1-10 nt. In a further embodiment, the 5'-overhang has a length of 1-6 nt. In an even further embodiment, the 5'-overhang has a length of 2-4 nt. In one embodiment, the 3'-overhang has a length of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nt.

[0175] The same principles and features discussed above also apply to the embodiment where the second strand is longer than the first strand (FIG. **2**F).

2.0. The Design of aiRNAs

[0176] For ease of understanding, the principles of the invention are sometimes explained and illustrated below using examples where, (a) in siRNA applications, the antisense strand is the longer strand of the aiRNA molecule; (b) in miRNA mimetic applications, the antisense strand with sequence similar to a mature miRNA is the longer strand of the aiRNA molecule; and (c) in miRNA inhibitor applications, the sense strand with sequence substantially complementary to the mature miRNA is the longer strand of the aiRNA molecule. However, the opposite situations can be true and are contemplated to be part of the invention, i.e., the antisense strand can be the shorter strand of the aiRNA in both the siRNA applications and miRNA mimetic applications, and the sense strand can be the shorter strand in miRNA inhibitor applications—the same features and principles described in other examples apply in those situations and are not necessarily repeated.

[0177] siRNAs and miRNAs are widely used as research tools, and developed as drug candidates. (see, e.g., Dykxhoorn, Novina & Sharp. *Nat. Rev. Mol. Cell Biol.* 4:457-467 (2003); Kim & Rossi, *Nature Rev. Genet.* 8:173-184 (2007); de Fougerolles, et al. Nature Rev. Drug Discov. 6:443-453 (2007); Czech, NEJM 354:1194-1195 (2006); and Mack, Nature Biotech. 25:631-638 (2007)). The duplex RNA molecules of the present invention, i.e., aiRNAs, can be derived from siRNAs and miRNAs known in the field.

[0178] The present invention provides a method of converting an siRNA or an miRNA into an aiRNA. The conversion results in a new duplex RNA molecule that has at least one property improved in comparison to the original molecule. The property can be size, efficacy, potency, the speed of onset, durability, synthesis cost, off-target effects, interferon response, or delivery.

[0179] In an embodiment, the original molecule is a duplex RNA molecule, such as an siRNA or a miRNA/miRNA* (guide/passenger) duplex. The duplex RNA molecule comprises an antisense strand (e.g., a guide strand) and a sense strand (e.g. a passenger strand) that form at least one double-stranded region. The method comprises changing the length of one or both strands so that the antisense strand is longer than the sense strand. In an embodiment, sense passenger strand is shortened. In another embodiment, the antisense guide strand is elongated. In an even further embodiment, the sense strand is shortened and the antisense strand is elongated. The antisense and sense RNA strands, intact or with changed size, can be synthesized, and then combined under conditions, wherein an aiRNA molecule is formed.

[0180] In a further embodiment, the method comprises changing the length of the antisene and/or sense strand so that the duplex RNA molecule is formed having at least one of a 3'-overhang of 1-6 nucleotides and a 5'-overhang of 1-6 nucleotides.

[0181] In an embodiment, the original molecule is a single-strand RNA molecule, such as a mature guide miRNA or a passenger miRNA. The method comprises synthesizing a shorter RNA strand that is substantially complementary to the single-strand template or a portion of it, and combining the synthesized strand with the original single-strand RNA molecule or a shortened version of it under hybridizing conditions, wherein an aiRNA molecule of assymetric strand lengths is formed. In an embodiment, the template RNA is the mature guide miRNA and the resulting aiRNA is a mimetic of it. In another embodiment, the template RNA is the passenger strand (miRNA*) in a miRNA/miRNA* duplex, and the resulting aiRNA is an inhibitor of the guide miRNA.

[0182] Alternatively, the duplex RNA molecules of the present invention can be designed de novo. A duplex RNA molecule of the present invention can be designed taking advantage of the design methods for siRNAs and miRNAs, such as the method of gene walk.

[0183] An RNA molecule of the present invention can be designed with bioinformatics approaches, and then tested in vitro and in vivo to determine its modulating efficacy against the target gene and the existence of any off-target effects. Based on these studies, the sequences of the RNA molecules can then be selected and modified to improve modulating efficacy against the target gene, and to minimize off-target effects. (see e.g., Patzel, *Drug Discovery Today* 12:139-148 (2007)).

2.1. Unmatched or Mismatched Region in the Duplex RNA Molecule

[0184] The two single strands of the aiRNA duplex can have at least one unmatched or imperfectly matched region containing, e.g., one or more mismatches. In one embodiment, the unmatched or imperfectly matched region is at least one end region of the RNA molecule, including an end region with a blunt end, an end region with a 3'-recess or a 5' overhang, and an end region with a 5' recess or a 3' overhang. As used herein, the end region is a region of the RNA molecule including one end and the neighboring area.

[0185] In an embodiment, the unmatched or imperfectly matched region is in a double-stranded region of the aiRNA molecule. In a further embodiment, the asymmetric RNA duplex has an unmatched bulge or loop structure.

2.2. Sequence Motifs in the Duplex RNA Molecule

[0186] In the design of an aiRNA molecule of the invention, the overall GC content may vary. In an embodiment, the GC content of the double-stranded region is 20-70%. In a further embodiment, the GC content of the double-stranded region is less than 50%, or preferably 30-50%, to make it easier for strand separation as the G-C pairing is stronger than the A-U pairing.

[0187] The nucleotide sequence at a terminal overhang, in some embodiments, e.g., the 5' terminal, can be designed independently from any template sequence (e.g., a target mRNA sequence), i.e., does not have to be substantially complementary to a target mRNA (in the case of an siRNA or miRNA mimetic) or a target miRNA (in the case of miRNA inhibitor). In one embodiment, the overhang, e.g., at the 5' or the 3', of the longer or antisense strand, has at least one A, U, or dT. In an embodiment, the overhang has at least one "AA", "UU" or "dTdT" motif, which have exhibited increased efficacy in comparison to some other motifs. In an embodiment, the 5' overhang of the longer or antisense strand has an "AA" motif. In another embodiment, the 3' overhang of the longer or antisense strand has a "UU" motif.

2.3. Nucleotide Substitution

[0188] One or more of the nucleotides in the RNA molecule of the invention can be substituted with deoxynucleotides or modified nucleotides or nucleotide analogues. The substitution can take place anywhere in the RNA molecule, e.g., one or both of the overhang regions, and/or a double-stranded region. In some cases, the substitution enhances a physical property of the RNA molecule such as strand affinity, solubility and resistance to RNase degradation or enhanced stability otherwise.

[0189] In one embodiment, the modified nucleotide or analogue is a sugar-, backbone-, and/or base-modified ribonucleotide. The backbone-modified ribonucleotide may have a modification in a phosphodiester linkage with another ribonucleotide. In an embodiment, the phosphodiester linkage in the RNA molecule is modified to include at least a nitrogen and/or sulphur heteroatom. In an embodiment, the modified nucleotide or analogue is an unnatural base or a modified base. In an embodiment, the modified nucleotide or analogue is inosine, or a tritylated base.

[0190] In a further embodiment, the nucleotide analogue is a sugar-modified ribonucleotide in which the 2'-OH group is replaced by a group selected from the group consisting of H, OR, R, halo, SH, SR, NH_2, NHR, NR_2, and CN, wherein each R is independently selected from the group consisting of C1-C6 alkyl, alkenyl and alkynyl, and halo is selected from the group consisting of F, Cl, Br and I.

[0191] In one embodiment, the nucleotide analogue is a backbone-modified ribonucleotide containing a phosphothioate group.

2.4. aiRNA as miRNA Mimetic

[0192] Novel constructs of double-stranded aiRNAs can be provided to compensate for the lack of or insufficient level of functioning, endogenous miRNAs in a subject. In a first approach, an endogenous double-stranded region involving a mature miRNA is selected as a template for the design of an aiRNA mimetic. Such a template can be any suitable endogenous precursor to the single stranded mature miRNA including the pri-miRNA, pre-miRNA and the guide/passenger (miRNA/miRNA*) duplex that results from the ribonuclease Dicer digestion of pre-miRNA. Referring to FIG. **18**A, these potential templates preserve secondary structures such as bulges from nucleotide mismatches within the double-stranded region that can be copied into the aiRNA design. Because such secondary structures may play a role in the gene silencing function of the mature miRNA, e.g., facilitation of strand separation, this approach advantageously preserves this possibility for the aiRNA.

[0193] In the specific embodiment shown in FIG. **18**A, the stem-loop structure shown at the bottom represents the pre-miRNA of human Let7a gene (pre-Let-7a) where the 22 nucleotides that eventually become the mature miRNA are underlined. In designing an aiRNA mimetic of the hsa-Let7a miRNA (the "Let-7a aiRNA oligo"), that underlined portion of the double-strand region is used as the basis for a 21-nt, longer strand of the aiRNA. A 15-nt stretch from the substantially but imperfectly complementary sequence in the double-strand region of the pre-Let-7a, which overall is a single-stranded stem-loop hairpin molecule, is used as the shorter strand to complete the aiRNA design. As shown in the figure, at least the bubble structure resulting from the "GU/UG" mismatch in the original pre-miRNA is conserved in the aiRNA design. In this particular example, the longer strand of the aiRNA has a 3-nt overhang on both the 3' and 5' ends.

[0194] Also, as described above in Section 2.0, a guide/passenger miRNA duplex can be converted into an aiRNA by changing strand length

[0195] In a second approach, a portion of the mature miRNA sequence is copied into the longer strand of the aiRNA, and a substantially complementary, preferably a perfectly complementary, shorter strand is generated without regard to any secondary structure that might have existed in the mature miRNA's natural precursors. FIG. **4**A shows, among other things, a mimetic of hsa-Let-7c miRNA ("aiLet-7c mimic"). The sequence for the mature human Let7c miRNA (miRBase (http://microma.sanger.ac.uk) Accession No. MIMAT0000064) is:

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hsa-Let-7c: 5'-UGAGGUAGUAGGUUGUAUGGUU
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The first 19 nucleotides (underlined in FIG. **19**A) are copied into the longer strand of the aiLet-7c mimic following an "AA" motif at the 5' end. And a perfectly complementary sequence consisting of 15 nucleotides are provided as the shorter strand, leaving a 3-nt overhang on both the 3' and 5' termini of the longer strand. The "AA" motif is added at the 5' end of the longer strand because, among the motifs tried, it appears to provide some advantage in enhancing aiRNA functionality. As shown in examples below, both approaches work. In an embodiment, the aiRNA mimetic adopts the "15/21" configuration. However, other aiRNA configurations can also be used here of course.

[0196] The present invention can be used to make mimetics of any given miRNA of any species including plants (e.g., *Arabidopsis*), worms (e.g., *C. elegans*), insects (e.g. *Drosophila*), and animals including mammalian species like mouse and human. A list of human miRNAs currently known and can be used to generate the RNA molecules of the present invention is provided in FIGS. **30** and **31**.

2.5. aiRNA as miRNA Inhibitor

[0197] Double-stranded aiRNAs can also be provided to inhibit endogenous miRNA, e.g., by reducing the level of active miRNAs in cells. Current antagomirs are based on single-stranded antisense oligonucleotides, which are notoriously unstable in eukaryotic cells. In contrast, the present aiRNA structural scaffold is double-stranded, therefore much more stable in cells. Its novel asymmetric design apparently works in effecting potent inhibition of target miRNAs inside mammalian cells as shown in Examples 7 below.

[0198] In an embodiment, a first strand of the aiRNA, preferably the longer strand, consists of sequence substantially complementary to at least a portion of the target miRNA. The other aiRNA strand, preferably the shorter strand, is then made to substantially complement, e.g., perfectly complement, the first strand in sequence. In an embodiment, the shorter strand is imperfectly, i.e., only partly complementary, to the longer strand in the double-strand region.

[0199] In an embodiment, the longer strand of the aiRNA construct is designed to be perfectly complementary to at least a portion of the target miRNA. For example, FIG. **19**A shows, among other things, three inhibitor constructs of hsa-Let-7c, miR-21, and miR-155, respectively. The corresponding target miRNA sequences for each inhibitor are shown in FIG. **19**B. Each inhibitor includes, following an "AA" motif at the 5' end, a sequence perfectly complementary to the first 19 nucleotides of the mature target miRNA. In the examples shown, the aiRNA constructs adopt the "15/21" configuration with the longer 21-nt strand having an overhang on both the 3' and 5' ends. Other aiRNA configurations can also be used here of course.

[0200] While not to be bound by theory, one possible mechanism for inhibition of miRNAs by the aiRNAs of the present invention is illustrated through FIG. **29** and as follows: inside the cell, strands of both the endogenous miRNA/miRNA* duplex and the introduced aiRNA duplex are constantly in a dynamic equilibrium under which condition some duplexes are constantly base-pairing together while others are constantly separating. The ratio, at any given time, between duplexes formed and unraveled, or, in other words, between an on-rate and off-rate, is largely dependent upon the affinity between the two strands forming the duplex. While not clear at this time, the longer strand of the aiRNA, possibly by virtue of its designed complementarity or the lack of structures that facilitate strand separation, once base-pairs with the target miRNA strand, exhibits stronger affinity for the target miRNA (i.e., a lower off-rate) than the endogenous passenger miRNA (miRNA*) strand. This can be especially true when there are mismatches or unmatched regions in the endogenous miRNA/miRNA* duplex that favors dissociation. As a result, over time, the longer strand of the aiRNA is able to compete against the endogenous passenger miRNA in forming longer-lasting duplexes with the mature guide miRNA, thus reducing the amount of unbound guide miRNAs at any given time that are available to effect intended

gene silencing. The length asymmetry of the aiRNA construct, in comparison to a symmetric construct, favors dissociation between the longer strand and the shorter strand, making more of the longer strands available to compete for the target miRNA strand.

[0201] In a preferred embodiment, the longer strand of the aiRNA has perfect base-pair complementarity with at least a portion of the targeted guide miRNA, but this does not have to be true—as long as the complementarity is sufficiently high for the longer strand to compete against the endogenous passenger miRNA in terms of forming a duplex with the guide miRNA.

[0202] The present invention can be used to make inhibitors of any given miRNA of any species including plants (e.g., *Arabidopsis*), worms (e.g., *C. elegans*), insects (e.g. *Drosophila*), and animals including mammalian species like mouse and human. A list of human miRNAs currently known and can be used to generate the RNA molecules of the present invention is provided in FIGS. **30** and **31**.

3. The Utilities

3.1. Research and Drug Discovery Tools

[0203] MicroRNAs serve gene-regulatory functions in cells; some have been found to associate with various types of human diseases including cancers, viral infections, etc. Therefore, miRNA mimetics and inhibitors can be used to study gene targets modulated by miRNAs, and the mechanisms and components of various miRNA pathways and their interactions.

[0204] Methods and constructs of the invention can be used to study miRNA pathways and related gene function in vitro and in vivo. RNA molecules of the invention can also be used to transfect cultured animal cells as a research tool in drug target/pathway identification and validation. For example, after transfecting or otherwise delivering the RNA molecules of the invention into host cells, these cells can be monitored for phenotypical or morphology changes that suggest some pathway of interest has been affected. Such phenotypical changes can involve numbers of nuclei, nuclei morphology, cell death, cell proliferation, DNA fragmentation, cell surface marker, and mitotic index, etc. In another example, interaction between a molecule/substrate in the host cell and the miRNA modulator (mimetic or inhibitor) transfected into the cell can be isolated or identified to discover potential therapeutic target. That target can be upstream and modulates the miRNA activity, or, the target can be downstream and its activity is modulated by the miRNA. Furthermore, the RNA molecules of the invention can be used to conduct drug target discovery and validation in vivo, e.g., in animal models with xenograft of diseased tissue or cell populations.

[0205] With evidence that a network or a number of miRNAs may target the same gene product or pathway (Sethupathy P. et al. *RNA* 12: 192-197 (2006)), multiple RNA molecules of the invention, including mimetics of different miRNAs, inhibitors of different miRNAs, and mixture of mimetics and inhibitors of different miRNAs can be used to study the interrelationship between various miRNA pathways including coregulation of the same gene product.

[0206] In terms of additional in vivo applications, since the present invention provides functional miRNA mimetics and inhibitors, after they are respectively introduced into a host body, a systems biology approach can be adopted in studying the effect of certain miRNA, which is being mimicked or inhibited, on different cell types and different tissues.

[0207] Further, with their ability to modulate post-transcriptional gene silencing mediated by miRNA, the RNA molecules of the present invention can be used to create gene "knockdown" in animal models as opposed to genetically engineered knockout models in order to study and validate gene functions.

[0208] The RNA molecule of the invention can be supplied as research reagents either in double-stranded duplex, or separated as single strands to be used in double-stranded form. Accordingly, the present invention also provides a kit that contains an RNA molecule of the invention in any of suitable forms including those described above.

3.2. Therapeutic Uses

[0209] The RNA molecules of the present invention can be used for the treatment and or prevention of various diseases, including the diseases summarized in Dykxhoorn, Novina & Sharp. *Nat. Rev. Mol. Cell Biol.* 4:457-467 (2003); Kim & Rossi, *Nature Rev. Genet.* 8:173-184 (2007); de Fougerolles, et al. *Nature Rev. Drug Discov.* 6:443-453 (2007); Czech, *NEJM* 354:1194-1195 (2006); and Mack, *Nature Biotech.* 25:631-638 (2007); Tong and Nemunaitis, *Cancer Gene Therapy* 15: 341-355 (2008).

[0210] In one embodiment, a mimetic of the present invention is used to reconstitute desired miRNA function to the normal level, thereby treating the disease or alleviating the symptom. In another embodiment, an inhibitor of the present invention is used to reduce or eliminate undesired miRNA activity, thereby treating diseases caused by overexpression or misexpression of the miRNA. Combinatorial use of multiple mimetics, multiple inhibitors, and a mixture of mimetic and inhibitor can also be used.

[0211] In an embodiment, the present invention is used as a cancer therapy or to prevent cancer by targeting one or more cancer-related genes. This method is effected by using miRNA mimetics and/or inhibitors of the invention to up-regulate tumor-suppressing genes, and/or by using miRNA inhibitors and/or mimetics of the invention to silence genes involved with cell proliferation or other cancer phenotypes.

[0212] In an embodiment, the therapeutic mimetics according to the invention mimic miRNAs that target oncogenes. Examples of such tumor-suppressing miRNAs may include: let-7 family, miR-15a, miR-16-1, miR-34a, miR-143, miR-145 and so on. Examples of various oncogenes targeted by these miRNAs include k-Ras and bcl-2. For example, k-Ras has been shown to be regulated by miRNA Let-7. These oncogenes are active and relevant in the majority of clinical cases. For example, k-Ras is aberrantly active in the majority of human colon cancer, pancreatic cancer, and non small cell lung cancers. Further, k-Ras mutation confers resistance to chemotherapy and current targeted therapy.

[0213] In an embodiment, the therapeutic inhibitors according to the invention inhibit miRNAs that target tumor-suppressor genes. Examples of such miRNAs may include miR-17/92 cluster, miR-21, miR-106, miR-155, miR-221, miR-222 and so on. Examples of tumor suppressing genes targeted by these miRNAs include ERK5 and p27 (kipi) (Tong and Nemunaitis, *Cancer Gene Therapy* 15: 341-355 (2008)).

[0214] These RNA molecules can also be used to modulate non-cancer genes targeted by the miRNAs being mimicked or inhibited. The RNA molecules of the invention can also be

used to treat or prevent ocular diseases, (e.g., age-related macular degeneration (AMD) and diabetic retinopathy (DR)); infectious diseases (e.g. HIV/AIDS, hepatitis B virus (HBV), hepatitis C virus (HCV), human papillomavirus (HPV), herpes simplex virus (HSV), RCV, cytomegalovirus (CMV), dengue fever, west Nile virus); respiratory diseases (e.g., respiratory syncytial virus (RSV), asthma, cystic fibrosis); neurological diseases (e.g., Huntingdon's disease (HD), amyotrophic lateral sclerosis (ALS), spinal cord injury, Parkinson's disease, Alzheimer's disease, pain); cardiovascular diseases; metabolic disorders (e.g., diabetes); genetic disorders; and inflammatory conditions (e.g., inflammatory bowel disease (1BD), arthritis, rheumatoid disease, autoimmune disorders), dermatological diseases, psychological disorders (e.g., bipolar disorder).

4. Manufacture and Use

[0215] 4.1. Making the aiRNA Molecules

[0216] The RNA molecules of the present invention can be made via any suitable means, including through chemical reactions and synthesis, through biological processes, and/or an enzyme-effected processes.

[0217] Chemical synthesis of RNA molecules of a given sequence is well known in the art. Biological processes for making RNA molecules are also well known. For example, a DNA expression vector, viral, eukaryotic, or bacterial, can be constructed with an appropriate promoter to transcribe a corresponding DNA sequence into the designed RNA sequence once transfected into host cells (e.g., bacteria). In an embodiment, the transcript may be a precursor to the final RNA duplex such that enzyme actions such as ribonuclease-effected site-specific cleavage is needed. For example, both strands of the aiRNA molecule of the invention may be transcribed into a single strand that needs to be cut into two separate strands to form the double-stranded RNA molecule of the invention.

[0218] One aspect of the invention is directed to an expression vector that includes a DNA sequence encoding part or all of the double-stranded RNA molecule of the present invention (e.g., one of the asymmetric strands), the DNA sequence being operably linked to an expression control sequence, e.g., a promoter. In an embodiment, the vector is single-stranded. In a further embodiment, two different vectors each encoding for a different strand of the RNA molecule of the invention are provided to co-transfect a cell, inside which the two expressed strands form a duplex. In another embodiment, the vector is double stranded and each strand contains the DNA sequence for a different strand of the RNA molecule operably linked to an expression control sequence. Further, the present invention provides a cell that includes such an expression vector. The cell can be a mammalian, avian or bacterial cell.

[0219] The two strands of the aiRNA molecule can be manufactured separately or at the same time, in any of the above processes. For example, in a biological process, two vectors can be constructed to express the shorter strand and the longer strand of aiRNA separately, or a single vector with two strands can be constructed to express the two strands of the aiRNA molecule simultaneously.

4.2. Modifications of the RNA Molecules

[0220] Naked RNA molecules are relatively unstable and can be degraded in vivo relatively quickly. Chemical modifications can be introduced to the RNA molecules of the present invention, including mimetics of siRNAs, of miRNAs and inhibitors of miRNAs, to improve their half-life and to further reduce the risk of non-specific effects of gene targeting, without reducing their biological activities.

[0221] The modifications of RNA molecules have been investigated to improve the stability of various RNA molecules, including antisense RNA, ribozyme, aptamer, and RNAi. (see, e.g., Chiu & Rana, *RNA* 9:1034-1048 (2003); Czaudema, et al, *Nucleic Acids Research* 31:2705-2716 (2003); Zhang H Y, et al, *Curr Top Med Chem.* 6:893-900 (2006); Kim & Rossi, *Nature Rev. Genet.* 8:173-184 (2007); de Fougerolles, et al. *Nature Rev. Drug Discov.* 6:443-453 (2007); and Schmit, *Nature Biotech.* 25:273-275 (2007); and Mack, Nature Biotech. 25:631-638 (2007)).

[0222] Any stabilizing modification known to one skill in the art can be used to improve the stability of the RNA molecules of the present invention. Within the RNA molecules of the present invention, chemical modifications can be introduced to the phosphate backbone (e.g., phosphorothioate linkages), the ribose (e.g., locked nucleic acids, 2'-deoxy-2'-fluorouridine, 2'-O-mthyl), and/or the base (e.g., 2'-fluoropyrimidines). Several examples of such chemical modifications are summarized below.

[0223] Chemical modifications at the 2' position of the ribose, such as 2'-O-mthylpurines and 2'-fluoropyrimidines, which increase resistance to endonuclease activity in serum, can be adopted to stabilize the RNA molecules of the present invention. The position for the introduction of the modification should be carefully selected to avoid significantly reducing the silencing potency of the RNA molecule. For example, the modifications on 5' end of the guide strand can reduce the silencing activity. On the other hand, 2'-O-methyl modifications can be staggered between the two RNA strands at the double-stranded region to improve the stability while reserving the gene silencing potency. The 2'-O-methyl modifications can also eliminate or reduce the interferon induction.

[0224] Another stabilizing modification is phosphorothioate (P=S) linkage. The introduction of phosphorothioate (P=S) linkage into the RNA molecules, e.g., at the 3'-overhang, can provide protection against exonuclease.

[0225] The introduction of deoxyribonucleotides into the RNA molecules can also reduce the manufacture cost, and increase stability.

[0226] In an embodiment, the 3'-overhang, 5'-overhang, or both are stabilized against degradation. In an embodiment, the RNA molecule contains at least one modified nucleotide or its analogue. In a further embodiment, the modified ribonucleotide is modified at its sugar, backbone, base, or any combination of the three.

[0227] In an embodiment, the nucleotide analogue is a sugar-modified ribonucleotide. In a further embodiment, the 2'-OH group of the nucleotide analogue is replaced by a group selected from H, OR, R, halo, SH, SR, NH2, NHR, NR2, or CN, wherein each R is independently C1-C6 alkyl, alkenyl or alkynyl, and halo is F, Cl, Br or I.

[0228] In an alternative embodiment, the nucleotide analogue is a backbone-modified ribonucleotide containing a phosphothioate group.

[0229] In an embodiment, the duplex RNA molecule contains at least one deoxynucleotide. In a further embodiment, the first strand comprises 1-6 deoxynucleotides. In an even further embodiment, the first strand comprises 1-3 deoxynucleotides. In another embodiment, the 3'-overhang comprises 1-3 deoxynucleotides. In a further embodiment, the

5'-overhang comprises 1-3 deoxynucleotides. In an alternative embodiment, the second strand comprises 1-5 deoxynucleotides.

[0230] In an embodiment, the duplex RNA molecule comprises a 3'-overhang or 5'-overhang that contains at least one deoxynucleotide. In another embodiment, the 3'-overhang and/or 5'-overhang of the RNA consists of deoxynucleotides.

[0231] In an embodiment, the duplex RNA molecule is conjugated to an entity. In a further embodiment, the entity is selected from the group consisting of peptide, antibody, polymer, lipid, oligonucleotide, and aptamer.

[0232] In another embodiment, the first strand and the second strand are joined by a chemical linker.

4.3. In Vivo Delivery of the RNA Molecules

[0233] One major obstacle for the therapeutic use of RNAi is the delivery of siRNA to the target cell (Zamore P D, Aronin N. *Nature Medicine* 9:266-8 (2003)). Various approaches have been developed for the delivery of RNA molecules, especially siRNA molecules (see, e.g., Dykxhoom, Novina & Sharp. *Nat. Rev. Mol. Cell Biol.* 4:457-467 (2003); Kim & Rossi, *Nature Rev. Genet.* 8:173-184 (2007); and de Fougerolles, et al. *Nature Rev. Drug Discov.* 6:443-453 (2007)). Any delivery approach known to one skilled in the art can be used for the delivery of the RNA molecules of the present invention.

[0234] Major issues in delivery include instability in serum, non-specific distribution, tissue barriers, and non-specific interferon response (Lu & Woodle, *Methods in Mol Biology* 437: 93-107 (2008)). Compared to their siRNA and miRNA counterparts, aiRNA molecules possess several advantages that should make a wider ranger of methods available for delivery purpose. First, aiRNAs can be designed to be smaller than their siRNA and miRNA counterparts, therefore, reducing or eliminating any interferon responses. Second, aiRNAs are more potent, faster-onsetting, more efficacious and last longer, therefore, less amount/dosage of aiRNAs is required to achieve a therapeutic goal. Third, aiRNA are double stranded and more stable than single-stranded antisense oligos and miRNAs, and they can be further modified chemically to enhance stability. Therefore, the RNA molecules of the invention can be delivered into a subject via a variety of systemic or local delivery routes. In some embodiments, molecules of the invention are delivered through systemic delivery routes include intra-venous (I.V.) and intra-peritoneal (ip). In other embodiments, molecules of the invention are delivered through local delivery routes, e.g., intra-nasal, intra-vitreous, intra-tracheal, intra-cerebral, intra-muscle, intra-articular, and intra-tumor.

[0235] Examples of the delivery technologies include direct injection of naked RNA molecules, conjugation of the RNA molecules to a natural ligand such as cholesterol, or an aptamer, liposome-formulated delivery, and non-covalently binding to antibody-protamine fusion proteins. Other carrier choices include positive charged carriers (e.g., cationic lipids and polymers) and various protein carriers. In one embodiment, the delivery of the molecules of the invention uses a ligand-targeted delivery system based on the cationic liposome complex or polymer complex systems (Woodle, et al. *J Control Release* 74: 309-311; Song, et al. *Nat Biotechnol.* 23(6): 709-717 (2005); Morrissey et al. *Nat Biotechnol.* 23(8): 1002-1007 (2005)).

[0236] In one embodiment, molecules of the invention are formulated with a collagen carrier, e.g., atelocollagen, for in vivo delivery. Atelocollagen has been reported to protect siRNA from being digested by RNase and to enable sustained release (Minakuchi, et al. *Nucleic Acids Res.* 32: e109 (2004); Takei et al. *Cancer Res.* 64: 3365-3370 (2004)). In another embodiment, molecules of the invention are formulated with nanoparticles or form a nanoemulsion, e.g., RGD peptide ligand targeted nanoparticles. It has been shown that different siRNA oligos can be combined in the same RGD ligand targeted nanoparticle to target several genes at the same time (Woodle et al. *Materials Today* 8 (suppl 1): 34-41 (2005)).

[0237] Viral vectors can also be used for the delivery of the RNA molecules of the present invention. In an embodiment, lentiviral vectors are used to deliver the RNA molecule transgenes that integrate into the genome for stable expression. In another embodiment, adenoviral and adeno-associated virus (AAV) are used to deliver the RNA molecule transgenes that do not integrate into the genome and have episomal expression.

[0238] Moreover, bacteria can be used for the delivery of the RNA molecules of the present invention. (see Xiang, Fruehauf, & Li, *Nature Biotechnology* 24:697-702 (2006)).

4.4. The Pharmaceutical Compositions and Formulations

[0239] The pharmaceutical compositions and formulations of the present invention can be the same or similar to the pharmaceutical compositions and formulations developed for siRNA, miRNA, and antisense RNA (see, e.g., Kim & Rossi, Nature Rev. Genet. 8:173-184 (2007); and de Fougerolles, et al. Nature Rev. Drug Discov. 6:443-453 (2007)), except for the RNA ingredient. The siRNA, miRNA, and antisense RNA in the pharmaceutical compositions and formulations can be replaced by the duplex RNA molecules of the present information. The pharmaceutical compositions and formulations can also be further modified to accommodate the duplex RNA molecules of the present information.

[0240] A "pharmaceutically acceptable salt" or "salt" of the disclosed duplex RNA molecule is a product of the disclosed duplex RNA molecule that contains an ionic bond, and is typically produced by reacting the disclosed duplex RNA molecule with either an acid or a base, suitable for administering to a subject. Pharmaceutically acceptable salt can include, but is not limited to, acid addition salts including hydrochlorides, hydrobromides, phosphates, sulphates, hydrogen sulphates, alkylsulphonates, arylsulphonates, acetates, benzoates, citrates, maleates, fumarates, succinates, lactates, and tartrates; alkali metal cations such as Na, K, Li, alkali earth metal salts such as Mg or Ca, or organic amine salts.

[0241] A "pharmaceutical composition" is a formulation containing the disclosed duplex RNA molecules in a form suitable for administration to a subject. In one embodiment, the pharmaceutical composition is in bulk or in unit dosage form. The unit dosage form is any of a variety of forms, including, for example, a capsule, an IV bag, a tablet, a single pump on an aerosol inhaler, or a vial. The quantity of active ingredient (e.g., a formulation of the disclosed duplex RNA molecule or salts thereof) in a unit dose of composition is an effective amount and is varied according to the particular treatment involved. One skilled in the art will appreciate that it is sometimes necessary to make routine variations to the dosage depending on the age and condition of the patient. The dosage will also depend on the route of administration. A variety of routes are contemplated, including oral, pulmonary, rectal, parenteral, transdermal, subcutaneous, intrave-

nous, intramuscular, intraperitoneal, intranasal, and the like. Dosage forms for the topical or transdermal administration of a duplex RNA molecule of this invention include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. In one embodiment, the active duplex RNA molecule is mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants that are required.

[0242] The present invention also provides pharmaceutical formulations comprising a duplex RNA molecule of the present invention in combination with at least one pharmaceutically acceptable excipient or carrier. As used herein, "pharmaceutically acceptable excipient" or "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Suitable carriers are described in "Remington: The Science and Practice of Pharmacy, Twentieth Edition," Lippincott Williams & Wilkins, Philadelphia, Pa., which is incorporated herein by reference. Examples of such carriers or diluents include, but are not limited to, water, saline, Ringer's solutions, dextrose solution, and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active duplex RNA molecule, use thereof in the compositions is contemplated. Supplementary active duplex RNA molecules can also be incorporated into the compositions.

[0243] In an embodiment, the pharmaceutically acceptable excipient, carrier, or diluent comprises a lipid for intravenous delivery. The lipid can be: phospholipids, synthetic phophatidylcholines, natural phophatidylcholines, sphingomyelin, ceramides, phophatidylethanolamines, phosphatidylglycerols, phosphatidic acids, cholesterol, cholesterol sulfate, and hapten and PEG conjugated lipids. The lipid may be in the form of nanoemulsion, micelles, emulsions, suspension, nanosuspension, niosomes, or liposomes. In an embodiment, the pharmaceutically acceptable excipient, carrier, or diluent is in a form of micellar emulsion, suspension, or nanoparticle suspension, and it further comprises an intravenously acceptable protein, e.g., human albumin or a derivative thereof, for intravenous delivery.

[0244] In an embodiment, the pharmaceutically acceptable excipient, carrier, or diluent comprises a waxy material for oral delivery. The waxy material may be mono-, di-, or triglycerides, mono-, di-fatty acid esters of PEG, PEG conjugated vitamin E (vitamin E TPGs), Gelucire and/or Gelucire 44/14. In an embodiment, the pharmaceutically acceptable excipient, e.g., Gelucire 44/14, is mixed with a surfactant, which can be Tween 80 or Tween 20. These embodiments of pharmaceutical compositions can be further formulated for oral administration. Methods for formulation are disclosed in PCT International Application PCT/US02/24262 (WO03/011224), U.S. Patent Application Publication No. 2003/0091639 and U.S. Patent Application Publication No. 2004/0071775, each of which is incorporated by reference herein.

[0245] A duplex RNA molecule of the present invention is administered in a suitable dosage faun prepared by combining a therapeutically effective amount (e.g., an efficacious level sufficient to achieve the desired therapeutic effect through inhibition of tumor growth, killing of tumor cells, treatment or prevention of cell proliferative disorders, etc.) of a duplex RNA molecule of the present invention (as an active ingredient) with standard pharmaceutical carriers or diluents according to conventional procedures (i.e., by producing a pharmaceutical composition of the invention). These procedures may involve mixing, granulating, and compressing or dissolving the ingredients as appropriate to attain the desired preparation. In another embodiment, a therapeutically effective amount of a duplex RNA molecule of the present invention is administered in a suitable dosage form without standard pharmaceutical carriers or diluents.

[0246] Pharmaceutically acceptable carriers include solid carriers such as lactose, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium stearate, stearic acid and the like. Exemplary liquid carriers include syrup, peanut oil, olive oil, water and the like. Similarly, the carrier or diluent may include time-delay material known in the art, such as glyceryl monostearate or glyceryl distearate, alone or with a wax, ethylcellulose, hydroxypropylmethylcellulose, methylmethacrylate or the like. Other fillers, excipients, flavorants, and other additives such as are known in the art may also be included in a pharmaceutical composition according to this invention.

[0247] The pharmaceutical compositions containing active duplex RNA molecules of the present invention may be manufactured in a manner that is generally known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes. Pharmaceutical compositions may be formulated in a conventional manner using one or more physiologically acceptable carriers comprising excipients and/or auxiliaries which facilitate processing of the active duplex RNA molecules into preparations that can be used pharmaceutically. Of course, the appropriate formulation is dependent upon the route of administration chosen.

[0248] A duplex RNA molecule or pharmaceutical composition of the invention can be administered to a subject in many of the well-known methods currently used for chemotherapeutic treatment. For example, for treatment of cancers, a duplex RNA molecule of the invention may be injected directly into tumors, injected into the blood stream or body cavities or taken orally or applied through the skin with patches. For treatment of psoriatic conditions, systemic administration (e.g., oral administration), or topical administration to affected areas of the skin, are preferred routes of administration. The dose chosen should be sufficient to constitute effective treatment but not so high as to cause unacceptable side effects. The state of the disease condition (e.g., cancer, psoriasis, and the like) and the health of the patient should be closely monitored during and for a reasonable period after treatment.

[0249] The duplex RNA molecule or pharmaceutical composition of the invention can be administered to a subject in any suitable dosing range, dosing frequencies and plasma concentration. In an embodiment, the subject is being treated with a pharmaceutical composition of the invention with an effective dosage amount of 1 ng to 1 g per day, 100 ng to 1 g per day, or 1 μg to 500 mg per day, and so on.

Examples

[0250] Examples are provided below to further illustrate different features of the present invention. The examples also

illustrate useful methodology for practicing the invention. These examples do not limit the claimed invention.

Methods and Materials

Cell Culture and Reagents

[0251] Hela, SW480, DLD1, HT29, and H1299 cells were obtained from ATCC, and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Invitrogen). Fresh peripheral blood mononuclear cells (PBMC) were obtained from All-Cells LLC and maintained in RPMI-1640 medium containing 10% FBS and pen/strep (Invitrogen). Small RNAs described in this study were synthesized by Dharmacon, Qiagen, or Integrated DNA technologies (Table 2) and annealed following the manufacturer's instructions (FIG. **3***a*). siRNAs targeting human Ago2, and Dicer (Ambion) were used at 100 nM. Transfections of the RNAs were performed using DharmaFECT1 (Dharmacon) at the indicated concentrations. Human Argonaute2 (Ago2) expression vector (OriGene) was transfected using Lipofectamine 2000 (Invitrogen). Serum stability was determined by incubation of aiRNA or siRNA duplex with 10% human serum (Sigma) for the indicated amount of time followed by non-denaturing TBE-acrylamide gel electrophoresis and ethidium bromide staining.

Northern Blot Analysis.

[0252] To determine levels of β-catenin, total RNA was extracted with TRIZOL (Invitrogen) from siRNA or aiRNA transfected Hela cells at various time points. 20 μg of total cellular RNA was loaded to each lane of a denaturing agarose gel. After electrophoresis, RNA was transferred to Hybond-XL Nylon membrane (Amersham Biosciences), UV crosslinked, and baked at 80° C. for 30 min. Probes detecting β-catenin and actin mRNA was prepared using Prime-It II Random Primer Labeling Kit (Stratagene) from β-catenin cDNA fragment (1-568 nt) and actin cDNA fragment (1-500 nt). To analyze small RNA RISC loading, siRNA or aiRNA were transfected into Hela cells 48 hours after transfection with pCMV-Ago2. Cells were lysed at the indicated timepoints and immunoprecipitated with Ago2 antibody. Immunoprecipitates were washed, RNA isolated from the complex by TRIZOL extraction, and loaded on a 15% TBE-Urea PAGE gel (Bio-Rad). Following electrophoreses, RNA was transferred to Hybond-XL Nylon membrane. mirVana miRNA Probe Kit (Ambion) was used to generate 5' ^{32}P labeled RNA probes. Antisense probe (5'-GUAGCUGAUAUUGAUGGACUU-3'). Sense probe (5'-UCCAUCAAUAUCAGC-3')

In Vitro Ago2-RISC Loading.

[0253] aiRNA or siRNA sense and anti-sense strands were ^{32}P end labeled using T4 kinase (Promega). End labeled RNAs were purified by phenol/chloroform/isoamyl alcohol, precipitated with EtOH, and resuspended in water. Labeled RNAs were then annealed to siRNA or aiRNA anti-sense strands as described. For in vitro lysates, Hela cells were transfected for 24 hours with human Ago2 expression vector, and S10 lysates generated essentially as described (Dignam et al., 1983). 5' sense strand or anti-sense strand labeled duplex aiRNA or siRNA was then added to the Ago2-S10 lysate. Following a 5 min incubation at 37° C., Ago2 was immunoprecipited as described, and Ago2-associated (pellet) and non-Ago2 associated (supernatant) fractions were separated on a 20% TBE-acrylamide gel and gel exposed to film to detect sense strand-Ago2 association. For aiRNA and siRNA competition experiments, up to 100 folds cold aiRNA and siRNA were used to compete with ^{32}P labeled aiRNA or siRNA to load to RISC. Briefly, S10 lysates were generated from Hela cells transfected with Ago2 expression vector as described. Labeled aiRNA or siRNA was then added to the S10 lysates followed immediately by addition of unlabeled aiRNA or siRNA. Reaction was incubated for 5 min at 37° C. and processed as described above.

qRT-PCR.

[0254] Cells transfected with siRNA, the indicated aiRNA, the indicated aiRNA anti-miRNA, or the commercially available miRNA inhibitor (Ambion) were harvested at the indicated time points following transfection. RNA was isolated with TRIZOL. For mRNAs, qRT-PCR performed using TaqMan one-step RT-PCR reagents on a StepOne real-time PCR and primer probe sets for the indicated mRNA (Applied Biosystems). Data is presented relative to control transfected cells and each sample is normalized to actin mRNA levels. For miRNAs, reverse transcription of miRNAs was performed using TaqMan microRNA reverse transcription kits (Applied Biosystems) and cDNA was subjected to real-time PCR using TaqMan microRNA assays (Applied Biosystems) on a StepOne real-time PCR machine (Applied Biosciences), each sample is normalized to U6snRNA. For the experiment in FIG. **14***d*, Stat3 constructs were created by cloning Stat3 cDNA (Origene) into either pcDNA3.1^{+} or pcDNA3.1^{-} at the HindIII-Xho1 sites. Stat3 forward or reverse expression vectors were then co-transfected into Hela cells with aiStat3 or siStat3 for 24 hours. Cells were then harvested, RNA isolated by TRIZOL, and qRT-PCR performed using TaqMan one-step RT-PCR reagents and primer probe sets for Stat3 or actin (Applied Biosystems). RT-PCR was performed on the same RNA samples using Superscript One-Step RT-PCR kit (Invitrogen) and Stat3 forward (5'-GGATCTAGAATCAGCTACAGCAGC-3') and Stat3 reverse (5'-TCCTCTAGAGGGCAATCTCCATTG-3') primers and actin forward (5'-CCATGGATGATGATATCGCC-3') and actin reverse (5'-TAGAAGCATTTGCGGTGGAC-3') primers.

RT-PCR.

[0255] Total RNA was prepared using the TRIZOL, and cDNA was synthesized using random primers with Thermoscript RT-PCR System (Invitrogen). PCR was run for 20 cycles using Pfx polymerase. Primers: ACTIN-1, 5' CCATGGATGATGATATCGCC-3'; ACTIN-2, 5'-TAGAAGCATTTGCGGTGGAC-3'; β-catenin-1, 5'-GACAATGGCTACTCAAGCTG-3'; β-catenin-2, 5'-CAGGTCAGTATCAAACCAGG-3'.

Western Blot

[0256] Cells were washed twice with ice-cold phosphate-buffered saline and lysed in lysis buffer (50 mM HEPES, pH 7.5, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM NaF, 2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of pepstatin, leupeptin, and aprotinin). 20 μg of soluble protein was separated by SDS-PAGE and transferred to PVDF membranes. Primary Antibodies against β-catenin, Nbs1, Survivin, p21, Rsk1, k-Ras, Stat3, PCNA, NQO1,

Actin (Santa Cruz), EF2, p70S6K, mTOR, PTEN (Cell Signaling Technology), Ago2 (Wako), Dicer (Novus), and Parp1 (EMD Biosciences) were used in this study. The antigen-antibody complexes were visualized by enhanced chemiluminescence (GE Biosciences).

RT-PCR and Western Blot Analysis on miRNAs

[0257] Hela cells were transfected with 100 nM of the indicated aiRNA or microRNA inhibitor. 24 hours after transfection, cells harvested for RNA using TRIZOL (Invitrogen) or for protein using whole cell extract buffer (50 mM HEPES, 2 mM magnesium chloride, 250 mM sodium chloride, 0.1 mM EDTA, 1 mM EGTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1× mammalian protease inhibitor cocktail [Sigma], 1× phosphatase inhibitor cocktails I and II [Sigma]) by incubation for 30 min on ice. Soluble proteins were separated by centrifugation at 13,000×g in a microcentrifuge, and supernatants were stored at −70° C. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and transferred to a polyvinylidene difluoride membrane by electroblotting. Primary antibodies used to detect k-Ras and actin (Santa Cruz Biotechnology) were incubated with the membrane followed by HRP-linked secondary antibody (GE Biosciences) and visualized by chemiluminesence (GE Biosciences). RT-PCR was performed on the RNA samples using Superscript One-Step RT-PCR kit (Invitrogen) and k-Ras forward (5'-AGTACAGTGCAATGAGGGACCAGT), k-Ras reverse (5'-AGCATCCTCCACTCTCTGTCTTGT), Actin forward (5'-CCATGGATGATGATATCGCC) and actin reverse (5'-TAGAAGCATTTGCGGTGGAC) primers.

5'-RACE Analysis

[0258] Total RNA (5 μg) from Hela cells treated with non-silencing aiRNA or aiRNA was ligated to GeneRacer™ RNA adaptor (Invitrogen, 5'-CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA-3') without any prior processing. Ligated RNA was reverse transcribed into cDNA using a random primer. To detect cleavage product, PCR was performed using primers complementary to the RNA adaptor (GeneRacer™ 5' Nested Primer: 5'-GGACACTGACATGGACTGAAGGAGTA-3') and β-catenin specific primer (GSP: 5'-CGCATGATAGCGTGTCTGGAAGCTT-3'). Amplification fragments were resolved on 1.4% agarose gel and sized using a 1-kb Plus DNA Ladder (Invitrogen). Specific cleavage site was further confirmed by DNA sequencing.

Interferon-Response Detection.

[0259] For the experiment in FIG. **15***a*, PBMC were incubated directly with 100 nM β-catenin siRNA or aiRNA. Total RNA was purified at 16 hours using TRIZOL, and levels of interferon responsive gene expression were determined by RT-PCR as described by the manufacturer (System Biosciences). For the experiment in FIG. **15***b*, Hela cells were mock transfected or transfected with 100 nM of the indicated aiRNA or siRNA for 24 hours. Total RNA was purified using TRIZOL and levels of interferon responsive gene expression were determined by RT-PCR. For microarray analysis, Hela cells were transfected with 100 nM aiRNA or siRNA. Total RNA was purified at 24 hours using TRIZOL, and RNA was used for hybridization to Human Genome U133 Plus 2.0 GeneChip (Affymetrix) according to the manufacturer's protocol (ExpressionAnalysis, Inc.). RNA from DharmaFECT 1 treated cells was used as control. To calculate transcript expression values, Microarray Suite 5.0 was used with quantile normalization, and transcripts with sufficient hybridization signals to be called present (P) were used in this study.

aiRNA and siRNA Sequences

[0260] Sequence and structure of aiRNA and siRNA duplexes were listed in Table 2. Location of point mutation is framed in the k-Ras aiRNA.

TABLE 2

slβ-catenin	⍰	slRsk1	⍰
alβ-catenin	⍰	alRsk1	⍰
slNbs1	⍰	siPCNA	⍰
alNbs1	⍰	aiPCNA	⍰
siEF2	⍰	siParp1	⍰
aiEF2	⍰	aiParp1	⍰
siStat3	⍰	siSurvivin	⍰
aiStat3	⍰	aiSurvivin	⍰
siPTEN	⍰	siNQO1	⍰
aiPTEN	⍰	aiNQO1	⍰
sip70S8K	⍰	slp21	⍰
aip70S8K	⍰	alp21	⍰
simTOR	⍰	alk-Ras	⍰
aimTOR	⍰		

⍰ indicates text missing or illegible when filed

In Life Evaluations

[0261] Daily examinations into the health status of each animal were also conducted. Body weights were checked every three days. Food and water was supplied daily according to the animal husbandry procedures of the facility. Treatment producing >20% lethality and or >20% net body weight loss were considered toxic. Results are expressed as mean tumor volume (mm^3) ±SE. P Values<0.05 are considered to be statistically relevant.

Animal Husbandry

[0262] Male or female athymic nude mice 4-5 weeks (Charles River Laboratories, Wilmington, Mass.), were acclimated to the animal housing facility for at least 1 week before study initiation. All of the experimental procedures utilized were consistent with the guidelines outlined by the American Physiology Society and the Guide for the Care and Use of Laboratory Animals and were also approved by the Institutional Animal Care and Use Committee of Boston Biomedical Inc. The animals were housed in groups of four in wood chip bedded cages in a room having controlled temperature (68° F.-72° F.), light (12-h light-dark cycle), and humidity (45-55%). The animals were allowed free access to water and food during the experiment.

Example 1

Asymmetric Interfering RNA (aiRNA) Causes Gene-Specific Silencing in Mammalian Cells

[0263] The siRNA structural scaffold is considered the essential configuration for incorporating into RISC and medi-

ating RNAi(Elbashir et al., 2001a; Elbashir et al., 2001b; Elbashir et al., 2001c; Rana, 2007; Zamore et al., 2000). However, very little is known about RNA duplex scaffold requirements for RISC incorporation and gene silencing. To investigate the structural scaffold requirements for an efficient RNAi mediator and RISC substrate, we first determined if RNA duplexes shorter than siRNAs could mediate gene silencing. The length of double stranded (ds) RNA is an important determinant of its propensity in activating protein kinase R (PKR)-mediated non-specific interferon responses, increased synthesis cost, and delivery challenges (Elbashir et al., 2001b; Sledz et al., 2003). We designed a series of short dsRNAs ranging from 12 to 21 by with 2 nucleotide 3' overhangs or blunt ends targeting different mammalian genes. No gene silencing was detected after the length was reduced below 19 by (data not shown), which is consistent with previous reports in *Drosophila Melanogaster* cell lysate(Elbashir et al., 2001b) and the notion that 19-21 bp is the shortest siRNA duplex that mediates RNAi (Elbashir et al., 2001a; Elbashir et al., 2001b; Elbashir et al., 2001c; Rana, 2007; Zamore et al., 2000).

[0264] We next tested if RNA duplexes of non-siRNA scaffold with an asymmetric configuration of overhangs can mediate gene silencing. The siRNA duplex contains a symmetrical sense strand and an antisense strand. While the duplex siRNA structure containing a 3' overhang is required for incorporation into the RISC complex, following Argonaute (Ago) mediated cleavage of the sense strand, the antisense strand directs cleavage of the target mRNA (Hammond et al., 2001; Matranga et al., 2005; Tabara et al., 1999). We sought to make asymmetric RNA duplexes of various lengths with overhangs at the 3' and 5' ends of the antisense strand.

[0265] Oligos with sequences shown in Table 3 were confirmed by 20% polyacrylamide gel after annealing. As shown in FIG. **3**A, each lane was loaded as follows: lane 1, 21nt/21nt; lane 2, 12nt (a)/21nt; lane 3, 12nt (b)/21nt; lane 4, 13nt/13nt; lane 5, 13nt/21nt; lane 6, 14nt/14nt; lane 7, 14nt (a)/21nt; lane 8, 14nt(b)/21nt; lane 9, 15nt/15nt; lane 10, 15nt/21nt.

TABLE 3

Oligos	Sequences	SEQ ID NO:
21nt/21nt	5'-**GUAGCUGAUAUUGAUGGAC**TT-3'	1
	3'-TT**CAUCGACUAUAACUACCUG**-5'	2
12nt/21nt (a)	5'-**UGAUAUUGAUGG**-3'	3
	3'-CAUCGACUAUAACUACCUGAA-5'	4
12nt/21nt (b)	5'-CUGAUAUUGAUG-3'	5
	3'-CAUCG**ACUAUAACUACC**UGAA-5'	4
13nt/21nt	5'-**CUGAUAUUGAUGG**-3'	6
	3'-CAUC**GACUAUAACUAC**CUGAA-5'	4
14nt/21nt (a)	5'-**GCUGAUAUUGAUGG**-3'	7
	3'-CAU**CGACUAUAACUACC**UGAA-5'	4
14nt/21nt (b)	5'-**CUGAUAUUGAUGGA**-3'	8
	3'-CAUC**GACUAUAACUACCU**GAA-5'	4
15nt/21nt	5'-**GCUGAUAUUGAUGGA**-3'	9
	3'-CAU**CGACUAUAACUACCU**GAA-5'	4

[0266] HeLa cells were plated at 200,000 cells/well into a 6 well culture plate. As shown in FIG. **3**B, 24 hours later they were transfected with scramble siRNA (lane 1), 21-bp siRNA targeted E2F1 (lane 2, as a control for specificity) or 21-bp siRNA targeted beta-catenin (lane 3, as a positive control), or the same concentration of aiRNA of different length mix: 12nt(a)/21nt (lane 4); 12nt (b)/21nt (lane 5); 13nt/21nt (lane 6); 14nt (a)/21nt (lane 7); 14nt (b)/21nt (lane 8); 15nt/21nt (lane 9). Cells were harvested 48 hours after transfection. Expression of β-catenin was determined by Western blot. E2F1 and actin are used as controls. The results demonstrate that asymmetric interfering RNA (aiRNA) causes gene-specific silencing in mammalian cells.

[0267] In order to determine the structural features of aiRNA important in aiRNA function, we generated multiple aiRNA oligonucleotides based on modification of the core 15/21 dual anti-sense overhang structure (Table 4). The aiRNAs, summarized in Table 4, contained modifications including, but not limited to, length of the sense and anti-sense strands, degree of sense and anti-sense overhangs, and RNA-DNA hybrid oligonucleotides.

[0268] Modification to the parental 15/21 aiRNA structure was done by altering the sense strand, anti-sense strand, or both (Table 4). Modified aiRNA duplexes were transfected into Hela cells at 50 nM for 48 hours. Western blots for β-catenin and actin were used to examine the degree of gene silencing compared to the parental 15/21 aiRNA and to the traditional siRNA structure. aiRNA modifications were also tested which contained dual sense strand overhangs. These oligonucleotides contain a 21 base sense strand paired to differing length anti-sense strands. In addition, we also examined the activity of aiRNA oligonucleotides that have been modified with DNA bases. DNA substitutions were done on both the anti-sense and sense strands (Table 3). RNA-DNA hybrid oligonucleotides tested contained 1 or more DNA substitutions in either the sense or anti-sense strand, or contained 21 base anti-sense RNA paired with indicated length of DNA sense strand. The gene silencing results of these various aiRNAs were shown in FIGS. **4** and **5**.

[0269] Taken together, these data provide structural clues to aiRNA function.

[0270] Regarding the sense strand, our data indicate that the length of 15 bases works well, while lengths between 14 and 19 bases remain functional. The sense strand can match any part of the anti-sense strand, provided that the anti-sense overhang rules are met. Replacement of a single RNA base with DNA at either the 5' or 3' end of the sense strand is tolerated and may even provide increased activity.

[0271] With respect to the anti-sense strand length, the length of 21 bases works well, 19-22 bases retains activity, and activity is decreased when the length falls below 19 bases or increases above 22 bases. The 3' end of the anti-sense strand requires an overhang of 1-5 bases with a 2-3 base overhang being preferred, blunt end shows a decrease in activity. Base pairing with the target RNA sequence is preferred, and DNA base replacement up to 3 bases is tolerated without concurrent 5' DNA base replacement. The 5' end of the anti-sense strand prefers a 0-4 base overhang, and does not require an overhang to remain active. The 5' end of the anti-sense strand can tolerate 2 bases not matching the target RNA sequence, and can tolerate DNA base replacement up to 3 bases without concurrent 3' DNA base replacement.

[0272] With respect to mismatched or chemically modified bases, we find that both mismatches and one or more chemically modified bases in either the sense or anti-sense strand is tolerated by the aiRNA structure.

TABLE 4

aiRNA sequences used for FIG. 4-5

aiRNA #	Generic Structure	Sequence
1	15-21 (NNN---NNN)	5'-GCUGAUAUUGAUGGA CAUCGACUAUAACUACCUGAA-5'
2	15-21a (NNNNNN---blunt)	5'-GAUAUUGAUGGACUU CAUCGACUAUAACUACCUGAA-5'
3	15-21b (blunt---NNNNNN)	5'-GUAGCUGAUAUUGAU CAUCGACUAUAACUACCUGAA-5'
4	15-21c (NNNN---NN)	5'-CUGAUAUUGAUGGAC CAUCGACUAUAACUACCUGAA-5'
5	15-21d (NN---NNNN)	5'-AGCUGAUAUUGAUGG CAUCGACUAUAACUACCUGAA-5'
7	15-18b (blunt cut 3'---NNN)	5'-GCUGAUAUUGAUGGA CGACUAUAACUACCUGAA-5'
8	15-21d (N---NNNNN)	5'-UAGCUGAUAUUGAUG CAUCGACUAUAACUACCUGAA-5'
9	15-21e (NNNNN---N)	5'-UGAUAUUGAUGGACU CAUCGACUAUAACUACCUGAA-5'
10	15-22a (NNNN---NNN)	5'-GCUGAUAUUGAUGGA UCAUCGACUAUAACUACCUGAA-5'
11	15-22b (NNN---NNNN)	5'-GCUGAUAUUGAUGGA CAUCGACUAUAACUACCUGAAA-5'
13	15-24a (NNNNN---NNNN)	5'-GCUGAUAUUGAUGGA UUCAUCGACUAUAACUACCUGUAA-5'
14	15-24b (NNNN---NNNNN)	5'-GCUGAUAUUGAUGGA UCAUCGACUAUAACUACCUGUCAA-5'
15	15-27 (NNNNNN---NNNNNN)	5'-GCUGAUAUUGAUGGA GUUCAUCGACUAUAACUACCUGUCAUA-5'
16	15-20a (NNN---NN)	5'-GCUGAUAUUGAUGGA CAUCGACUAUAACUACCUGA-5'
17	15-20b (NNNN---N)	5'-GCUGAUAUUGAUGGA UCAUCGACUAUAACUACCUG-5'
18	15-20c (NN---NNN)	5'-GCUGAUAUUGAUGGA AUCGACUAUAACUACCUGAA-5'
21	15-19c (NNNN-blunt)	5'-GCUGAUAUUGAUGGA UCAUCGACUAUAACUACCU-5'
22	15-18a (NN---N)	5'-GCUGAUAUUGAUGGA AUCGACUAUAACUACCUG-5'
23	15-18b (NNN-blunt)	5'-GCUGAUAUUGAUGGA CAUCGACUAUAACUACCU-5'
24	15-18c (blunt---NNN)	5'-GCUGAUAUUGAUGGA CGACUAUAACUACCUGAA-5'
25	15-17a (NN---blunt)	5'-GCUGAUAUUGAUGGA AUCGACUAUAACUACCU-5'
26	15-17b (blunt---NN)	5'-GCUGAUAUUGAUGGA CGACUAUAACUACCUGA-5'
29	14-20 (NNN---NNN)	5'-GCUGAUAUUGAUGG CAUCGACUAUAACUACCUGA-5'
30	14-19a (NNN---NN)	5'-GCUGAUAUUGAUGG CAUCGACUAUAACUACCUG-5'

TABLE 4-continued

aiRNA sequences used for FIG. 4-5

aiRNA #	Generic Structure	Sequence
31	14-19b (NN---NNN)	5'-GCUGAUAUUGAUGG AUCGACUAUAACUACCUGA-5'
33	14-18b (NNN---N)	5'-GCUGAUAUUGAUGG CAUCGACUAUAACUACCU-5'
34	16-21a (NNN---NN)	5'-GCUGAUAUUGAUGGAC CAUCGACUAUAACUACCUGAA-5'
35	16-21b (NN---NNN)	5'-AGCUGAUAUUGAUGGA CAUCGACUAUAACUACCUGAA-5'
36	17-21 (NN---NN)	5'-AGCUGAUAUUGAUGGAC CAUCGACUAUAACUACCUGAA-5'
37	18-21a (NN---N)	5'-AGCUGAUAUUGAUGGACU CAUCGACUAUAACUACCUGAA-5'
38	18-21b (N---NN)	5'-UAGCUGAUAUUGAUGGAC CAUCGACUAUAACUACCUGAA-5'
39	18-21c (NNN---blunt)	5'-GCUGAUAUUGAUGGACUU CAUCGACUAUAACUACCUGAA-5'
40	19-21a (NN---blunt)	5'-AGCUGAUAUUGAUGGACUU CAUCGACUAUAACUACCUGAA-5'
41	18-21b (blunt---NNN)	5'-GUAGCUGAUAUUGAUGGA CAUCGUCUAUAACUACCUGAA-5'
42	19-21c (N---N)	5'-UAGCUGAUAUUGAUGGACU CAUCGACUAUAACUACCUGAA-5'
43	20-21a (N---blunt)	5'-UAGCUGAUAUUGAUGGACUU CAUCGACUAUAACUACCUGAA-5'
44	20-21b (blunt---N)	5'-GUAGCUGAUAUUGAUGGACU CAUCGACUAUAACUACCUGAA-5'
45	Mismatch and miRNA	5'-GCUGAUAUUGAAGGA CAUCGACUAUAACUACCUGAA-5'
46	5' end homologous to target	5'-GCUGAUAUUGAUGGA CAUCGACUAUAACUACCUGUC-5'
47	NNNNNNNNNNNNNNN 3'NNNNNNNNNNNNNNNNNNNDDD-5'	5'-GCUGAUAUUGAUGGA CAUCGACUAUAACUACCUgaa-5'
48	NNNNNNNNNNNNNNN 3'DDDNNNNNNNNNNNNNNNNNN-5'	5'-GCUGAUAUUGAUGGA catCGACUAUAACUACCUGAA-5'
49	NNNNNNNNNNNNNNN 3'DDDNNNNNNNNNNNNNNNDDD-5'	5'-GCUGAUAUUGAUGGA catCGACUAUAACUACCUgaa-5'
51	DNDNNNNNNNDNDND 3'NNNNNNNNNNNNNNNNNNNNN-5'	5'-gCtGAUAUUGaUgGa CAUCGACUAUAACUACCUGAA-5'
52	DNNNNNNNNNNNNNN 3'NNNNNNNNNNNNNNNNNNNNN-5'	5'-gCUGAUAUUGAUGGA CAUCGACUAUAACUACCUGAA-5'
53	NNNNNNNNNNNNNND 3'NNNNNNNNNNNNNNNNNNNNN-5'	5'-GCUGAUAUUGAUGGa CAUCGACUAUAACUACCUGAA-5'
54		5'-UAGCUGAUAUUGAUG UUCAUCGACUAUAACUACCUG-5'
55		5'-GUAGCUGAUAUUGAUGGA UUCAUCGACUAUAACUACCUG-5'
56		5'-AGCUGAUAUUGAUGGA UUCAUCGACUAUAACUACCUG-5'

TABLE 4-continued

aiRNA sequences used for FIG. 4-5

aiRNA #	Generic Structure	Sequence
57	DNNNNNNNNNNNNNN	5'-gCUGAUAUUGAUGGA
	3'DDDNNNNNNNNNNNNNNNNNN-5'	catCGACUAUAACUACCUGAA-5'
58	DNNNNNNNNNNNNNN	5'-gCUGAUAUUGAUGGA
	3'NNNNNNNNNNNNNNNNNNDDD-5'	CAUCGACUAUAACUACCUgaa-5'
59	NNNNNNNNNNNNNND	5'-GCUGAUAUUGAUGGa
	3'DDDNNNNNNNNNNNNNNNNNN-5'	catCGACUAUAACUACCUGAA-5'
60	NNNNNNNNNNNNNND	5'-GCUGAUAUUGAUGGa
	3'NNNNNNNNNNNNNNNNNNDDD-5'	CAUCGACUAUAACUACCUgaa-5'
61	NNNNNNNNNNNNNNN	5'-UAGCUGAUAUUGAUGGACU
	3'DDDNNNNNNNNNNNNNNNNN-5	catCGACUAUAACUACCUGAA-5'
62	NNNNNNNNNNNNNNN	5'-UAGCUGAUAUUGAUGGACU
	3'NNNNNNNNNNNNNNNNNNDDD-5'	CAUCGACUAUAACUACCUgaa-5'

In table 4, A, U, G, C represent nucleotides, while a, t, g, c represent deoxynucleotides.

Example 2

Mechanism of Gene Silencing Triggered by aiRNA

[0273] To investigated the mechanism of gene knockdown induced by aiRNA, we first determined if the gene silencing by aiRNA occurs at translational or mRNA level. Northern blot analysis of β-catenin in cells transfected with 10 nM of the 15 by aiRNA showed that the aiRNA reduced mRNA levels by over 95% within 24 hours and the decrease lasted more than 4 days (FIG. **6***a*), suggesting that aiRNA mediates gene silencing at the mRNA level. The reduction of β-catenin mRNA induced by aiRNA was substantially more rapid, efficacious and durable than by siRNA (FIG. **6***a*). We further determined if the 15 by aiRNA catalyzed the site-specific cleavage of β-catenin mRNA. Total RNA isolated from cells transfected with the 15 by aiRNA was examined by rapid amplification of cDNA ends (5'-RACE) and PCR for the presence of the β-catenin mRNA cleavage fragments (FIG. **6***b*). We detected β-catenin cleavage fragments at 4 and 8 hours following aiRNA transfection (FIG. **6***c*). Sequence analysis showed that cleavage was taking place within the aiRNA target sequence between bases 10 and 11 relative to the 5' end of the aiRNA antisense strand (FIG. **6***d*). No such cleavage fragments were observed following transfection with a scrambled aiRNA (FIG. **6***c*). These results demonstrate that aiRNA induced potent and efficacious gene silencing through sequence-specific cleavage of the target mRNA.

[0274] We next determined whether the novel asymmetric scaffold of aiRNA can be incorporated into the RISC. RNAi is catalyzed by RISC enzyme complex with an Argonaute protein (Ago) as the catalytic unit of the complex (Liu et al., 2004; Matranga et al., 2005). To determine if aiRNA is incorporated into the Ago/RISC complex, we immunoprecipitated myc-tagged human Ago1 from cells expressing myc-tagged Ago1 (Siolas et al., 2005) after cells were transfected with aiRNA. Small RNAs associated with the RISC complex were detected by northern blotting of Ago immunoprecipitates. Northern blot analysis revealed that the aiRNA entered the RISC complex with high efficiency (FIG. **6***e*). These data suggest the asymmetric scaffold of aiRNA can be efficiently incorporated into RISC.

[0275] Since aiRNA induced more efficient gene silencing than siRNA, we tested if aiRNA can give rise to RISC complex more efficiently than siRNA. As shown in FIG. **6***e*, aiRNA-Ago2/RISC complexes formed faster and more efficient than the siRNA-Ago2/RISC complexes, with more aiRNA contained in the RISC complex than the corresponding siRNA (FIG. **6***e* and FIG. **7**A). Of note, siRNA displayed a typical pattern (21) that is consistent with formation of secondary structures by siRNA (FIG. **6***e* and FIG. **7**). In contrast, aiRNA displayed a single band, suggesting that the shorter length of aiRNA may reduce or eliminate the secondary structure formation as occurred with siRNA.

[0276] Further, the asymmetric configuration of aiRNA may facilitate the formation of active RISC with antisense strand and reduce the ineffective RISC formed with the sense strand (Ref. 16). Our data proved this is true as shown in FIG. **7**B, no sense strand can be detected in the RISC complex. FIG. **8**A also demonstrates that while the anti-sense strand of the aiRNA strongly associates with Ago 2, the sense-strand does not. In contrast, both the anti-sense and sense strand of the siRNA associate with Ago 2. These data suggest that aiRNA has higher efficiency in forming RISC than siRNA in cells, which may underlie the superior gene silencing efficiency of aiRNA.

[0277] In addition, it has been shown that the sense strand of siRNA is required to be cleaved in order to be functional. Therefore, we tested if the same requirement is true for aiRNA. To do that, the nucleotide at position 8 or 9 of the aiRNA sense strand was modified with 2'-O-methyl to make it uncleavable. Our results show that the aiRNAs with the uncleavable sense strand are still functional (FIG. **8**B), demonstrating aiRNA is quite different than siRNA in terms of their mechanism.

[0278] Further we asked if there is any different loading pocket for aiRNA and siRNA. We used cold aiRNA or siRNA to compete with the radioactively labelled siRNA or aiRNA for the RISC complex (FIG. **9**). Surprisingly, the results show that cold aiRNA does not compete with the siRNA for RISC complex (FIG. **9**B) and cold siRNA does not compete with

aiRNA for the RISC complex either (FIG. **9**C). These data indicate that aiRNA and siRNA may load to different pockets of RISC complex.

[0279] Together, the data above suggest that aiRNA represents the first non-siRNA scaffold that is incorporated into RISC, providing a novel structural scaffold that interacts with RISC. The difference of the RISC loading of aiRNA and siRNA is illustrated in our model shown in FIG. **10**. Briefly, because of the asymmetric property, only the anti-sense strand is selected to stay in the RISC complex and results in a 100% efficiency in strand selection. In contrast, siRNA is structurally symmetric. Both anti-sense strand and sense strand of the siRNA has a chance to be selected to stay in the RISC complex and therefore siRNA has an inefficient strand selection and at the same time may cause non-specific gene silencing due to the sense strand RISC complex.

Example 3

aiRNA Mediates a More Rapid, Potent, Efficacious, and Durable Gene Silencing than siRNA

[0280] To compare aiRNA with siRNA in gene silencing properties, we first determined the optimal aiRNA structure for gene silencing.

[0281] The siRNA duplex contains a symmetrical sense strand and an antisense strand. While the duplex siRNA structure containing a 3' overhang is required for incorporation into the RISC complex, following Argonaute (Ago) mediated cleavage of the sense strand, the antisense strand directs cleavage of the target mRNA(Hammond et al., 2001; Matranga et al., 2005; Tabara et al., 1999). We sought to make asymmetric RNA duplexes of various lengths with overhangs at the 3' and 5' ends of the antisense strand. We designed one set of such asymmetrical RNA duplexes of 12 to15 by with 3' and 5' antisense overhangs to target β-catenin (FIG. **11**A), an endogenous gene implicated in cancer and stem cells (Clevers, 2006). An optimized siRNA of the standard configuration has been designed to target β-catenin for triggering RNAi (Xiang et al., 2006). All aiRNAs against β-catenin were designed within the same sequence targeted by the siRNA (FIG. **11**A). The results showed that the optimal gene silencing achieved was with the 15 by aiRNA (FIG. **11**B). Therefore, we used 15 by aiRNA to be compared with 21-mer siRNA duplex in the subsequent experiments.

[0282] To our surprise, we found that aiRNA induced potent and highly efficacious reduction of β-catenin protein while sparing the non-targeted control genes actin (FIG. **11**C).

[0283] We next examined the onset of gene silencing by aiRNA and siRNA targeting β-catenin. The sequence of the aiRNA and siRNA used is shown in FIG. **11**A. As shown in FIG. **12**, aiRNA has a more rapid onset (FIGS. **12**C and D) and also a better efficacy (FIGS. **12**B and D).

[0284] We also compared the gene silencing effects of aiRNA and siRNA on various targets and multiple human cell lines. The aiRNAs were designed to target genes of different functional categories including Stat3 (FIG. **13***b*), NQO1 (FIG. **12***d*), elongation factor 2 (EF2) (FIG. **13***c*), Nbs1 (FIG. **14***b*), Survivin (FIG. **14***b*), Parp1 (FIG. **14***b*), p21 (FIG. **14***b*), Rsk1 (FIG. **14***c*), PCNA (FIG. **14***c*), p70S6K (FIG. **14***c*), mTOR (FIG. **14***c*), and PTEN (FIG. **14***c*), besides β-catenin (FIG. **13***a*) at the same sequences that have been targeted with siRNA with low efficiency (Rogoff et al., 2004). As shown in FIGS. **13** and **14**, aiRNA is more efficacious than siRNA in silencing Stat3, β-catenin, Rsk1, p70S6K, Nbs1, mTOR, and EF2, and is as efficacious as siRNA in silencing NQO1, PCNA, Survivin, PTEN, Parp1, and p21. Since the target sequences were chosen based on the optimization for siRNA, it is possible that the efficacy and potency of aiRNA can be further increased by targeting sites that are optimized for aiRNA. In addition, our data also shows that aiRNA is more efficacious than siRNA against b-catenin in multiple cell lines including Hela (FIG. **13***a*), H1299 (FIG. **14***a*, left panel) and Dld1 (FIG. **14***a*, right panel).

[0285] Taken together, these data demonstrate that aiRNA is more efficacious, potent, rapid-onset, and durable than siRNA in mediating gene silencing in mammalian cells.

Example 4

Specificity of Gene Silencing Medicated by aiRNA

[0286] We next investigated the specificity of gene silencing mediated by aiRNAs. We first analyzed aiRNAs that target the wildtype k-Ras allele. DLD1 cells contain wildtype k-Ras while SW480 cells contain mutant k-Ras that has a single base pair substitution (FIG. **14***d*). Transfection of DLD1 cells with aiRNA targeting wildtype k-Ras showed effective silencing, but no silencing of mutant k-Ras was observed in the SW480 cells. These data demonstrate that aiRNA mediates allele specific gene silencing.

[0287] The activation of an interferon-like response is a major non-specific mechanism of gene silencing. A primary reason that siRNAs are used for gene silencing is that the dsRNA of shorter than 30 by has reduced ability to activate the interferon-like response in mammalian cells (Bernstein et al., 2001; Martinez and Tuschl, 2004; Sledz et al., 2003). We tested if aiRNA showed any signs of activating the interferon-like response in mammalian cells. RNA collected from PBMC cells transfected with aiRNA against β-catenin and Hela cells transfected with aiRNA against EF2 or Survivin was analyzed by RT-PCR for interferon inducible genes. We found that aiRNA transfection showed no increase by RT-PCR of any of the interferon inducible genes tested, while levels of targeted mRNAs were reduced relative to control transfected cells (FIGS. **15***a* and *b*). Microarray analysis was also performed to compare the changes in the expression of known interferon response related genes induced by aiRNA and miRNA. As shown in FIG. **15***c*, much less changes were observed for aiRNA compared to siRNA.

[0288] In addition, as mentioned above, sense strand-RISC complex may cause non-specific gene silencing. To compare aiRNA and siRNA on the non-specific gene silencing mediated by sense-strand-RISC complex, cells were co-transfected with aiRNA or siRNA and either a plasmid expressing Stat3 (sense RNA) or a plasmid expressing antisense Stat3 (antisense RNA). Cells were harvested and RNA collected at 24 hours post transfection and relative levels of Stat3 sense or antisense RNA were determined by quantitative real time PCR or RT-PCR (inserts). The results show that aiRNA has no effect on the antisense Stat3 mRNAs while siRNA does (FIG. **15***d*). This result demonstrate aiRNA completely abolish the undesired non-specific gene-silencing mediated by the sense strand-RISC complex.

[0289] In summary, we have shown that aiRNA is a novel class of gene-silencing inducers, the non-siRNA type and the smallest structural scaffold for RISC substrates and RNAi mediators (FIG. **15***f*). Our data suggest that aiRNA works through RISC, the cellular RNAi machinery. After incorpo-

ration into RISC, aiRNA mediates sequence-specific cleavage of the mRNA between base 10 and 11 relative to the 5' end of the aiRNA antisense strand. The asymmetrical configuration of aiRNA can interact more efficiently with RISC than siRNA. Consistent with high RISC binding efficiency, aiRNA is more potent, efficacious, rapid-onset, and durable than siRNA in mediating gene-specific silencing against genes tested in our study. While previous studies have proposed a role of Dicer in facilitating efficient RISC formation, our data suggest aiRNA can give rise to active RISC complexes with high efficiency independent of Dicer-mediated processing.

[0290] The key feature of this novel RNA duplex scaffold is antisense overhangs at the 3' and 5' ends. The 12-15 by aiRNA are the shortest RNA duplex known to induce RNAi. While long dsRNAs triggered potent gene silencing in *C. elegans* and *Drosophila Melanogaster,* gene-specific silencing in mammalian cells was not possible until siRNA duplexes were used. The siRNA scaffold, as defined by Dicer digestion, is characterized by symmetry in strand lengths of 19-21 by and 3' overhangs (Bernstein et al., 2001), which has been considered the essential structure for incorporating into RISC to mediate RNAi. Therefore, optimization efforts for RNAi inducers have been focused on siRNA precursors, which are invariably larger than siRNA (Soutschek et al., 2004; Zhang and Farwell, 2007). Our data suggest that siRNA is not the essential scaffold for incorporating into RISC to mediate RNAi. The aiRNAs of different lengths displayed a spectrum of gene silencing efficacy and RISC incorporation efficiency, offering unique opportunity for understanding the mechanism of RISC incorporation and activation. Research is needed to further understand the structure-activity relationship of aiRNAs in RISC incorporation and RNAi induction, which should help establish a rational basis for optimizing aiRNAs with regards to target sequence selection, length, structure, chemical composition and modifications for various RNAi applications.

Example 5

aiRNA is More Efficacious than siRNA In Vivo

[0291] To investigate if aiRNA is efficacious in vivo and to compare it with siRNA, we tested the effects of aiRNA and siRNA in human colon cancer xenograft models.

[0292] Human Colon Cancer is the second leading cause of cancer death in the U.S. The Wnt β-catenin signaling pathway is tightly regulated and has important functions in development, tissue homeostasis, and regeneration. Deregulation of Wnt/β-catenin signaling is frequently found in various human cancers. Eighty percent of colorectal cancers alone reveal activation of this pathway by either inactivation of the tumor-suppressor gene adenomatous polyposis coli or mutation of the proto-oncogene β-catenin.

[0293] Activation of Wnt/β-catenin signaling has been found to be important for both initiation and progression of cancers of different tissues. Therefore, targeted inhibition of Wnt/β-catenin signaling is a rational and promising new approach for the therapy of cancers of various origins.

[0294] In vitro, by ribozyme-targeting we have demonstrated the reduction of β-catenin expression in human colon cancer SW480 cells and associated induction of cell death, indicating that β-catenin expression is rate-limiting for tumor growth in vitro.

[0295] SW480 human colon cancer cells were inoculated subcutaneously into female athymic nude mice (8×10^6 cells/mouse) and allowed to form palpable tumors. In this study, dosing began when the tumors reached approximately 120 mm^3. Animals were treated intravenously (iv) with 0.6 nmol PET-complexed β-catenin siRNAs, PEI-complexed β-catenin aiRNAs or a PEI-complexed unrelated siRNA as a negative control daily. The animals received a total of 10 doses of siRNA, aiRNA or control. Tumors were measured throughout treatment. As shown in FIG. **16**, intravenous treatment with siRNA and aiRNA as a monotherapy at 0.6 nmol mg/kg significantly inhibited tumor growth. The % T/C value of siRNA was calculated to be 48.8% with a p value of 0.0286. The treatment with the β-catenin-specific aiRNAs, however, resulted in a much more potent reduction in tumor growth. The % T/C value was calculated to be 9.9% with a p value of 0.0024. There was no significant change in body weight due to iv administration of the siRNA, aiRNA or control. These data suggest that the systemic in vivo application of aiRNAs through PEI complexation upon targeting of the β-catenin offers an avenue for the development of highly efficient, specific and safe agents for therapeutic applications for patients with colon cancer.

[0296] In addition, we also tested the effects of aiRNA and siRNA in HT29 human colon cancer xenograft model. HT29 human colon cancer cells were inoculated subcutaneously into female athymic nude mice (6×10^6 cells/mouse) and allowed to form palpable tumors. In this study, dosing began when the tumors reached approximately 200 mm^3. Animals were treated intravenously (iv) with 0.6 nmol PEI-complexed β-catenin siRNAs, PEI-complexed β-catenin aiRNAs or a PEI-complexed unrelated siRNA as a negative control every other day. The animals received a total of 8 doses of siRNA, aiRNA or control. Tumors were measured throughout treatment. As shown in FIG. **17**, intravenous treatment with siRNA and aiRNA as a monotherapy at 0.6 nmol mg/kg significantly inhibited tumor growth. The % T/C value of siRNA was calculated to be 78% with a p value of 0.21. Again, the treatment with the β-catenin-specific aiRNAs resulted in an even more potent reduction in tumor growth. The % T/C value was calculated to be 41% with a p value of 0.016. There was no significant change in body weight due to iv administration of the siRNA, aiRNA or control. These data second that the systemic in vivo application of aiRNAs through PEI complexation upon targeting of the β-catenin offers an avenue for the development of highly efficient, specific and safe agents for therapeutic applications for patients with colon cancer.

[0297] Together, the aiRNA may significantly improve broad RNAi applications. The siRNA-based therapeutics have met with challenges, including limited efficacy, delivery difficulty, interferon-like responses and manufacture cost (de Fougerolles et al., 2007; loins et al., 2007; Rana, 2007). The improved efficacy, potency, durability, and smaller size of aiRNAs may help or overcome these challenges since aiRNA is smaller and may need less material for its delivery. Therefore, aiRNA represents new and smallest RNA duplexes that enter RISC and mediates gene silencing of better efficacy, potency, onset of action, and durability than siRNA in mammalian cells, holding significant potential for broad RNAi applications in gene function study and RNAi-based therapies.

Example 6

aiRNAs Function as Mimetic miRNAs

[0298] Micro-RNAs (miRNA) are an additional regulator of gene expression with some similarity to siRNAs. However,

miRNA primarily regulates gene expression through a mechanism(s) distinct from siRNA-mediated target cleavage. Silencing by miRNAs occurs through interaction of the miRNA with the 3' untranslated region (UTR) of the target RNA that leads to translation inhibition and/or target RNA degradation. Unlike single target specificity of siRNA, a single miRNA can regulate expression of multiple targets.

[0299] The aiRNA structure was tested for its ability to function as a miRNA (amiRNA). Specifically in this example, an aiRNA (Let-7a aiRNA) was constructed to have a 21-nt longer strand and a 15-nt shorter strand forming a double stranded region of 15 nt flanked by a 3-nt 3' overhang and a 3-nt 5' overhang, both on the longer strand. The aiRNA was constrcuted as a mimetic of the Let-7a miRNA. As shown in FIG. **18**A, the entire aiRNA construct (boxed area) was derived directly from a portion of the stem-loop structure of the pre-miRNA of Let-7a (pre-Let-7a) and preserves the secondary structure, a bulge resulting from a 2-bp mismatch, in the endogenous pre-miRNA structure. The boxed area was selected such that the longer strand of the aiRNA contains most of the mature guide miRNA of Let-7a, the sequence of which is underlined in the pre-Let-7a structure.

[0300] The sequence structure of a positive control Let7a miRNA oligo, a double-stranded RNA molecule of symmetrical strand length (23 nt) and including the same portion of the Let-7a miRNA sequence (underlined) as Let-7a aiRNA is also shown in FIG. **18**A. Hela and Dld1 cells were transfected with the miRNA duplex or the aiRNA duplex at 100 nM for 24 hours at which time RNA was isolated and RT-PCR was performed to detect levels of k-Ras mRNA, a known silencing target of Let-7a miRNA. As shown in FIG. **18**B, both the aiRNA duplex (labeled as aiLet7) and the miRNA duplex (labeled as miLet7) led to a decrease in k-Ras mRNA in both cell lines. These data suggest that aiRNA can function as a mimetic in the miRNA pathway to alter gene expression.

[0301] We next determined whether a miRNA mimetic aiRNA could alter protein levels of known miRNA targets. The k-Ras gene can be regulated by members of the Let7 miRNA family. We therefore determined if k-Ras mRNA and/or protein was modulated following transfection with the miRNA mimetic aiRNA. The k-Ras mRNA levels were determined by RT-PCR and the protein levels of k-Ras were determined by western blot analysis (FIG. **19**). The k-Ras mRNA levels were down regulated by the Let7 mimetic aiRNA (FIG. **20**A), and the protein levels were also down regulated by the Let7 mimetic aiRNA (FIG. **20**B).

Example 7

aiRNAs Function as miRNA Inhibitors

[0302] To see if aiRNA structure could be used to create inhibitors of miRNAs, we designed aiRNAs against hsa-Let-7c, hsa-miR-21, and hsa-miR-155. Their sequences are shown in FIG. **19**A. The full length of mature hsa-Let-7c, hsa-miR-21, and hsa-miR-155 are shown in FIG. **19**B. RT-PCR and western blot analyses were performed to analyze the effect of the aiRNA-Let7c inhibitor on the expression of k-Ras, one of the targets of Let-7c. As shown in FIG. **20**A, k-Ras mRNA level was down regulated by aiRNA-Let7c mimic and was up regulated by aiRNA-Let7c inhibitor. Similar results were observed at the protein level as shown in FIG. **20**B.

[0303] Relative levels of Let-7c in Hela cells treated with aiRNA-Let7c inhibitor (FIG. **21**A), miR-21 in MCF-7 cells treated with aiRNA-miR21 inhibitor (FIG. **21**B), or miR-155 in FaDu cells treated with aiRNA-miR-155 inhibitor (FIG. **21**C) were also determined by qRT-PCR. As shown in FIG. **21**, these aiRNAs can potently inhibit their specific target—the endogenous corresponding miRNAs. Note that MCF7 cells contain relatively high levels of miR-21 (FIG. **22**A) and FaDu cells contain relatively high levels of miR-155 (FIG. **22**B). These cell lines were therefore used to test whether transfection of the aiRNA miRNA inhibitor could reduce the levels of miR-21 or miR-155 in MCF7 and FaDu cells, respectively.

[0304] We next compared the efficacy of aiRNA miRNA inhibitors to the commercially available miRNA inhibitors (Ambion). Transfection of 100 nM of either the aiRNA miRNA inhibitor or the Ambion inhibitor resulted in a decrease in the levels of Let-7c at 24 hours (FIG. **23**), miR-21 at 72 hours (FIG. **24**), and miR-155 at 72 hours (FIG. **25**) following transfection. In all cases, the aiRNA miRNA showed comparable or enhanced efficacy.

[0305] We next compared the potency of the aiRNA miRNA inhibitors to the commercially available inhibitors from Ambion. Cells were transfected with aiRNA or miRNA inhibitor targeting Let-7c (FIG. **26**), miR-21 (FIG. **27**), or miR-155 (FIG. **28**) at 1, 10, and 100 nM for 24 hours. Quantitative RT-PCR (qRT-PCR) analyses were then performed to determine levels of miRNA remaining, relative to non-targeting control aiRNA transfected cells. The aiRNA miRNA showed similar potency to the commercial inhibitor at the 10 and 100 nM doses. At the 1 nM dose, aiRNA showed enhanced potency, in the case of miR-21, similar potency, in the case of miR-155, or reduced potency, in the case of Let-7c.

[0306] Together, these data demonstrate that the aiRNA structure can function as both an inhibitor of endogenous miRNA that may show enhanced efficacy and/or duration of gene silencing compared to the antagomir, and as a mimic of miRNA function, causing the repression of endogenous miRNA target gene expression.

[0307] All references cited herein are incorporated herein by reference in their entirety and for all purposes to the same extent as if each individual publication or patent or patent application is specifically and individually indicated to be incorporated by reference in its entirety for all purposes. To the extent publications and patents or patent applications incorporated by reference contradict the disclosure contained in the specification, the specification is intended to supersede and/or take precedence over any such contradictory material.

[0308] All numbers expressing quantities of ingredients, reaction conditions, analytical results and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters set forth in the specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should be construed in light of the number of significant digits and ordinary rounding approaches.

[0309] Modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only and are not meant to be limiting in any way. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 43

uaaaggugaa gauau 15

<210> SEQ ID NO 44
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 44

aauauaucuu caccuuuagc u 21

<210> SEQ ID NO 45
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 45

ccguguuuga uuuggauuuu u 21

<210> SEQ ID NO 46
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

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<400> SEQUENCE: 46

aaauccaaau caaacacggu u 21

<210> SEQ ID NO 47
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 47

uguuugauuu ggauu 15

<210> SEQ ID NO 48
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 48

aaaaauccaa aucaaacacg g 21

<210> SEQ ID NO 49
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 49

gcagaauugu caagggauau u 21

<210> SEQ ID NO 50
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 50

uaucccuuga caauucugcu u 21

<210> SEQ ID NO 51
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 51

gaauugucaa gggau 15

<210> SEQ ID NO 52
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 52

aauaucccuu gacaauucug c 21

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<210> SEQ ID NO 53
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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 53

ggaaauugga acacaguuuu u 21

<210> SEQ ID NO 54
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 54

aaacuguguu ccaauuuccu u 21

<210> SEQ ID NO 55
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 55

aauuggaaca caguu 15

<210> SEQ ID NO 56
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 56

aaaaacugug uuccaauuuc c 21

<210> SEQ ID NO 57
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<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 57

uggagaugcu guuguaauuu u 21

<210> SEQ ID NO 58
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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 58

aauuacaaca gcaucuccau u 21

<210> SEQ ID NO 59
<211> LENGTH: 15
<212> TYPE: RNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 59

agaugcuguu guaau 15

<210> SEQ ID NO 60
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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 60

aaaauuacaa cagcaucucc a 21

<210> SEQ ID NO 61
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 61

guggcgaaga agaaaucuau u 21

<210> SEQ ID NO 62
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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 62

uagauuucuu cuucgccacu u 21

<210> SEQ ID NO 63
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 63

gcgaagaaga aaucu 15

<210> SEQ ID NO 64
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 64

aauagauuuc uucuucgcca c 21

<210> SEQ ID NO 65
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA
<220> FEATURE:

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<221> NAME/KEY: misc_feature
<222> LOCATION: (20)..(21)
<223> OTHER INFORMATION: n = deoxythymidine

<400> SEQUENCE: 65

aaggagauca acauuuucan n 21

<210> SEQ ID NO 66
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (20)..(21)
<223> OTHER INFORMATION: n = deoxythymidine

<400> SEQUENCE: 66

ugaaaauguu gaucuccuun n 21

<210> SEQ ID NO 67
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 67

aggagaucaa cauuu 15

<210> SEQ ID NO 68
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (20)..(21)
<223> OTHER INFORMATION: n = deoxythymidine

<400> SEQUENCE: 68

ugaaaauguu gaucuccuun n 21

<210> SEQ ID NO 69
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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (20)..(21)
<223> OTHER INFORMATION: n = deoxythymidine

<400> SEQUENCE: 69

gccgcagacc uugugauaun n 21

<210> SEQ ID NO 70
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA
<220> FEATURE:

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<221> NAME/KEY: misc_feature
<222> LOCATION: (20)..(21)
<223> OTHER INFORMATION: n = deoxythymidine

<400> SEQUENCE: 70

auaucacaag gucugcggcn n 21

<210> SEQ ID NO 71
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 71

gcagaccuug ugaua 15

<210> SEQ ID NO 72
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 72

aaauaucaca aggucugcgg c 21

<210> SEQ ID NO 73
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 73

aggcccgcuc uacaucuucu u 21

<210> SEQ ID NO 74
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 74

gaagauguag agcgggccuu u 21

<210> SEQ ID NO 75
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 75

cccgcucuac aucuu 15

<210> SEQ ID NO 76
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

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<400> SEQUENCE: 76

aagaagaugu agagcgggcc u 21

<210> SEQ ID NO 77
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 77

ggagcuguug gcgua 15

<210> SEQ ID NO 78
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 78

ggagcuguug gcgua 15

<210> SEQ ID NO 79
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (20)..(21)
<223> OTHER INFORMATION: n = deoxythymidine

<400> SEQUENCE: 79

guagcugaua uugauggacn n 21

<210> SEQ ID NO 80
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (20)..(21)
<223> OTHER INFORMATION: n = deoxythymidine

<400> SEQUENCE: 80

guccaucaau aucagcuacn n 21

<210> SEQ ID NO 81
<211> LENGTH: 12
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 81

ugauauugau gg 12

<210> SEQ ID NO 82
<211> LENGTH: 21
<212> TYPE: RNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 82

aaguccauca auaucagcua c 21

<210> SEQ ID NO 83
<211> LENGTH: 12
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 83

cugauauuga ug 12

<210> SEQ ID NO 84
<211> LENGTH: 13
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 84

cugauauuga ugg 13

<210> SEQ ID NO 85
<211> LENGTH: 14
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 85

gcugauauug augg 14

<210> SEQ ID NO 86
<211> LENGTH: 14
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 86

cugauauuga ugga 14

<210> SEQ ID NO 87
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 87

gcugauauug augga 15

<210> SEQ ID NO 88
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

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<400> SEQUENCE: 88

gauauugaug gacuu 15

<210> SEQ ID NO 89
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 89

guagcugaua uugau 15

<210> SEQ ID NO 90
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 90

cugauauuga uggac 15

<210> SEQ ID NO 91
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 91

agcugauauu gaugg 15

<210> SEQ ID NO 92
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 92

uagcugauau ugaug 15

<210> SEQ ID NO 93
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 93

ugauauugau ggacu 15

<210> SEQ ID NO 94
<211> LENGTH: 22
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 94

aaguccauca auaucagcua cu 22

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<210> SEQ ID NO 95
<211> LENGTH: 22
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 95

aaaguccauc aauaucagcu ac 22

<210> SEQ ID NO 96
<211> LENGTH: 24
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 96

aauguccauc aauaucagcu acuu 24

<210> SEQ ID NO 97
<211> LENGTH: 24
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 97

aacuguccau caauaucagc uacu 24

<210> SEQ ID NO 98
<211> LENGTH: 27
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 98

auacugucca ucaauaucag cuacuug 27

<210> SEQ ID NO 99
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 99

aguccaucaa uaucagcuac 20

<210> SEQ ID NO 100
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 100

guccaucaau aucagcuacu 20

<210> SEQ ID NO 101
<211> LENGTH: 20
<212> TYPE: RNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 101

aaguccauca auaucagcua 20

<210> SEQ ID NO 102
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 102

uccaucaaua ucagcuacu 19

<210> SEQ ID NO 103
<211> LENGTH: 18
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 103

guccaucaau aucagcua 18

<210> SEQ ID NO 104
<211> LENGTH: 18
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 104

uccaucaaua ucagcuac 18

<210> SEQ ID NO 105
<211> LENGTH: 18
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 105

aaguccauca auaucagc 18

<210> SEQ ID NO 106
<211> LENGTH: 17
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 106

uccaucaaua ucagcua 17

<210> SEQ ID NO 107
<211> LENGTH: 17
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

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<400> SEQUENCE: 107

aguccaucaa uaucagc 17

<210> SEQ ID NO 108
<211> LENGTH: 14
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 108

gcugauauug augg 14

<210> SEQ ID NO 109
<211> LENGTH: 16
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 109

gcugauauug auggac 16

<210> SEQ ID NO 110
<211> LENGTH: 17
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 110

agcugauauu gauggac 17

<210> SEQ ID NO 111
<211> LENGTH: 18
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 111

agcugauauu gauggacu 18

<210> SEQ ID NO 112
<211> LENGTH: 18
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 112

uagcugauau ugauggac 18

<210> SEQ ID NO 113
<211> LENGTH: 18
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 113

gcugauauug auggacuu 18

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<210> SEQ ID NO 114
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 114

agcugauauu gauggacuu 19

<210> SEQ ID NO 115
<211> LENGTH: 18
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 115

guagcugaua uugaugga 18

<210> SEQ ID NO 116
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 116

aaguccauca auaucugcua c 21

<210> SEQ ID NO 117
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 117

uagcugauau ugauggacu 19

<210> SEQ ID NO 118
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 118

uagcugauau ugauggacuu 20

<210> SEQ ID NO 119
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 119

guagcugaua uugauggacu 20

<210> SEQ ID NO 120
<211> LENGTH: 15
<212> TYPE: RNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 120

gcugauauug aagga 15

<210> SEQ ID NO 121
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 121

cuguccauca auaucagcua c 21

<210> SEQ ID NO 122
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(2)
<223> OTHER INFORMATION: n = deoxyadenosine
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (3)..(3)
<223> OTHER INFORMATION: n = deoxyguanosine

<400> SEQUENCE: 122

nnnuccauca auaucagcua c 21

<210> SEQ ID NO 123
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (19)..(19)
<223> OTHER INFORMATION: n = deoxythymidine
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: n = deoxyadenosine
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (21)..(21)
<223> OTHER INFORMATION: n = deoxycytosine

<400> SEQUENCE: 123

aaguccauca auaucagcnn n 21

<210> SEQ ID NO 124
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(2)
<223> OTHER INFORMATION: n = deoxyadenosine
<220> FEATURE:

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<221> NAME/KEY: misc_feature
<222> LOCATION: (3)..(3)
<223> OTHER INFORMATION: n = deoxyguanosine
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (19)..(19)
<223> OTHER INFORMATION: n = deoxythymidine
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: n = deoxyadenosine
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (21)..(21)
<223> OTHER INFORMATION: n = deoxycytosine

<400> SEQUENCE: 124

nnnuccauca auaucagcnn n 21

<210> SEQ ID NO 125
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: n = deoxyguanosine
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (3)..(3)
<223> OTHER INFORMATION: n = deoxythymidine
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (11)..(11)
<223> OTHER INFORMATION: n = deoxyadenosine
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (13)..(13)
<223> OTHER INFORMATION: n = deoxyguanosine
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (15)..(15)
<223> OTHER INFORMATION: n = deoxyadenosine

<400> SEQUENCE: 125

ncngauauug nungn 15

<210> SEQ ID NO 126
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<212> TYPE: RNA
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<220> FEATURE:
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<222> LOCATION: (1)..(1)
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<400> SEQUENCE: 126

ncugauauug augga 15

<210> SEQ ID NO 127
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<221> NAME/KEY: misc_feature
<222> LOCATION: (15)..(15)
<223> OTHER INFORMATION: n = deoxyadenosine

<400> SEQUENCE: 127

gcugauauug auggn 15

<210> SEQ ID NO 128
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 128

guccaucaau aucagcuacu u 21

<210> SEQ ID NO 129
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
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<220> FEATURE:
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<222> LOCATION: (1)..(15)
<223> OTHER INFORMATION: n = A, U, C, G

<400> SEQUENCE: 129

nnnnnnnnnn nnnnn 15

<210> SEQ ID NO 130
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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Strand of duplex RNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(3)
<223> OTHER INFORMATION: n = dA,dT,dG,dC
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (4)..(21)
<223> OTHER INFORMATION: n = A,U,G,C

<400> SEQUENCE: 130

nnnnnnnnnn nnnnnnnnnn n 21

<210> SEQ ID NO 131
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(18)
<223> OTHER INFORMATION: n=A,U,G.C
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (19)..(21)
<223> OTHER INFORMATION: n=dA,dT,dG.dC

<400> SEQUENCE: 131

nnnnnnnnnn nnnnnnnnnn n 21

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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA
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<222> LOCATION: (1)..(3)
<223> OTHER INFORMATION: n=dA,dT,dG,dC
<220> FEATURE:
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<222> LOCATION: (4)..(18)
<223> OTHER INFORMATION: n is a, c, g, or u
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (19)..(21)
<223> OTHER INFORMATION: n=dA,dT,dG,dC

<400> SEQUENCE: 132

dddnnnnnnn nnnnnnnndd d 21

<210> SEQ ID NO 133
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<212> TYPE: RNA
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<222> LOCATION: (1)..(1)
<223> OTHER INFORMATION: n=dA,dT,dG,dC
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<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: n is a, c, g, or u
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (3)..(3)
<223> OTHER INFORMATION: n=dA,dT,dG,dC
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (4)..(10)
<223> OTHER INFORMATION: n is a, c, g, or u
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (11)..(11)
<223> OTHER INFORMATION: n=dA,dT,dG,dC
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (12)..(12)
<223> OTHER INFORMATION: n is a, c, g, or u
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (13)..(13)
<223> OTHER INFORMATION: n=dA,dT,dG,dC
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (14)..(14)
<223> OTHER INFORMATION: n is a, c, g, or u
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (15)..(15)
<223> OTHER INFORMATION: n=dA,dT,dG,dC

<400> SEQUENCE: 133

nnnnnnnnnn nnnnn 15

<210> SEQ ID NO 134
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<400> SEQUENCE: 134

nnnnnnnnnn nnnnnnnnnn n 21

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<223> OTHER INFORMATION: Strand of duplex RNA
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<223> OTHER INFORMATION: n=dA,dT,dG,dC
<220> FEATURE:
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<223> OTHER INFORMATION: n=A,U,G,C

<400> SEQUENCE: 135

nnnnnnnnnn nnnnn 15

<210> SEQ ID NO 136
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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<223> OTHER INFORMATION: n=dA,dT,dG,dC

<400> SEQUENCE: 136

nnnnnnnnnn nnnnn 15

<210> SEQ ID NO 137
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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
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<222> LOCATION: (1)..(19)
<223> OTHER INFORMATION: n=A,U,G,C

<400> SEQUENCE: 137

nnnnnnnnnn nnnnnnnnn 19

<210> SEQ ID NO 138
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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 138

ugagguagua gguuguauag uu 22

<210> SEQ ID NO 139
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<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

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ugagguagua gguuguaugg uu 22

<210> SEQ ID NO 140
<211> LENGTH: 22
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 140

uagcuuauca gacugauguu ga 22

<210> SEQ ID NO 141
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<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 141

uuaaugcuaa ucgugauagg ggu 23

<210> SEQ ID NO 142
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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 142

acuauacaau cuacugucuu ucc 23

<210> SEQ ID NO 143
<211> LENGTH: 23
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 143

ugagguagua gguuguauag uuu 23

<210> SEQ ID NO 144
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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 144

auacaaucua cuguc 15

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<210> SEQ ID NO 145
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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 145

ugagguagua gguuguauag u 21

<210> SEQ ID NO 146
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 146

acaaccuacu accuc 15

<210> SEQ ID NO 147
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 147

aaugagguag uagguuguau g 21

<210> SEQ ID NO 148
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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 148

gguaguaggu uguau 15

<210> SEQ ID NO 149
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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 149

aacauacaac cuacuaccuc a 21

<210> SEQ ID NO 150
<211> LENGTH: 15
<212> TYPE: RNA
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<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 150

aucagacuga uguug 15

<210> SEQ ID NO 151
<211> LENGTH: 21
<212> TYPE: RNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 151

aaucaacauc agucugauaa g 21

<210> SEQ ID NO 152
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 152

augcuaaucg ugaua 15

<210> SEQ ID NO 153
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 153

aacuaucacg auuagcauua a 21

<210> SEQ ID NO 154
<211> LENGTH: 79
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Strand of duplex RNA

<400> SEQUENCE: 154

ugggaugagg uaguagguug uauaguuuua gggucacacc accacuggga gauaacuaua 60

caaucuacug ucuuuccua 79

1. A mimetic of a microRNA (miRNA), comprising:

a double stranded RNA molecule comprising a first strand of a first length and a second strand of a second and shorter length, said first strand having a sequence substantially the same as at least a portion of said miRNA, said first and second strands being substantially complementary to each other such that they form at least one double stranded region, wherein said RNA molecule further comprises a terminal overhang of 1-10 nucleotides; and

wherein said mimetic is adapted to mimic said miRNA in modulating expression of at least one gene.

2. The mimetic of claim **1** wherein said miRNA is a guide strand.

3. The mimetic of claim **1** wherein said miRNA is a mature miRNA.

4. The mimetic of claim **1** wherein said miRNA is an endogenous miRNA duplex comprising a mature miRNA and a substantially complementary passenger strand, and wherein said second strand of said mimetic has a sequence substantially the same as at least a portion of said passenger strand.

5. The mimetic of claim **1** further comprising at least one mismatched or unmatched nucleotide in sequence between said first and second strands.

6-13. (canceled)

14. The mimetic of claim **1** further comprising both a 3' overhang and a 5' overhang on said first strand.

15. The mimetic of claim **14** wherein both said 3' and 5' overhangs are of 1-3 nucleotides.

16-17. (canceled)

18. The mimetic of claim **1** wherein said first strand has a length of 15-30 nucleotides, and said second strand has a length of 12-29 nucleotides.

19. The mimetic of claim **1** wherein said first strand has a length of 15-28 nucleotides and said second strand has a length from 12-26 nucleotides.

20. The mimetic of claim **1** wherein said first strand has a length of 19-25 nucleotides and said second strand has a length of 12-24 nucleotides.

21. The mimetic of claim **1** wherein said first strand has a length of 19-23 nucleotides and said second strand has a length of 14-20 nucleotides.

22. (canceled)

23. The mimetic of claim **1** wherein said terminal overhang is stabilized against degradation.

24. The mimetic of claim **1** further comprising a nick in at least one of said first and second strands.

25. The mimetic of claim **1** wherein the double-stranded region comprises a gap of one or more unpaired nucleotides.

26. The mimetic of claim **1** further comprising a modified nucleotide or a nucleotide analogue.

27. The mimetic of claim **26** wherein said modified nucleotide or analogue is a sugar-, backbone-, and/or base-modified ribonucleotide.

28-30. (canceled)

31. The mimetic of claim **27**, wherein the at least one modified nucleotide or analogue is inosine, or a tritylated base.

32. The mimetic of claim **26**, wherein the nucleotide analogue is a sugar-modified ribonucleotide in which the 2'-OH group is replaced by a group selected from the group consisting of H, OR, R, halo, SH, SR, NH_2, NHR, NR_2, and CN, wherein each R is independently selected from the group consisting of C1-C6 alkyl, alkenyl and alkynyl, and halo is selected from the group consisting of F, Cl, Br and I.

33. The mimetic of claim **26**, wherein the nucleotide analogue is a backbone-modified ribonucleotide containing a phosphothioate group.

34. The mimetic of claim **1** further comprising a deoxynucleotide.

35-36. (canceled)

37. The mimetic of claim **1**, wherein said first strand shares the same seed region as said miRNA.

38. The mimetic of claim **1** wherein the GC content of the double stranded region is about 20-60%.

39. The mimetic of claim **1** wherein said first strand comprises a 5' overhang with at least one nucleotide selected from the group consisting of A, U, and dT.

40. The mimetic of claim **1**, further being conjugated to an entity selected from the group consisting of peptide, antibody, polymer, lipid, oligonucleotide, cholesterol and aptamer.

41. The mimetic of claim **1** wherein said double stranded RNA molecule is synthetic or isolated.

42. The mimetic of claim **1** wherein said double stranded RNA molecule is transcribed from a recombinant vector or its progeny.

43. The mimetic of claim **1** adapted to modulate at least 20% the expression of said at least one gene.

44. The mimetic of claim **1** where said miRNA is of the Let7 family.

45. The mimetic of claim **1** comprising one of the following duplex sequence:

```
Sense: 5'-AUACAAUCUACUGUC
Antisense: 5'-UGAGGUAGUAGGUUGUAUAGU,
and

Sense: 5'-ACAACCUACUACCUC
Antisense: 5'-AAUGAGGUAGUAGGUUGUAUG.
```

46. A mimetic of a mature microRNA (miRNA) comprising:

a double stranded RNA molecule comprising a first strand of 15-28 nucleotides and a second shorter strand of 12-26 nucleotides, said first strand having a sequence substantially the same as at least a portion of said mature miRNA, said first and second strands being substantially complementary to each other such that they form at least one double stranded region, wherein said first strand further comprises both a 3' overhang of 1-8 nucleotides and a 5' overhang of 1-8 nucleotides,

wherein said mimetic is adapted to mimic said mature miRNA in modulating expression of at least one gene.

47. The mimetic of claim **46** wherein said second strand has a sequence substantially the same as at least a portion of a passenger RNA strand that forms an endogenous duplex with the mature miRNA.

48. The mimetic of claim **46** further comprising at least one mismatched or unmatched nucleotide in sequence between said first and second strands.

49. The mimetic of claim **48** wherein a loop is formed by said at least one mismatched or unmatched nucleotide.

50. The mimetic of claim **46** further comprising a deoxynucleotide.

51. The mimetic of claim **46** wherein at least one of said 3' and 5' overhang is stabilized against degradation.

52. The mimetic of claim **46** further comprising a nick in at least one of said first and second strands.

53. The mimetic of claim **46** wherein the double-stranded region comprises a gap of one or more unpaired nucleotides.

54. The mimetic of claim **46** further comprising a modified nucleotide or a nucleotide analogue.

55-146. (canceled)

* * * * *