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Inoue et al.

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(54) **CONSTRUCTION OF PROTEIN-RESPONSIVE SHRNA/RNAI CONTROL SYSTEM USING RNP MOTIF**

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(73) Assignee: **Japan Science and Technology Agency**, Saitama (JP)

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(2), (4) Date: **Jul. 13, 2011**

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PCT Pub. Date: **Jun. 17, 2010**

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(30) **Foreign Application Priority Data**

Dec. 9, 2008 (JP) 2008-312951

(51) **Int. Cl.**

C12Q 1/68	(2006.01)
C12P 19/34	(2006.01)
C12N 15/63	(2006.01)
C07H 21/02	(2006.01)
C07H 21/04	(2006.01)

(52) **U.S. Cl.**

USPC **435/6**; 435/91.1; 435/91.31; 435/320.1; 435/375; 435/455; 530/350; 536/23.1; 536/24.5

(58) **Field of Classification Search**

USPC 435/6, 91.1, 91.31, 320.1, 455, 3, 75, 435/375; 514/44; 536/23.1, 24.5; 530/350

See application file for complete search history.

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Turner et al, RNA, vol. 11, pp. 1192-1200 (2005).*
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Osamu Nagae et al. , "Jinko RNAi/RNP o Mochiita Hito Gan Saibo deno Hon'yaku Seigyo System no Kochiku", Dai 80 Kai The Japanese Biochemical Society Taikai Dai 30 Kai Annual Meeting of the Molecular Biology Society of Japan Godo Taikai Koen Yoshishu, 2007, 4P-1321.
An, C. -I. et al., Artificial control of gene expression in mammalian cells by modulating RNA interference through aptamer-small molecule interaction, RNA, 2006, vol. 12, No. 5, pp. 710-716.
Hirohisa Ono et al., "RNA—Tanpakushitsu Sogo Sayo Motif L7Ae-BoxC/D o Mochiita Nanoscale Kozotai no Sekkei Oyobi Kochiku", RNA Meeting, Jul. 23, 2008, vol. 10th, p. 144.
Shun'ichi Kashida et al., "RNP Sogo Sayo o Mochiita Tanpakushitsu Otogata shRNA System no Kochiku to Apoptosis Keiro no Seigyo", Dai 82 Kai The Japanese Biochemical Society Taikai Program•Koen Yoshishu, Sep. 25, 2009, 4T15a-10.
Turner, B. et al., Induced fit of RNA on binding the L7Ae protein to the kink-turn motif, RNA, 2005, vol. 11, No. 8, p. 1192-1200.
International Search Report issued Mar. 9, 2010 in corresponding International Application No. PCT/JP2009/070580, 2 pages.

* cited by examiner

Primary Examiner — Jane Zara

(74) *Attorney, Agent, or Firm* — Meunier Carlin & Curfman, LLC

(57) **ABSTRACT**

An object of the present invention is to provide an RNAi control system using an RNA-protein interaction motif. The present invention provides an shRNA comprising: a guide strand having a sequence complementary to a target sequence; a passenger strand which forms a duplex with the guide strand; and a linker strand which links the guide strand and the passenger strand, wherein the linker strand comprises an RNP-derived protein-binding motif sequence. The present invention also provides an RNAi control system comprising: the shRNA; and an RNP-derived protein which specifically binds to a protein-binding motif sequence in the shRNA.

9 Claims, 17 Drawing Sheets

FIG.1

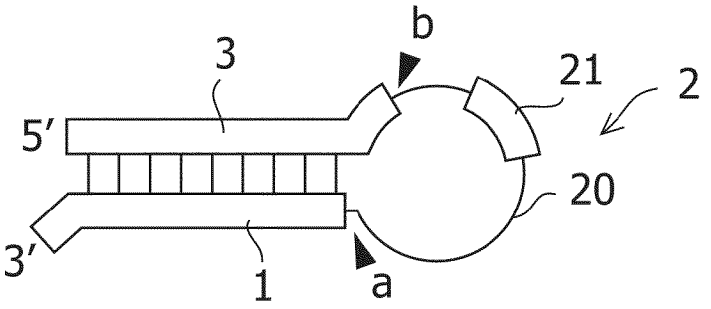


FIG.2(A)

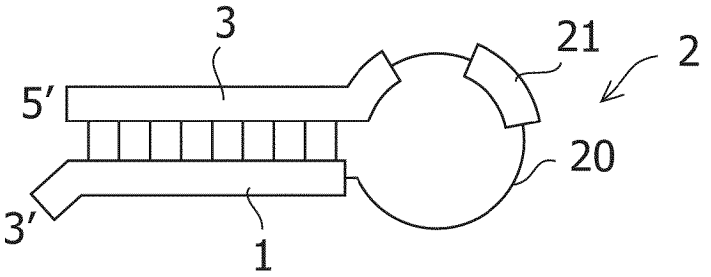


FIG.2(B)

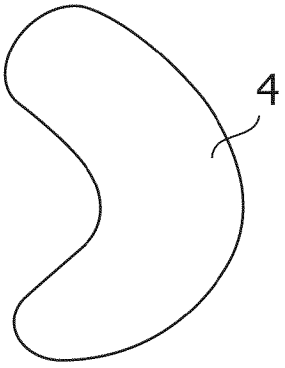


FIG.2(C)

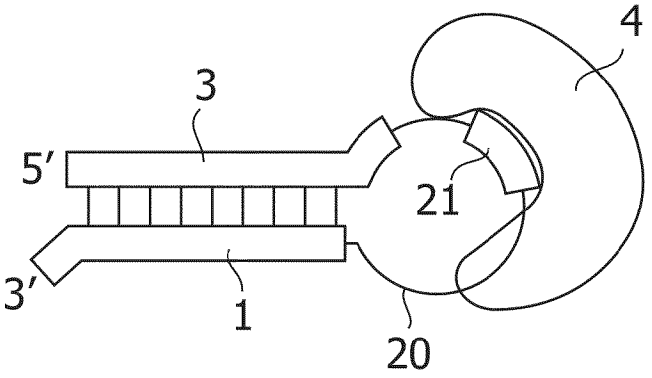
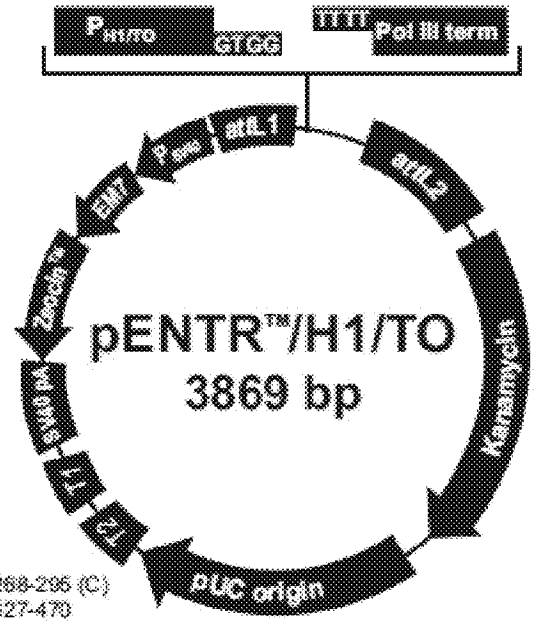


FIG.3(A)



Comments for pENTR™/H1/TO
3869 nucleotides

rrnB T2 transcription terminator: bases 268-295 (C)
rrnB T1 transcription terminator: bases 427-470
SV40 polyadenylation signal: bases 513-842 (C)
Zeocin™ resistance gene: bases 772-1146 (C)
EM7 promoter: bases 1147-1213 (C)
SV40 early promoter and origin: bases 1268-1576 (C)
M13 forward (-20) priming site: bases 1648-1663
attL1: bases 1680-1779 (C)
H1/TO promoter: bases 1836-1935
$tetO_2$ site: bases 1885-1903
TATA box: bases 1906-1910
$tetO_2$ site: bases 1913-1931
H1 forward priming site: bases 1856-1875
5' overhang: bases 1932-1935 (C)
5' overhang: bases 1936-1939
Pol III transcription terminator: bases 1936-1941
attL2: bases 1994-2093
M13 reverse priming site: bases 2134-2150
Kanamycin resistance gene: bases 2283-3072
pUC origin: bases 3193-3866

(C) = complementary strand

FIG.3(B)

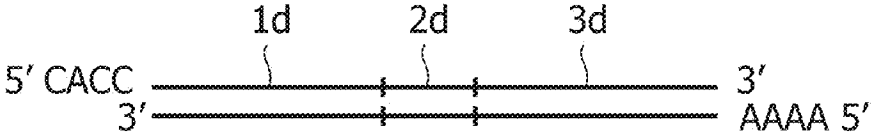


FIG.4

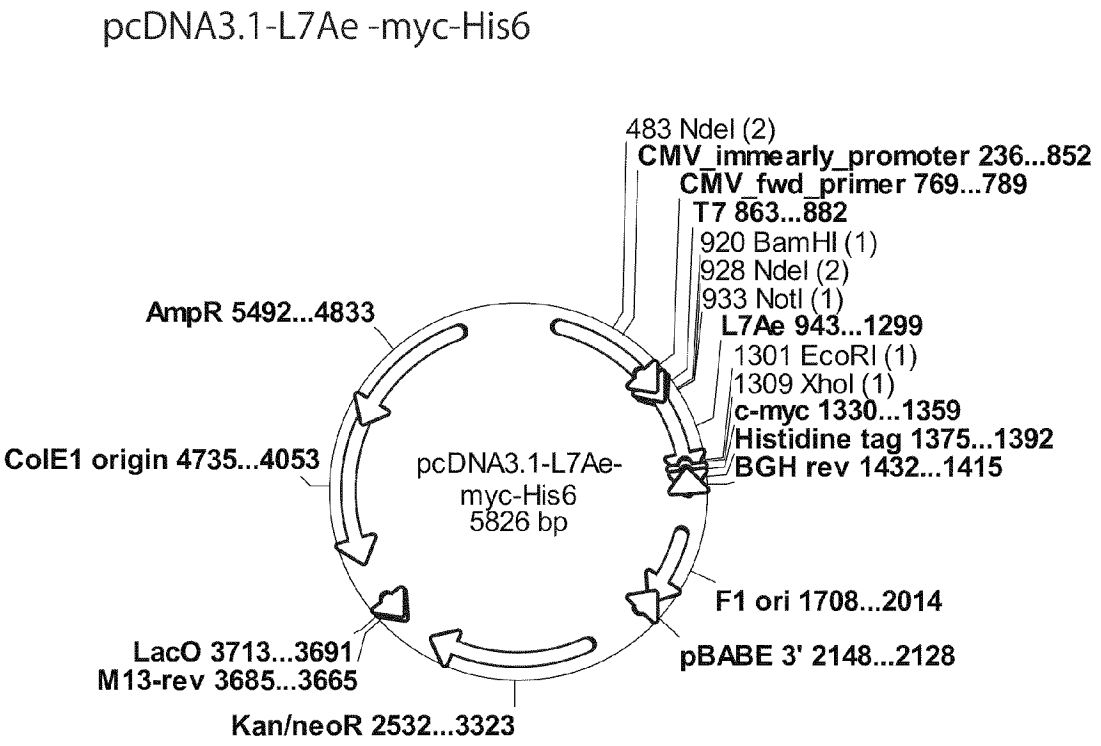

pcDNA3.1-L7Ae -myc-His6
483 NdeI (2)
CMV_immearly_promoter 236...852
CMV_fwd_primer 769...789
T7 863...882
920 BamHI (1)
928 NdeI (2)
933 NotI (1)
L7Ae 943...1299
1301 EcoRI (1)
1309 XhoI (1)
c-myc 1330...1359
Histidine tag 1375...1392
BGH rev 1432...1415
F1 ori 1708...2014
pBABE 3' 2148...2128
Kan/neoR 2532...3323
LacO 3713...3691
M13-rev 3685...3665
ColE1 origin 4735...4053
AmpR 5492...4833
pcDNA3.1-L7Ae-
myc-His6
5826 bp

FIG.5(A)

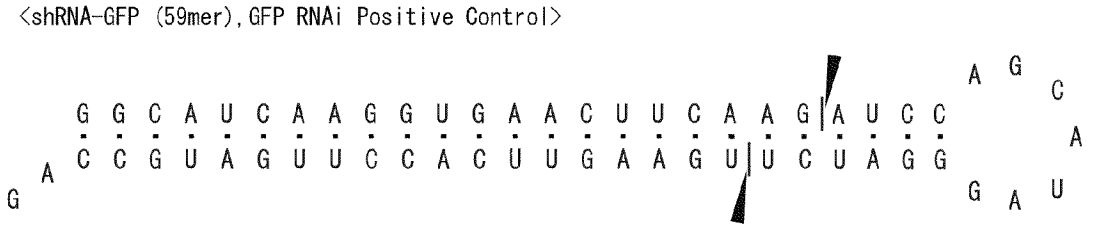


FIG.5(B)

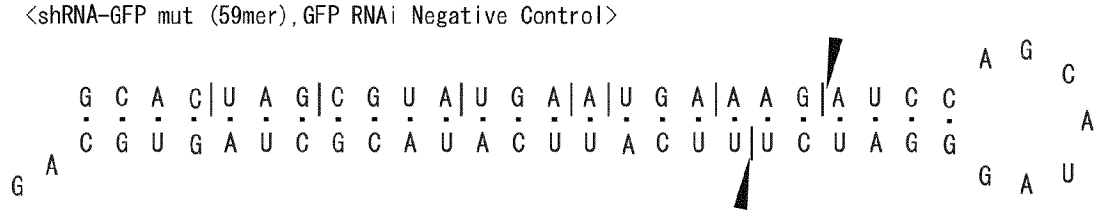


FIG.5(C)

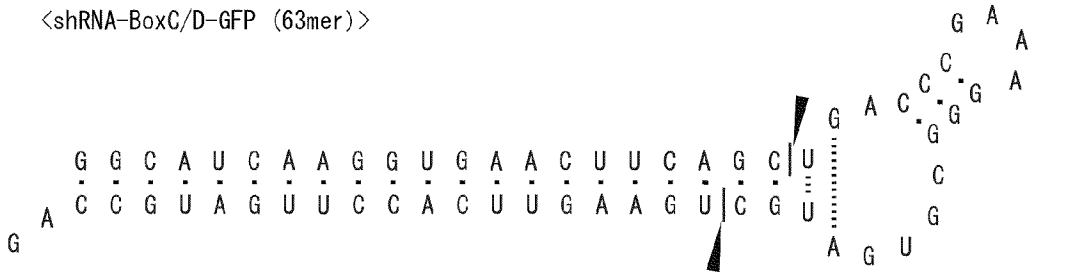


FIG.5(D)

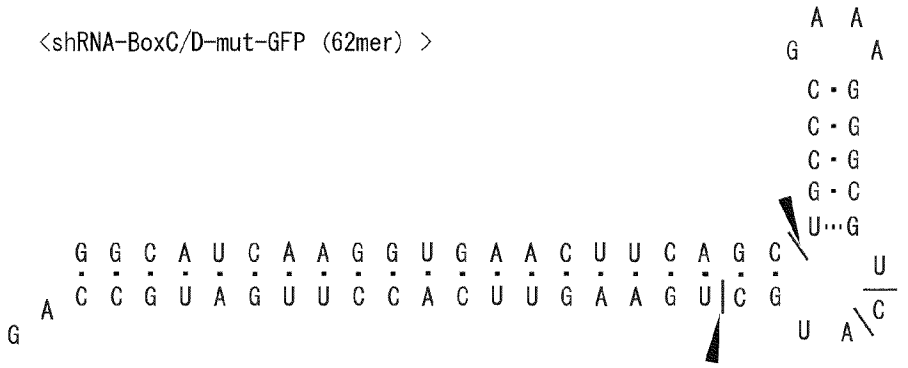


FIG.6

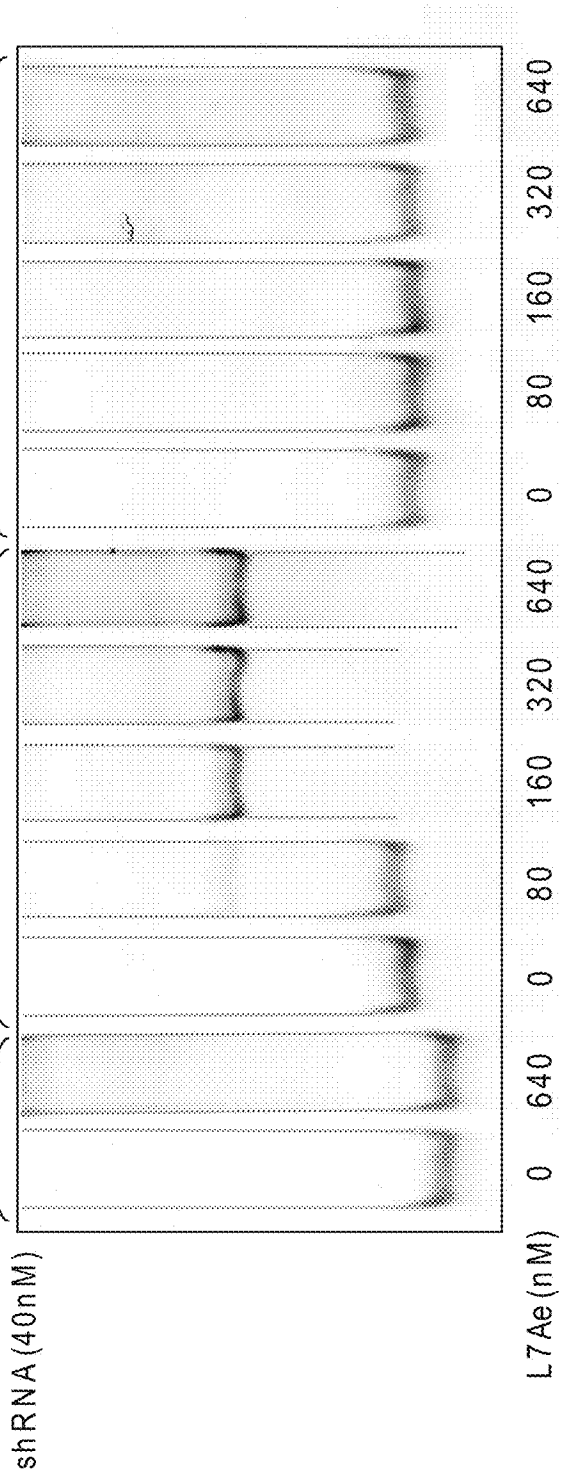

shRNA-GFP
shRNA-BoxC/D-GFP
shRNA-BoxC/D mut-GFP
shRNA (40nM)
L7Ae (nM)
0
640
0
80
160
320
640
0
80
160
320
640

FIG.7

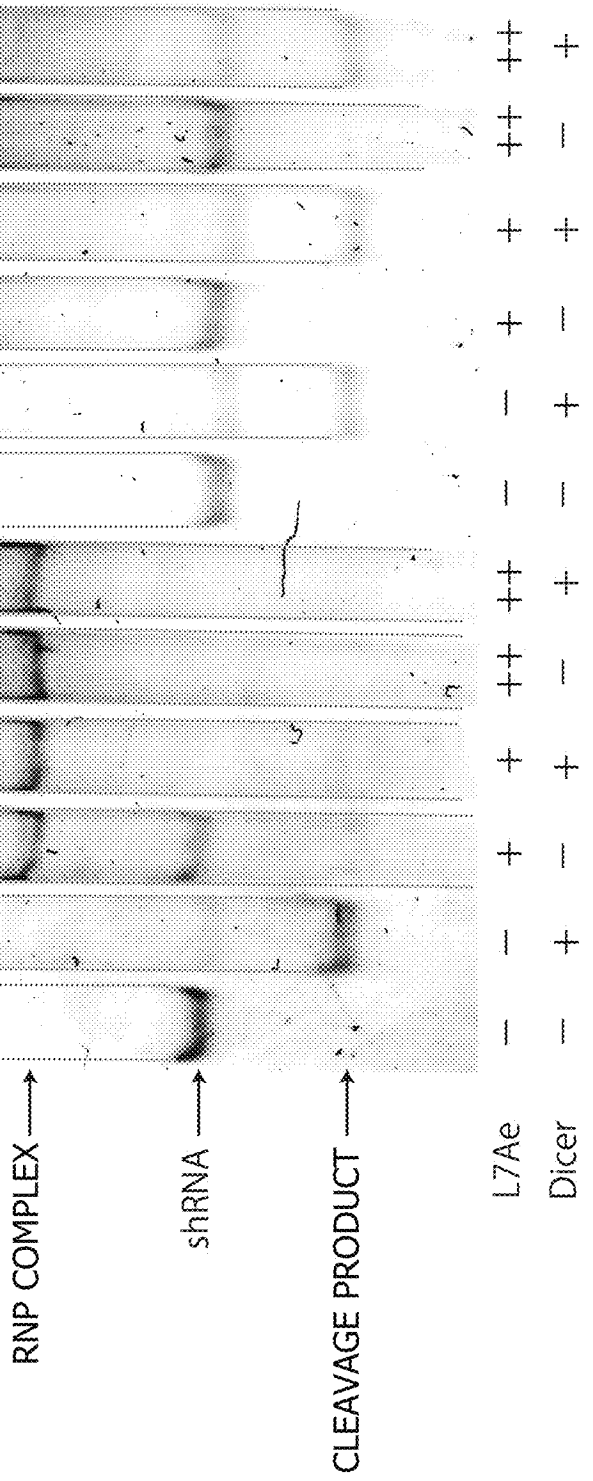

shRNA-BoxC/D-GFP
shRNA-BoxC/D-GFP-mut
RNP COMPLEX
shRNA
CLEAVAGE PRODUCT
L7Ae
Dicer
- - + + - - + + - - + +
- + - + - + - + - + - +

FIG.8

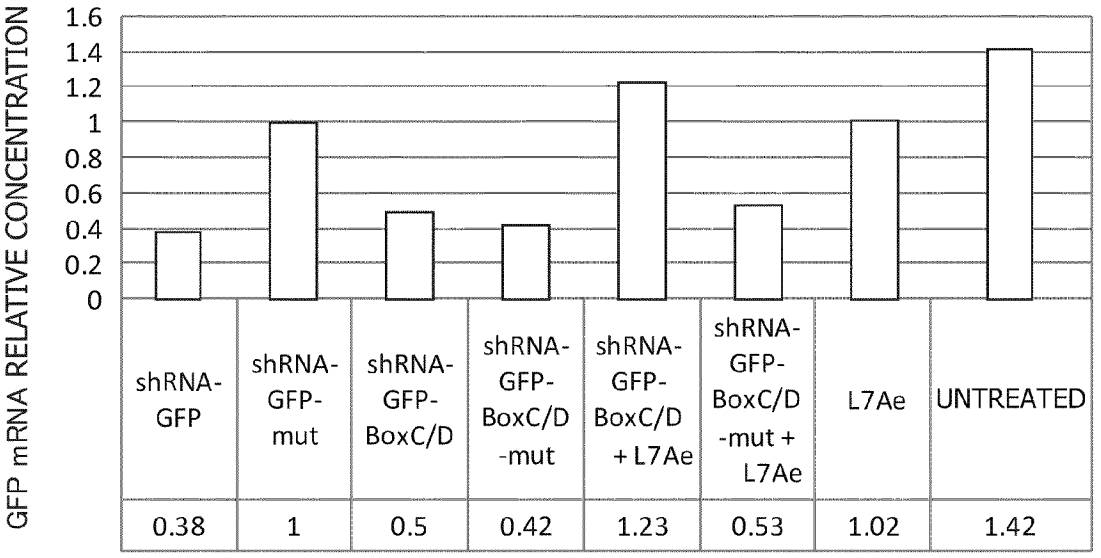

GFP mRNA RELATIVE CONCENTRATION
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
shRNA-GFP
shRNA-GFP-mut
shRNA-GFP-BoxC/D
shRNA-GFP-BoxC/D-mut
shRNA-GFP-BoxC/D+L7Ae
shRNA-GFP-BoxC/D-mut+L7Ae
L7Ae
UNTREATED
0.38
1
0.5
0.42
1.23
0.53
1.02
1.42

FIG.9

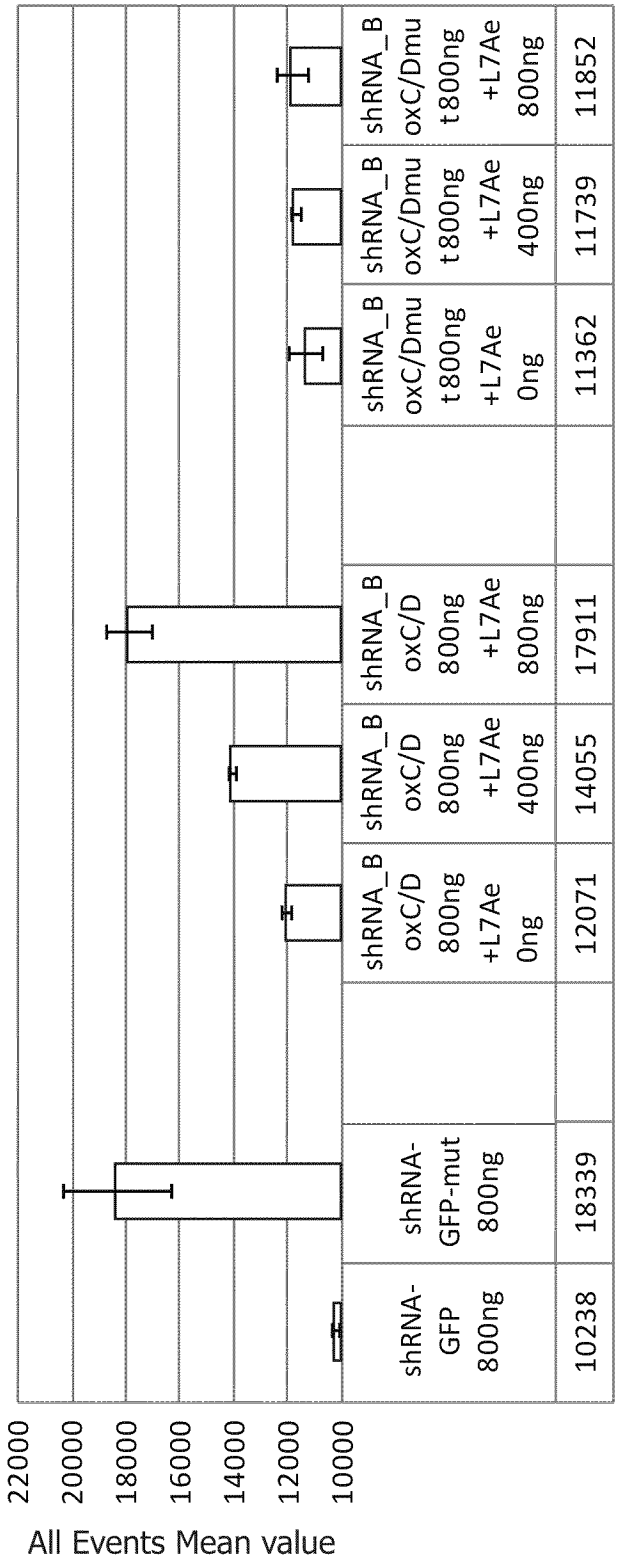

22000
20000
18000
16000
14000
12000
10000
All Events Mean value
shRNA-GFP 800ng
shRNA-GFP-mut 800ng
shRNA_B oxC/D 800ng +L7Ae 0ng
shRNA_B oxC/D 800ng +L7Ae 400ng
shRNA_B oxC/D 800ng +L7Ae 800ng
shRNA_B oxC/Dmu t800ng +L7Ae 0ng
shRNA_B oxC/Dmu t800ng +L7Ae 400ng
shRNA_B oxC/Dmu t800ng +L7Ae 800ng
10238
18339
12071
14055
17911
11362
11739
11852

FIG.10

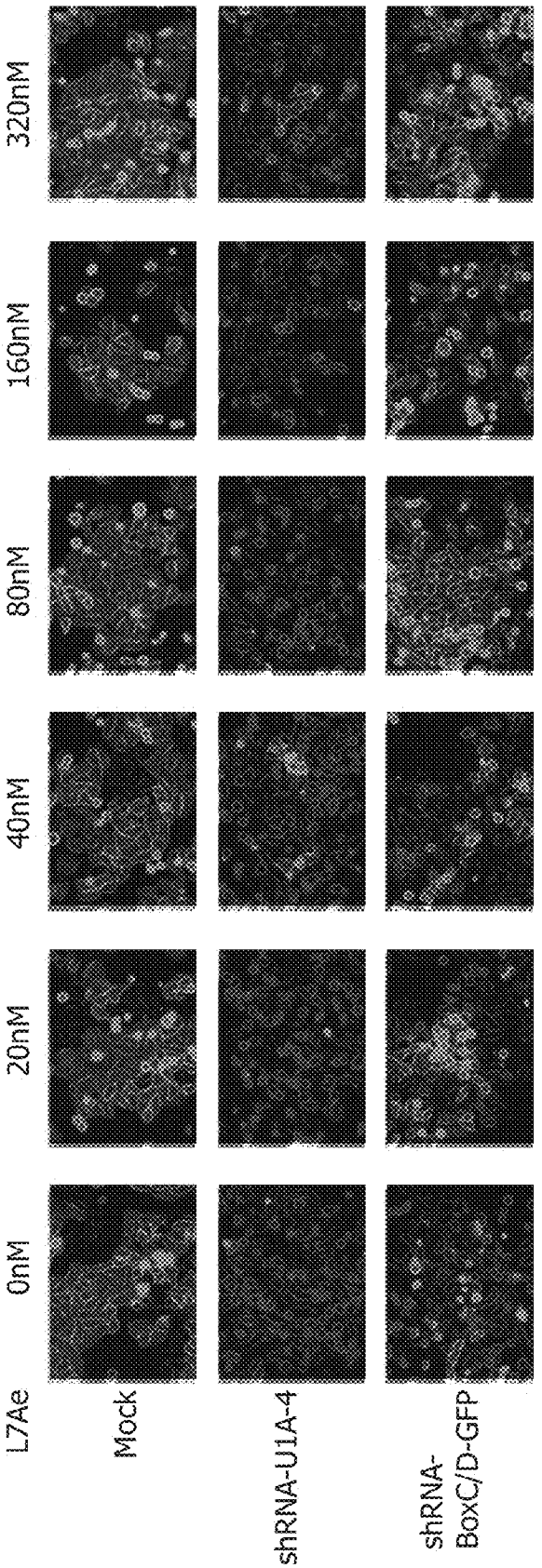

L7Ae
0nM
20nM
40nM
80nM
160nM
320nM
Mock
shRNA-U1A-4
shRNA-BoxC/D-GFP

FIG.11(A)
Mock

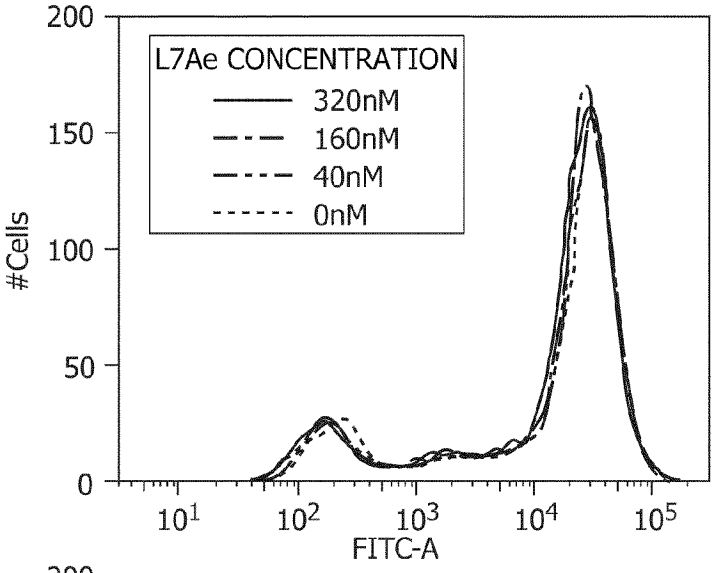


FIG.11(B)
shRNA-U1A-4

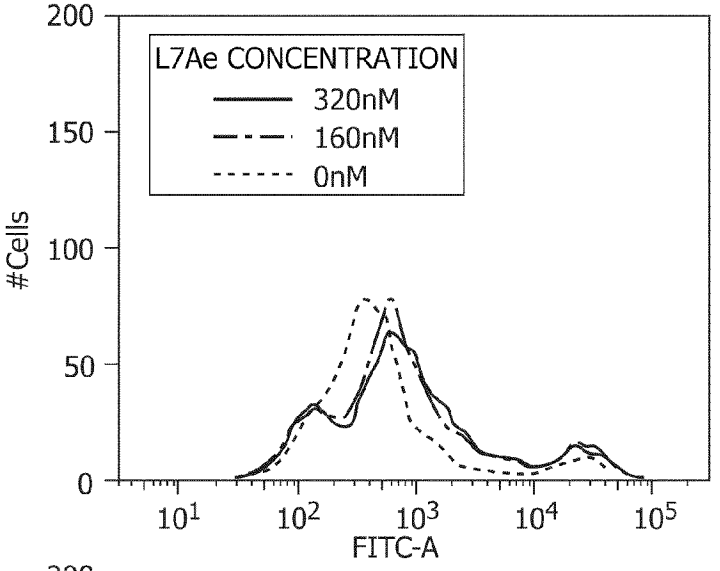


FIG.11(C)
shRNA-BoxC/D-GFP

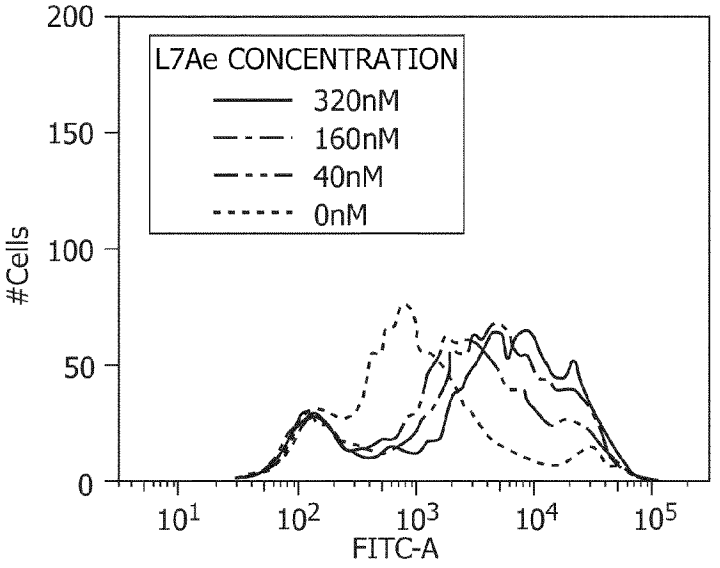


FIG.12

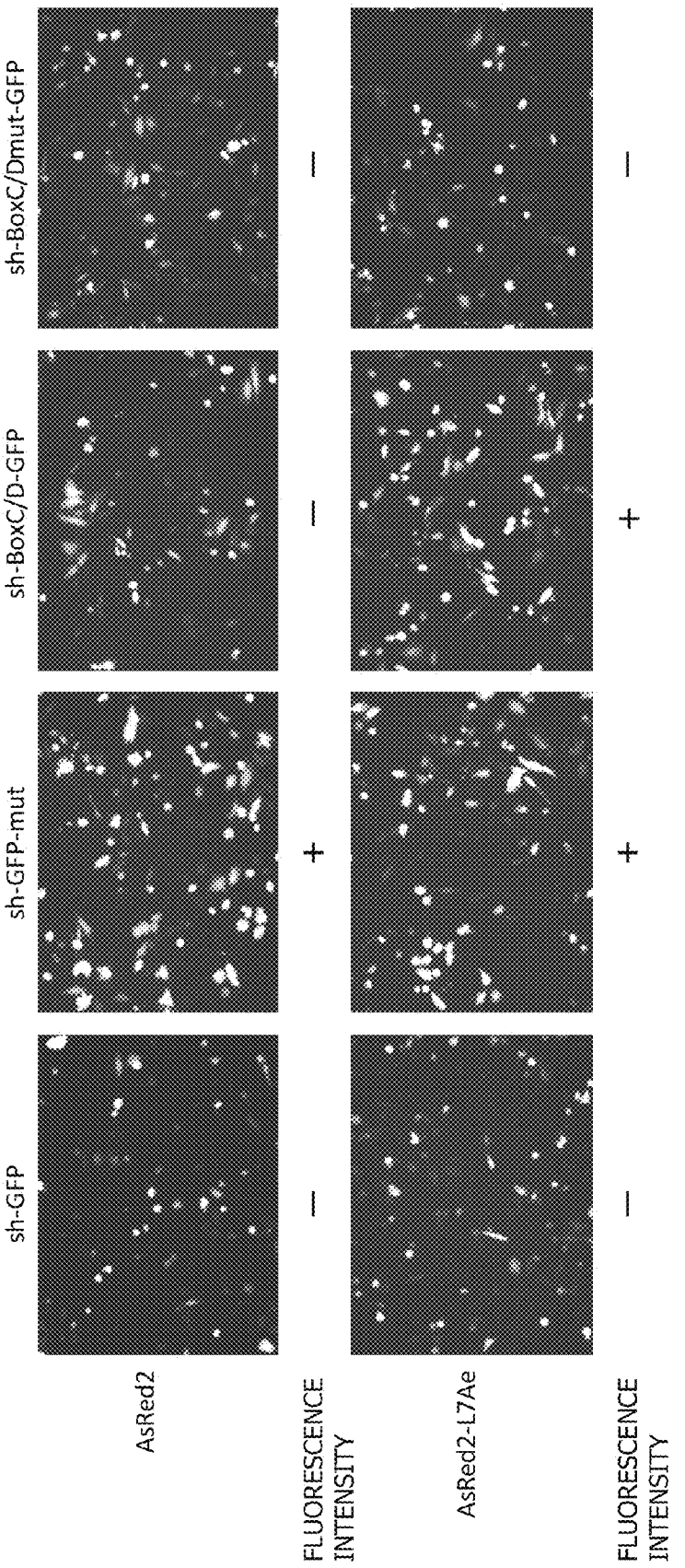

sh-GFP
sh-GFP-mut
sh-BoxC/D-GFP
sh-BoxC/Dmut-GFP
AsRed2
AsRed2-L7Ae
FLUORESCENCE INTENSITY
FLUORESCENCE INTENSITY
−
+
−
+
−
−
+
+

FIG.13(A)

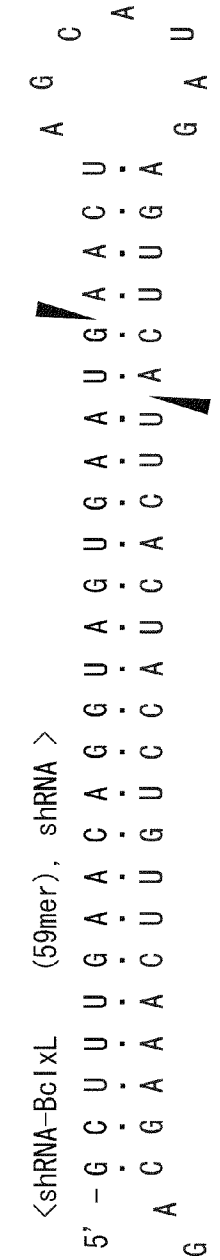


FIG.13(B)

FIG.13(C)

FIG.14

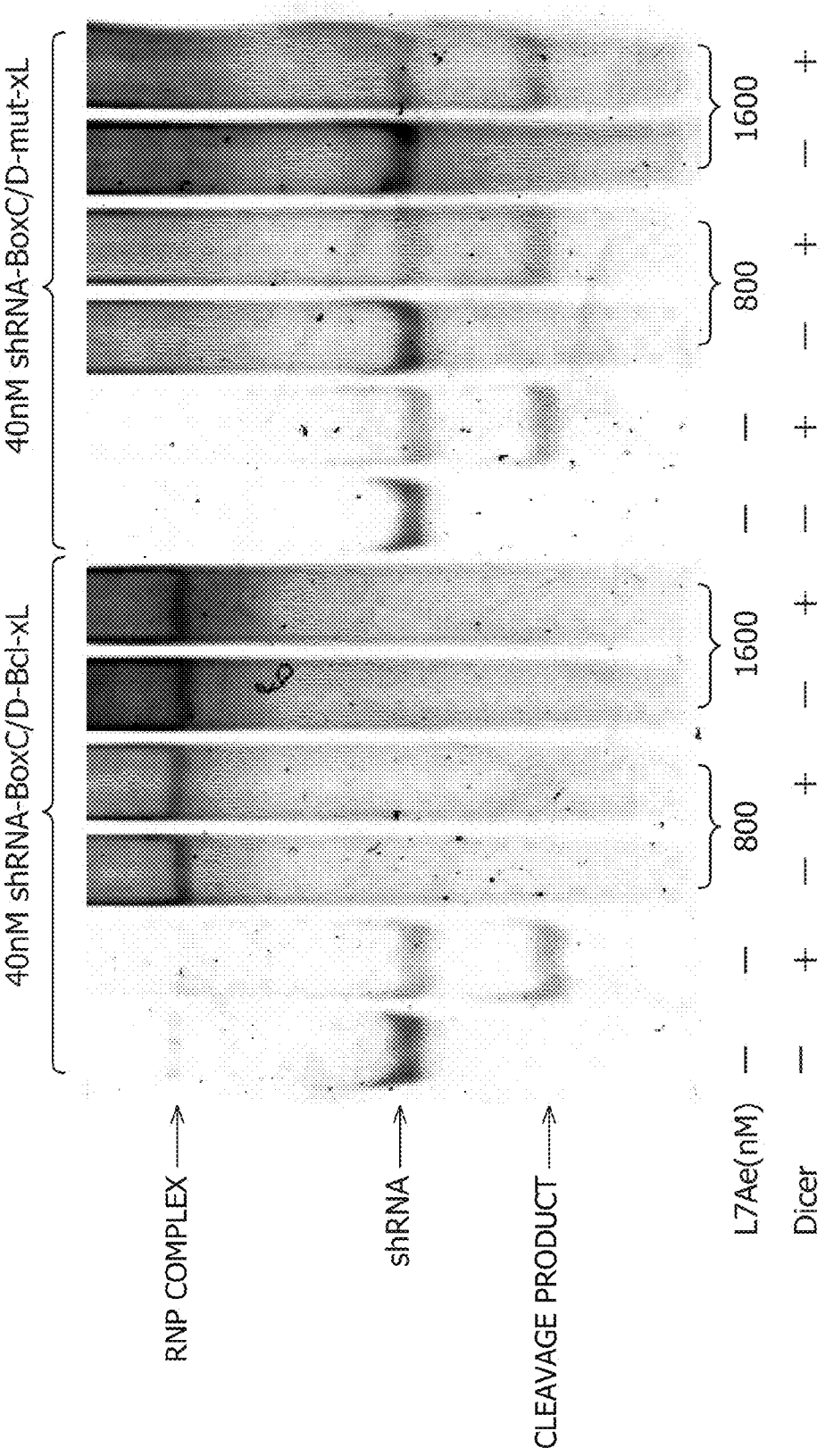

40nM shRNA-BoxC/D-Bcl-xL
40nM shRNA-BoxC/D-mut-xL
RNP COMPLEX
shRNA
CLEAVAGE PRODUCT
L7Ae(nM)
Dicer
800
1600
800
1600

FIG.15

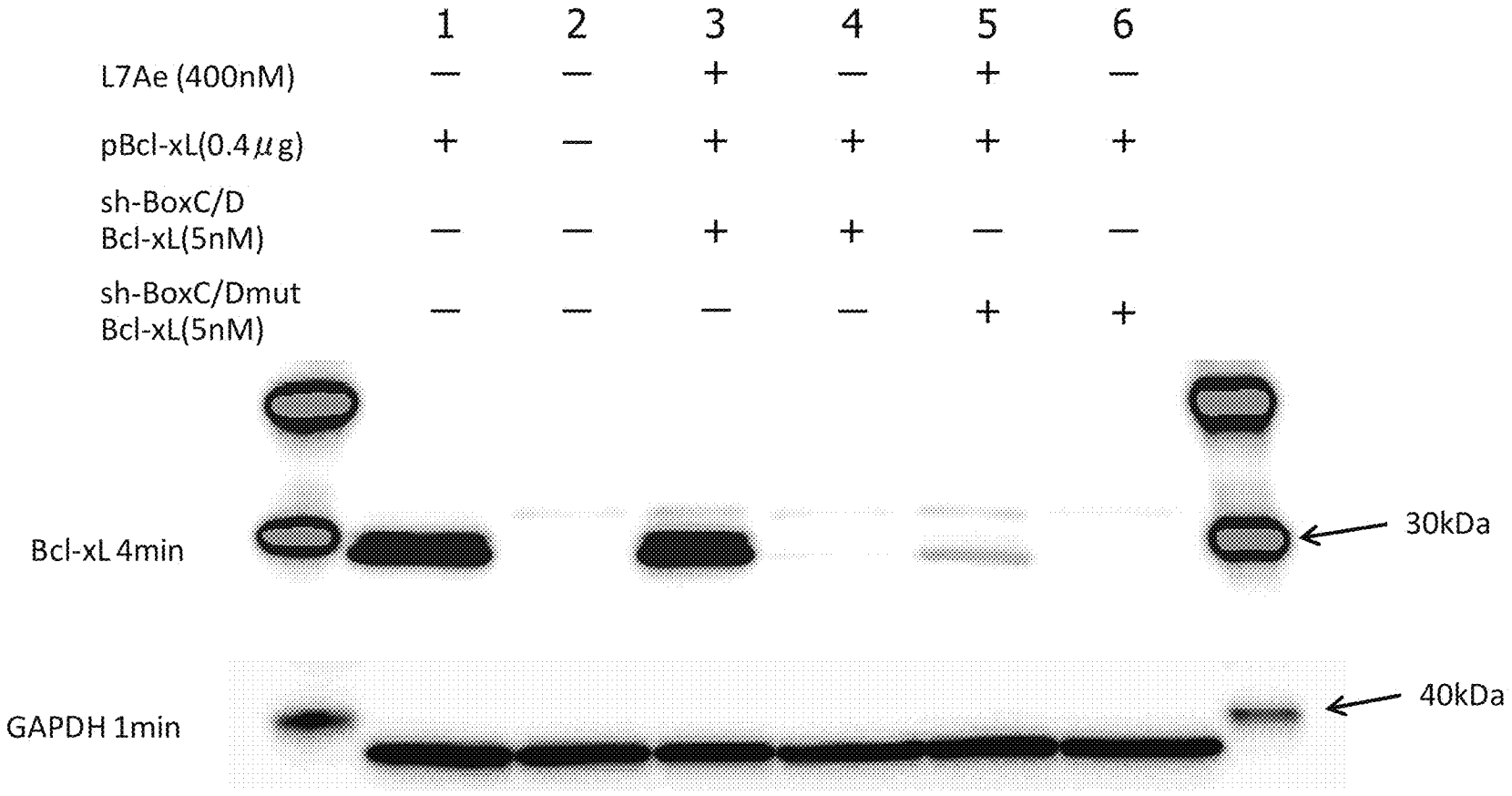

1
2
3
4
5
6
L7Ae (400nM)
— — + — + —
pBcl-xL(0.4μg)
+ — + + + +
sh-BoxC/D Bcl-xL(5nM)
— — + + — —
sh-BoxC/Dmut Bcl-xL(5nM)
— — — — + +
Bcl-xL 4min
30kDa
GAPDH 1min
40kDa

FIG.16

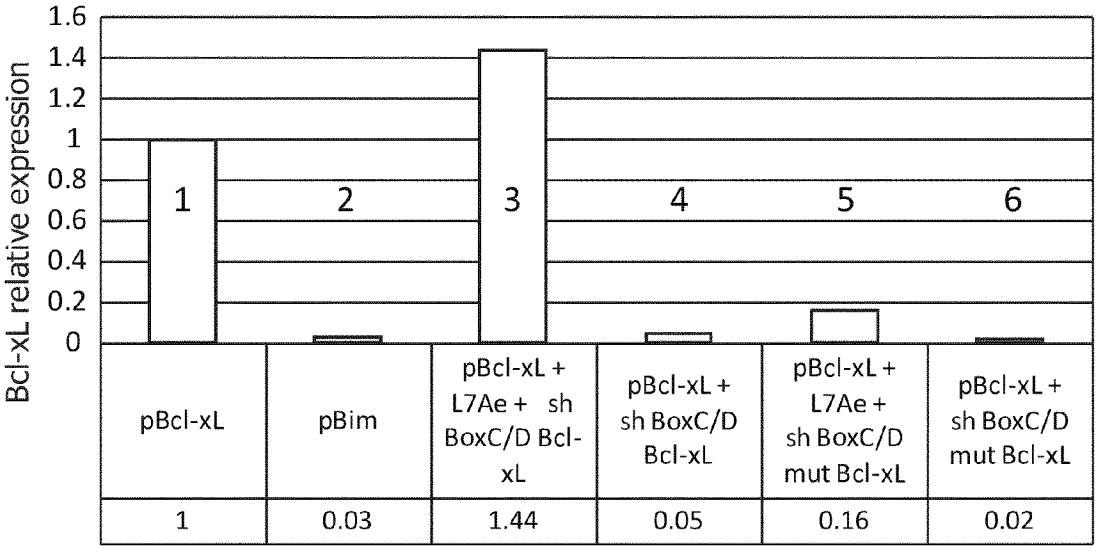

Bcl-xL relative expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
pBcl-xL
pBim
pBcl-xL + L7Ae + sh BoxC/D Bcl-xL
pBcl-xL + sh BoxC/D Bcl-xL
pBcl-xL + L7Ae + sh BoxC/D mut Bcl-xL
pBcl-xL + sh BoxC/D mut Bcl-xL
1
0.03
1.44
0.05
0.16
0.02

FIG.17

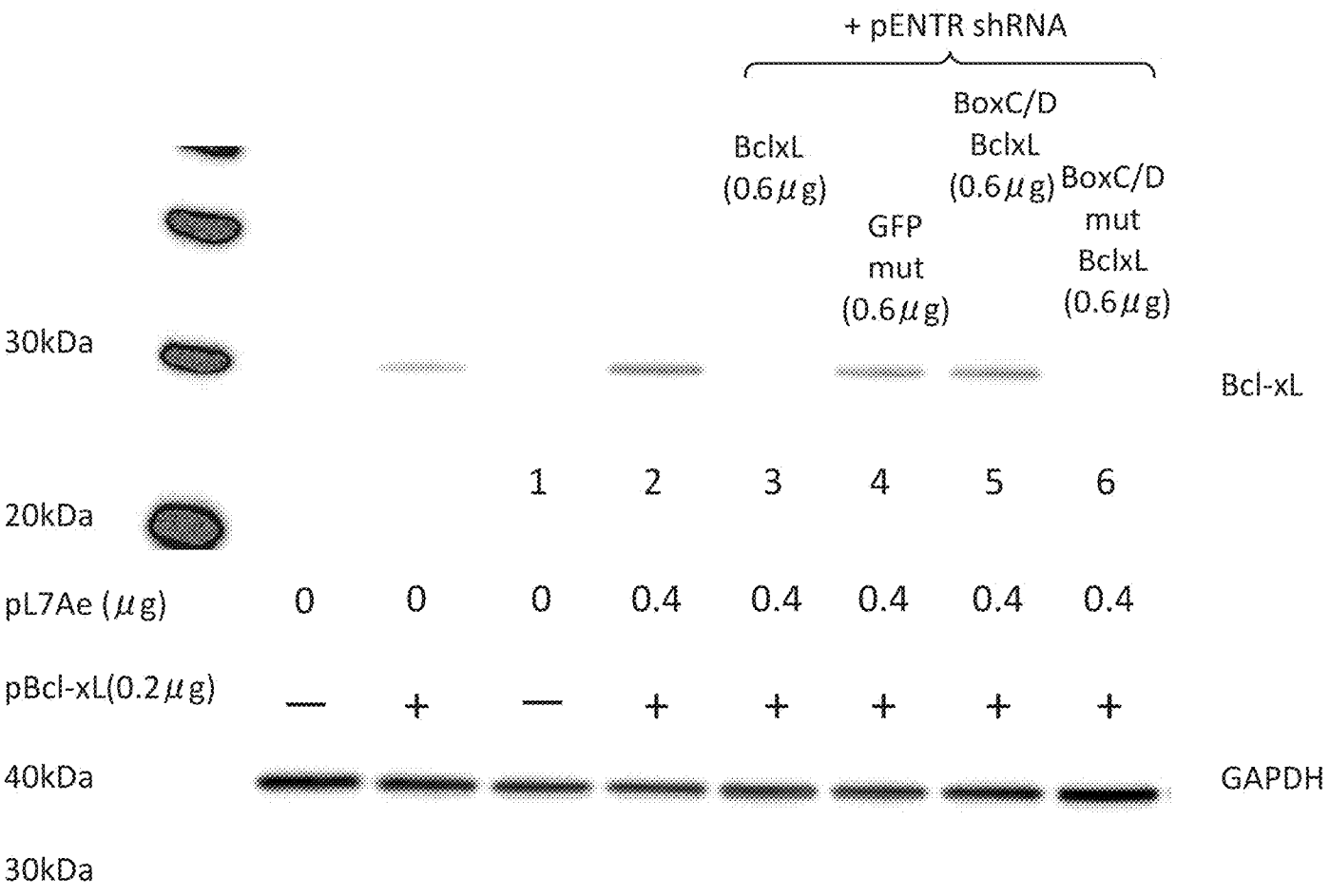
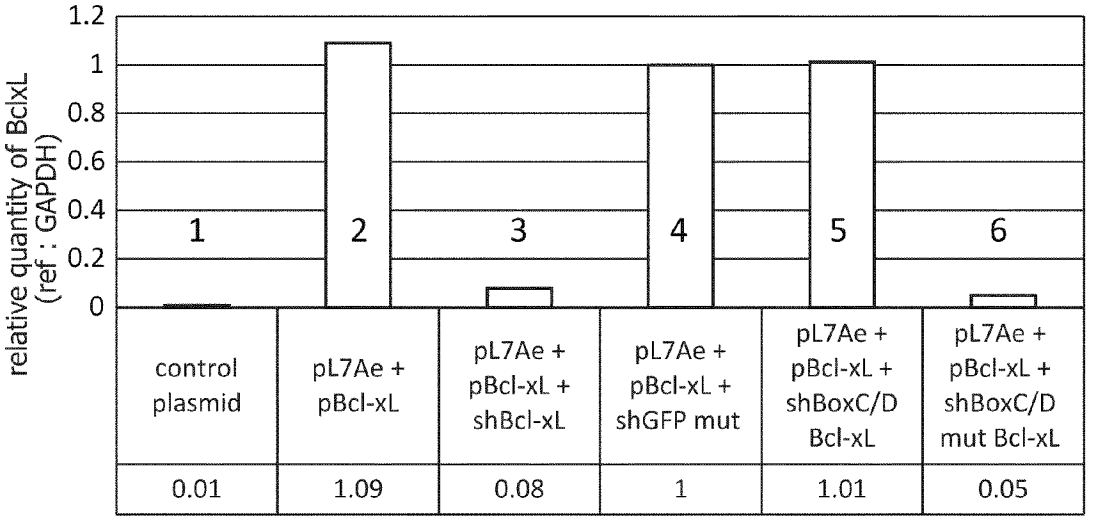

+ pENTR shRNA
BclxL (0.6μg)
GFP mut (0.6μg)
BoxC/D BclxL (0.6μg)
BoxC/D mut BclxL (0.6μg)
30kDa
Bcl-xL
20kDa
1 2 3 4 5 6
pL7Ae (μg) 0 0 0 0.4 0.4 0.4 0.4 0.4
pBcl-xL(0.2μg) — + — + + + + +
40kDa
GAPDH
30kDa

FIG.18


relative quantity of BclxL (ref : GAPDH)
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
control plasmid
pL7Ae + pBcl-xL
pL7Ae + pBcl-xL + shBcl-xL
pL7Ae + pBcl-xL + shGFP mut
pL7Ae + pBcl-xL + shBoxC/D Bcl-xL
pL7Ae + pBcl-xL + shBoxC/D mut Bcl-xL
0.01
1.09
0.08
1
1.01
0.05

CONSTRUCTION OF PROTEIN-RESPONSIVE SHRNA/RNAI CONTROL SYSTEM USING RNP MOTIF

TECHNICAL FIELD

The present invention relates to the construction of a protein-responsive shRNA and RNAi control system using an RNP motif.

BACKGROUND ART

RNA interference (hereinafter, referred to as RNAi) is a phenomenon in which translation is transiently inhibited by cleaving mRNA in a sequence-specific manner. An approach called knockdown, which causes this RNAi by introducing an RNA duplex such as short hairpin RNA (hereinafter, referred to as shRNA), has been established in various organism species. RNAi has been diffused widely, including study on its application to medical treatment, in a period as short as 10 years from its discovery as the convenient and potent approach of transiently inhibiting gene expression. However, its mechanisms or introduction techniques still remain to be evolved. Moreover, at this time, the theme of RNAi centers on studies for making knockdown strongly effective or the development of delivery techniques for delivering RNA to a site of interest.

Chung et al. have prepared artificial RNA in which the binding site of theophylline known as a caffeine-like low-molecular-weight compound has been introduced in the loop portion of shRNA, and have revealed that RNAi is inhibited in a theophylline concentration-dependent manner (see Non-Patent Document 1).

Non-Patent Document 1: Chung-Il An, Vu B. Trinh, and Yohei Yokobayashi, RNA, May 2006; 12: 710-716

DISCLOSURE OF THE INVENTION

Problems to be Solved by the Invention

An object of the present invention is to provide an RNAi control system using RNP for the purpose of newly designing and preparing a translational control mechanism using RNAs or proteins as inhibitors and incorporating such an artificial translational control system into a living body.

Means for Solving the Problems

The present invention has been achieved for attaining the object. Specifically, according to one embodiment, the present invention relates to an shRNA comprising: a guide strand having a sequence complementary to a target sequence; a passenger strand which forms a duplex with the guide strand; and a linker strand which links the guide strand and the passenger strand, wherein the linker strand comprises an RNP-derived protein-binding motif sequence. In the present specification, this shRNA is also referred to as a sensor shRNA.

It is preferred that the RNP-derived protein-binding motif sequence should be a Box C/D sequence.

According to an alternative aspect, the present invention relates to an RNAi control system comprising: the sensor shRNA; and an RNP-derived protein which specifically binds to a protein-binding motif sequence in the shRNA.

According to an alternative aspect, the present invention relates to an RNAi control system comprising: a vector for expression of the sensor shRNA; and a vector for expression of an RNP-derived protein which specifically binds to a protein-binding motif sequence in the shRNA.

According to a further alternative aspect, the present invention relates to an RNAi control method comprising the steps of: contacting the sensor shRNA with an RNP-derived protein which specifically binds to a protein-binding motif sequence in the shRNA, in a solution; and introducing the solution containing the shRNA and the protein into a cell.

According to a further alternative aspect, the present invention relates to an intracellular RNAi control method comprising the steps of: introducing a vector for expression of the sensor shRNA into a cell; introducing a vector for expression of an RNP-derived protein which specifically binds to a protein-binding motif sequence in the shRNA, into the cell; and causing their expressions from the vector for expression of the shRNA and the vector for expression of the protein.

According to a further alternative aspect, the present invention provides an RNAi control system responsive to a protein expressed in a cell, the system comprising: the sensor shRNA in which RNP-derived protein-binding motif sequence is a sequence specifically binding to the protein expressed in the cell, or a vector for expression of the shRNA, and also provides an RNAi control method responsive to a protein expressed in a cell, the method comprising the step of: introducing the sensor shRNA in which RNP-derived protein-binding motif sequence is a sequence specifically binding to the protein expressed in the cell, or a vector for expression of the shRNA, into a cell.

According to a further alternative aspect, the present invention provides the RNAi control system which controls the expression of an apoptosis regulatory protein wherein the target sequence of the shRNA is Bcl-xL mRNA, and also provides an artificial protein information conversion system using the shRNA, wherein information of a protein specifically binding to an RNP-derived protein-binding motif sequence is converted to information of a protein encoded by an RNA of the target sequence of the shRNA.

Advantageous Effects of the Invention

As an advantageous effects of the present invention, use of the sensor shRNA described above enables RNAi control such that RNAi is inhibited in a manner dependent on a protein specifically binding to the shRNA. This means that in the presence of the sensor shRNA, use of a particular protein as an input signal can inhibit the RNAi of particular mRNA and relatively increase the amount of proteins expressed from the particular mRNA. The sensor shRNA according to the present invention used in combination with the particular protein is useful in the construction of biosensors for quantifying the expression of intracellular marker proteins without destroying cells or artificial gene circuits capable of activating the translation of proteins of interest in response to the expression level of marker proteins. For example, the present invention produces significant advantageous effects that lead to the treatment of diseases such as cancer or Alzheimer's disease by activating apoptosis-inducing proteins in response to the expression of cancer marker proteins.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** schematically shows an shRNA according to the first embodiment;

FIG. **2**(A) schematically shows an shRNA constituting an RNAi control system according to the second embodiment, FIG. **2**(B) schematically shows an RNP-derived protein 4 constituting the RNAi control system according to the second

embodiment, and FIG. **2**(C) schematically shows a complex of the shRNA and the protein 4 in a system in which the shRNA and the protein 4 coexist;

FIG. **3**(A) shows a pENTR (trademark)/H1/TO vector sold by Invitrogen Corp., FIG. **3**(B) schematically shows a DNA duplex inserted to the pENTR (trademark)/H1/TO vector;

FIG. **4** shows a preparation example of a protein expression vector;

FIG. **5**(A) shows the secondary structure sequence of shRNA-GFP for EGFP knockdown (SEQ ID NO:10), FIG. **5**(B) shows the secondary structure sequence of shRNA-GFP-mut used as a negative control that does not cause EGFP knockdown (SEQ ID NO:12), FIG. **5**(C) shows the secondary structure sequence of shRNA-Box C/D-GFP that is expected to specifically bind at the Box C/D sequence to an L7Ae protein (SEQ ID NO:9), and FIG. **5**(D) shows the secondary structure sequence of shRNA-Box C/D-mut-GFP that does not bind to L7Ae (SEQ ID NO:11). In FIGS. **5**(A) to **5**(D), the wedge-shaped mark represents the position of cleavage by Dicer;

FIG. **6** shows the binding of shRNA-GFP, shRNA-Box C/D-GFP, and shRNA-Box C/D mut-GFP to L7Ae by gel shift assay;

FIG. **7** shows results of the inhibition of Dicer cleavage of shRNA-Box C/D-GFP by L7Ae using an in-vitro reconstituted Dicer system;

FIG. **8** is a graph showing results of RT-PCR-analyzing RNAi inhibition by L7Ae;

FIG. **9** is a graph showing results of FACS-analyzing RNAi inhibition by L7Ae;

FIG. **10** is a superimposed image of fluorescence and phase-contrast images of intracellular GFP in Example2;

FIG. **11** is a graph showing results of FACS-analyzing fluorescence intensity distribution in Example 2;

FIG. **12** is a fluorescence image of EGFP showing the inhibitory effect of AsRed2-L7Ae on the knockdown function of shRNA-Box C/D-GFP;

FIG. **13**(A) shows the secondary structure sequence of shRNA-Bc1xL for Bc1xL knockdown (SEQ ID NO:47), FIG. **13**(B) shows the secondary structure sequence of shRNA-Box C/D-Bc1xL that is expected to specifically bind at the Box C/D sequence to an L7Ae protein (SEQ ID NO:45), and FIG. **13**(C) shows the secondary structure sequence of shRNA-Box C/D mut-Bc1xL that does not bind to L7Ae (SEQ ID NO:59). In FIGS. **13**(A) to **13**(C), the arrowheads indicate Dicer cleavage site;

FIG. **14** shows results of the inhibition of Dicer cleavage of shRNA-Box C/D-Bc1-xL and shRNA-Box C/D mut-Bc1-xL by L7Ae using an in-vitro reconstituted Dicer system;

FIG. **15** shows intracellular Bc1-xL expression;

FIG. **16** shows results of adding up the intensities of detected Bc1-xL bands;

FIG. **17** shows intracellular Bc1-xL expression; and

FIG. **18** shows results of adding up the intensities of detected Bc1-xL bands.

DESCRIPTION OF SYMBOLS

1 guide strand
2 linker strand
20 base sequence
21 protein-binding motif sequence
3 passenger strand
4 protein
a Dicer cleavage site
b Dicer cleavage site
1*d* guide strand-encoding DNA sequence
2*d* linker strand-encoding DNA sequence
3*d* passenger strand-encoding DNA sequence

BEST MODE FOR CARRYING OUT THE INVENTION

Hereinafter, the present invention will be described in detail with reference to embodiments. However, the present invention is not intended to be limited to the description below.

In recent years, various noncoding RNAs that function in vivo have been discovered, and their roles have received attention. However, these RNAs often form a complex with a protein (RNP) in vivo. Thus, artificial RNP has been expected as a novel nanoblock capable of controlling cell functions. Naturally occurring RNP is found to form many complexes using an RNA-protein interaction motif (RNP motif) composed of a relatively short sequence. For example, HIV Rev proteins interact with high affinity with RNA motifs that recognize Rev. Thus, RNP has been expected to be used as a research material for synthetic biology (field in which biological molecules or the systems of life are constituted through the procedures of artificially creating biological molecules to induce new technologies).

To develop a system for controlling the translation of proteins of interest in response to proteins expressed in cells, the present inventors have come up with the idea that RNA interference is controlled using this RNA-protein interaction motif, thereby controlling the translation of proteins of interest. Based on this idea, the present invention has been completed.

According to a first embodiment, the present invention provides an shRNA comprising: a guide strand having a sequence complementary to a target sequence; a passenger strand which forms a duplex with the guide strand; and a linker strand which links the guide strand and the passenger strand, wherein the linker strand comprises an RNP-derived protein-binding motif sequence.

The shRNA according to the first embodiment is schematically shown in FIG. **1**. The shRNA according to the first embodiment comprises a guide strand **1**, a linker strand **2**, and a passenger strand **3** in this order from the 3' end. The linker strand **2** has an RNP-derived protein-binding motif sequence **21** in the strand.

The guide strand **1** may be an RNA nucleotide sequence of approximately 21 bases to 26 bases and may be located at the 3' end in the shRNA. The guide strand **1** has a sequence complementary to a particular sequence of mRNA to be controlled (hereinafter, referred to as a target sequence). The mRNA to be controlled can be selected appropriately by those skilled in the art according to the purpose. Examples of the mRNA to be controlled include, but not limited to, mRNAs of apoptosis-inducing genes, mRNAs of apoptosis-suppressing genes, and mRNAs of cancer marker genes. More specific examples of the mRNA to be controlled include GFP mRNA, BimEL mRNA, and Bc1-xL mRNA. Moreover, in these mRNAs, a sequence used as the target sequence can be selected appropriately by those skilled in the art by using design software based on information about the constitution and type of its primary gene sequence in view of inhibited off-target effect and the more efficiently inhibited expression of the target gene. The guide strand must be completely complementary to the target sequence. This is to cause the RNAi effect. At least 2 bases of the 3'-terminal in the guide strand **1** may form overhang that does not form complementary strands with the passenger strand. The guide strand **1** is a portion that becomes siRNA after cleavage by Dicer.

The passenger strand **3** may be an RNA nucleotide sequence of approximately 21 bases to 26 bases and may be located at the 5' end of the shRNA. When the passenger strand **3** is, for example, of 21 bases, bases 3 to 21 from the 3' end of the passenger strand **3** have a sequence that forms complementary strands with bases 3 to 21 from the 3' end of the guide strand **1**. When the passenger strand **3** is composed of 21 bases, usually, the guide strand is also composed of 21 bases. The numbers of bases constituting the passenger strand **3** and the guide strand **1** may be 22, 23, 24, 25, or 26. In either case, the numbers of bases of the passenger strand **3** and the guide strand **5** are usually equal. Moreover, as shown in FIG. **1**, base 3 from the 3' end to the base at the 5' end of the passenger strand **3** has a sequence that forms complementary strands with the base at the 5' end to base 3 from the 3' end of the guide strand **1**. In this case, the passenger strand may be permitted to contain a 1-base to 2-base mismatch to the guide strand. After cleavage by Dicer, the passenger strand **3** may have, at the 3' end, overhang of at least 2 bases that forms complementary strands neither with a portion of the guide strand **1** nor with a portion of the linker strand. The passenger strand **3** may be also a portion that becomes siRNA after cleavage by Dicer.

The linker strand **2** serves as a linker between the guide strand **1** and the passenger strand **3**. The linker strand **2** may be bound to the 5' end of the guide strand **1** and the 3' end of the passenger strand **3**. In other words, the linker strand **2** may be a portion that is cleaved off from the guide strand **1** and the passenger strand **3** after cleavage by Dicer. The linker strand **2** may constitute the major part of the nonhybridized loop portion, as shown in the drawing, in the sensor shRNA according to the present invention. A portion of the nonhybridized loop portion may be derived from a portion of the 3' end of the passenger strand **3**. Alternatively, the linker strand **2** may constitute the whole nonhybridized loop portion, and a few bases at the 3' end of the linker strand **2** and a few bases at the 5' end of the linker strand **2** may form a portion of the stem portion of the hairpin structure through hybridization therebetween, though this structure is not shown in the drawings.

The linker strand **2** may comprise a base sequence **20** and an RNP-derived protein-binding motif sequence **21**. In this context, the base sequence **20** in the present invention refers to a portion of the sequence constituting the linker strand and a portion that is not derived from the RNP-derived protein-binding motif sequence **21**. In another embodiment, the linker strand **2** may be composed only of the RNP-derived protein-binding motif sequence **21**. When the linker strand **2** comprises the base sequence **20** and the RNP-derived protein-binding motif sequence **21** or when the linker strand **2** is composed only of the RNP-derived protein-binding motif sequence **21**, the linker strand **2** may have, in its sequence, a sequence that does not form complementary strands, and this sequence may constitute the loop portion of the shRNA. The sequence that does not form complementary strands may be of 4 to 20 bases, preferably 4 to 11 bases, and the types of the bases are not limited. Preferable examples of the sequence that does not directly form complementary strands include, but not limited to, 5'-AGCAUAG-3' and 5'-GAAA-3'.

The 3'-terminal 2 bases of the linker strand **2** may form complementary strands with the 3'-terminal 2 bases of the passenger strand **3**. Furthermore, bases 3 to 7 bases may from the 3' end of the linker strand **2** may form complementary strands with bases 1 to 4 from the 5' end of the linker strand **2**. In this case, the number of bases forming complementary strands is 4. However, this number is not limited to 4 and can be determined between 1 and 8 bases.

When the protein-binding motif sequence **21** is introduced to the base sequence **20**, the introduction position of the protein-binding motif sequence **21** is not limited and may be a range which maintains recognition by Dicer described later. Moreover, when the linker strand is free from the base sequence, the protein-binding motif sequence **21** can be bound directly to the guide strand **1** and the passenger strand **3**.

In this context, examples of the protein-binding motif sequence **21** include nucleotide sequences derived from RNA-protein complex interaction motifs (RNP motifs), and nucleotide sequences mutated from these nucleotide sequences. In the present invention, the nucleotide sequences derived from RNA-protein complex interaction motifs encompass: nucleotide sequences known as the RNA sequences of RNA-protein interaction motifs in known natural RNA-protein complexes; and nucleotide sequences as the RNA sequences of artificial RNA-protein complex interaction motifs obtained by an in-vitro evolution method. The RNA-protein complexes are a large number of associates of proteins and RNAs that have been confirmed in vivo, and are 3D objects having a complicated structure. The nucleotide sequences derived from natural RNA-protein complex interaction motifs are usually composed of approximately 10 to 80 bases and known to form specific binding in a noncovalent manner, i.e., through a hydrogen bond, with a particular amino acid sequence of a particular protein. Such nucleotide sequences derived from natural RNA-protein complex interaction motifs can be selected from Tables 1 and 2 below and the database: http://gibk26.bse.kyutech.acjp/jouhou/image/dna-protein/rna/ma.html that is available on the website. The protein-binding motif sequence **21** as an RNA-protein interaction motif-derived nucleotide sequence preferably used in the present embodiment is a sequence that can be recognized by Dicer described below in detail to cause RNAi when incorporated in the shRNA. For conformational conditions, it is preferred that such a protein-binding motif sequence **21** should form a characteristic RNA tertiary structure comprising nonnatural base pairs and be highly specific for a protein binding to this site. Moreover, it is preferred that the RNA-protein interaction motif should have Kd of, but not limited to, approximately 0.1 nM to approximately 1 μM.

TABLE 1

RNA name	Protein name	Kd	Document
5S RNA (*Xenopus laevis oocyte*)	5R1	0.64 ± 0.10 nM	Nat Struct Biol. 1998 Jul; 5(7): 543-6
5S RNA (*Xenopus laevis oocyte*)	5R2	0.35 ± 0.03 nM	Nat Struct Biol. 1998 Jul; 5(7): 543-6
dsRNA	B2	1.4 ± 0.13 nM	Nat Struct Mol Biol. 2005 Nov; 12(11): 952-7
RNA splicing motif with UGCAUGU element	Fox-1	0.49 nM at 150 mM salt	EMBO J. 2006 Jan 11; 25(1): 163-73.
TGE	GLD-1	9.2 ± 2 nM	J Mol Biol. 2005 Feb 11; 346(1): 91-104.

TABLE 1-continued

RNA name	Protein name	Kd	Document
sodB mRNA	Hfq	1.8 nM	EMBO J. 2004 Jan 28; 23(2): 396-405.
RyhB (siRNA)	Hfq	1500 nM	Annu Rev Microbiol. 2004; 58: 303-28
mRNA	HuD	0.7 ± 0.02 nM	Nat Struct Biol. 2001 Feb; 8(2): 141-5
S domain of 7S RNA	human SRP19		RNA. 2005 Jul; 11(7): 1043-50. Epub 2005 May 31
Large subunit of SRP RNA	human SRP19	2 nM	Nat Struct Biol. 2001 Jun; 8(6): 515-20
23S rRNA	L1		Nat Struct Biol. 2003 Feb; 10(2): 104-8
23S rRNA	L11		Nat Struct Biol. 2000 Oct; 7(10): 834-7
5S rRNA	L18		Biochem J. 2002 May 1; 363(Pt 3): 553-61
23S rRNA	L20	13 ± 2 nM	J Biol Chem. 2003 Sep 19; 278(38): 36522-30.
Own mRNA site1	L20	88 ± 23 nM	J Biol Chem. 2003 Sep 19; 278(38): 36522-30.
Own mRNA site2	L20	63 ± 23 nM	Mol Microbiol. 2005 Jun; 56(6): 1441-56
23S rRNA	L23		J Biomol NMR. 2003 Jun; 26(2): 131-7
5S rRNA	L25		EMBO J. 1999 Nov 15; 18(22): 6508-21
Own mRNA	L30		Nat Struct Biol. 1999 Dec; 6(12): 1081-3.
mRNA	LicT		EMBO J. 2002 Apr 15; 21(8): 1987-97
Own mRNA	MS2 coat	39 ± 5 nM	FEBS J. 2006 Apr; 273(7): 1463-75
Stem-loop RNA motif	Nova-2		Cell. 2000 Feb 4; 100(3): 323-32
SL2	Nucleocapsid	110 ± 50 nM	J Mol Biol. 2000 Aug 11; 301(2): 491-511
Pre-rRNA	Nucleolin		EMBO J. 2000 Dec 15; 19(24): 6870-81
	p19	0.17 ± 0.02 nM	Cell. 2003 Dec 26; 115(7): 799-811
Box C/D	L7Ae	0.9 ± 0.2 nM	RNA. 2005 Aug; 11(8): 1192-200.

TABLE 2

RNA name	Protein name	Kd	Document
siRNA with the characteristic two-base 3' overhangs	PAZ (PiWi Argonaut and Zwille)		Nat Struct Biol. 2003 Dec; 10(12): 1026-32.
dsRNA	Rnase III		Cell. 2006 Jan 27; 124(2): 355-66
HIV-1 RRE (IIB)	RR1-38	3-8 nM	Nat Struct Biol. 1998 Jul; 5(7): 543-6
Own mRNA	S15	5 nM	EMBO J. 2003 Apr 15; 22(8): 1898-908
16S rRNA	S15	6 nM	Nat Struct Biol. 2000 Apr; 7(4): 273-277.
Own mRNA	S15	43 nM	EMBO J. 2003 Apr 15; 22(8): 1898-908
16S rRNA	S4	6.5 μM in 4° C., 1.7 nM in 42° C.	J Biol Chem. 1979 Mar 25; 254(6): 1775-7
16S rRNA	S4	18 μM	J Biol Chem. 1979 Mar 25; 254(6): 1775-7
16S rRNA	S8	26 ± 7 nM	J Mol Biol. 2001 Aug 10; 311(2): 311-24
mRNA	S8	200 nM	RNA. 2004 Jun; 10(6): 954-64
mRNA	SacY	1400 nM	EMBO J. 1997 Aug 15; 16(16): 5019-29
SnRNA	Sm		Cold Spring Harb Symp Quant Biol. 2006; 71: 313-20.
tmRNA	SmpB	21 ± 7 nM	J Biochem (Tokyo). 2005 Dec; 138(6): 729-39
TD3 of tmRNA	SmpB	650 nM	J Biochem (Tokyo). 2005 Dec; 138(6): 729-39
U1 snRNA	snRNP U1A	0.032 ± 0.007 nM (salt dependence)	Nat Struct Biol. 2000 Oct; 7(10): 834-7
S domain of 7S RNA	SRP54	500 nM	RNA. 2005 Jul; 11(7): 1043-50.
TAR	Tat	200-800 nM	Nucleic Acids Res. 1996 Oct 15; 24(20): 3974-81
BIV TAR	Tat	1.3 nM or 8 nM or 60 nM (depending on difference in Mg)	Mol Cell. 2000 Nov; 6(5): 1067-76
$tRNA^{Thr}$	ThrRS	500 nM	Nat Struct Biol. 2002 May; 9(5): 343-7
thrS mRNA operator	ThrRS	10 nM	Trends Genet. 2003 Mar; 19(3): 155-61
Single stranded mRNA	TIS11d		Nat Struct Mol Biol. 2004 Mar; 11(3): 257-64.
PSTVd	Virp1	500 nM	Nucleic Acids Res. 2003 Oct 1; 31(19): 5534-43
RNA hairpin; Smaug recognition element (SRE)	Vts1p	30 nM	Nat Struct Mol Biol. 2006 Feb; 13(2): 177-8.
□ BoxB	□ N	90 nM	Cell. 1998 Apr 17; 93(2): 289-99

The nucleotide sequences derived from artificial RNA-protein complex interaction motifs are the RNA nucleotide sequences of RNA-protein interaction motifs in artificially designed RNA-protein complexes. Such nucleotide sequences are usually composed of approximately 10 to 80 bases and designed to form specific binding in a noncovalent manner, i.e., through a hydrogen bond, with a particular amino acid sequence of a particular protein. Examples of such nucleotide sequences derived from artificial RNA-protein complex interaction motifs include, but not limited to, RNA aptamers specifically binding to the apoptosis-inducing protein Bc1-2 family, and RNA aptamers specifically recognizing cancer cell surface antigens. Moreover, nucleotide sequences listed in Table 3 below are also known, and these can also be used as the RNA-protein complex interaction motif-derived nucleotide sequence **2** of the present invention.

TABLE 3

RNA name	Protein name	Kd	Document
Rev aptamer 5	Rev	190 nM	RNA. 2005 Dec; 11(12): 1848-57
Aptamer	p50	5.4 ± 2.2 nM	Proc Natl Acad Sci USA. 2003 Aug 5; 100(16): 9268-73.
BMV Gag aptamer	BMV Gag	20 nM	RNA. 2005 Dec; 11(12): 1848-57

TABLE 3-continued

RNA name	Protein name	Kd	Document
BMV Gag aptamer	CCMV Gag	260 nM	RNA. 2005 Dec; 11(12): 1848-57
CCMV Gag aptamer	CCMV Gag	280 nM	RNA. 2005 Dec; 11(12): 1848-57
CCMV Gag aptamer	BMV Gag	280 nM	RNA. 2005 Dec; 11(12): 1848-57

The artificial RNA-protein complexes can be prepared by using a molecular design method and an in-vivo evolution method in combination. The in-vivo evolution method can produce aptamers or ribozymes by selecting functional RNAs from molecular libraries having various sequence diversities, and repeating the reactions of amplification and transcription of their genes (DNAs). Thus, RNA-protein interaction motifs adapted to RNP having a functional structure of interest can be extracted in advance from natural RNP molecules by molecular design or prepared artificially by the in-vitro evolution method. In the present embodiment, for the RNA-protein complex interaction motif-derived nucleotide sequence **2**, it is preferred that the RNA-protein complex from which the nucleotide sequence is derived should have a dissociation constant Kd of approximately 0.1 nM to approximately 1 μM. Specific examples of the protein-binding motif sequence **21** include a Box CD sequence: 5'-GGGCGUGAUGCGAAAGCUGACCC-3' (SEQ ID NO: 2), which is a nucleotide sequence binding to L7Ae (SEQ ID NO: 1) (Moore T et al., Structure Vol. 12, pp. 807-818 (2004)) known to participate in RNA modifications such as RNA methylation or pseudouridylation.

The constitution of the shRNA according to the present embodiment can be obtained by molecular design. The sensor shRNA of the present embodiment can be obtained, for example, by introducing a protein-binding motif sequence to a sequence portion forming the linker strand, based on the sequence of known natural or nonnatural shRNA, or by replacing a protein-binding motif sequence for a sequence portion forming the linker strand, based on the sequence of known natural or nonnatural shRNA. In this procedure, the type and introduction position of the protein-binding motif sequence can be determined in view of the appropriate placement of RNP of interest such that the function of the Dicer protein can be inhibited.

Alternatively, the nucleotide sequence of the guide strand can be determined according to the desired target sequence to design the linker strand and the passenger strand by computer-aided molecular modeling. In this procedure, particular attention may be paid to the correct formation of a duplex structure by the guide strand and the passenger strand.

The shRNA according to the first embodiment may be stably present through the formation of the hairpin structure shown in FIG. **1** under physiological conditions involving pH 6.5 to 8.0 and a temperature of 4 to 42° C., preferably pH 7.3 to 7.5 and a temperature of 4 to 37° C. When the shRNA according to the first embodiment may be present in this form in vivo, this shRNA is recognized by an RNA duplex-cleaving enzyme Dicer. Then, the shRNA may be cleaved at positions shown by arrowheads a and b in FIG. **1** to form an RNA duplex of approximately 19 to 24 bases in the length of each strand having a 2-base protruding end. As a result, the guide RNA complementary to the target mRNA can be transferred from RLC to RISC to inhibit the translation of the mRNA to be controlled by its cleavage.

Thus, the shRNA according to the first embodiment is characterized in that the shRNA in the form shown in the drawing, i.e., in the form in which the protein-binding motif sequence **21** is unbound to the particular protein, functions in the same way as known natural shRNA.

Next, according to the second embodiment, the present invention provides an RNAi control system comprising: the shRNA according to the first embodiment; and an RNP-derived protein which specifically binds to a protein-binding motif sequence in the shRNA.

The shRNA constituting the RNAi control system according to the present embodiment is schematically shown in FIG. **2**(A), and an RNP-derived protein **4** is schematically shown in FIG. **2**(B). The shRNA is as described in the first embodiment, and its description is omitted here. The same reference numerals will be used to designate the same components as those in FIG. **1**.

The protein **4** shown in FIG. **2**(B) is a protein that is derived from RNP and specifically binds to the protein-binding motif sequence **21** on the shRNA. Accordingly, this protein **4** can be determined in a manner specific for a sequence selected as the protein-binding motif sequence **21**. Specifically, when Box C/D (SEQ ID NO: 2) is selected as the protein-binding motif sequence **21**, the protein **4** is L7Ae (SEQ ID NO: 1). The protein **4** may also be a fusion protein containing the protein specifically binding to the protein-binding motif sequence **21** or may be a protein having an additional peptide added to the protein specifically binding to the protein-binding motif sequence **21**. This is because the protein **4** needs only to be capable of inhibiting recognition by Dicer described below.

In the state shown in FIG. **2**(A), the shRNA according to the present embodiment functions in the same way as known natural shRNA, as described in the first embodiment, in the absence of the protein **4** to cause RNAi such that the translational function of particular mRNA is inhibited.

Next, the states of the shRNA according to the present embodiment and the protein **4** in a system in which these molecules coexist will be described. FIG. **2**(C) schematically shows the shRNA and the protein 4 in the system in which the shRNA and the protein 4 coexist. The shRNA and the protein 4 may be stably present in the form of an RNP complex formed by specific binding under physiological conditions involving pH 6.5 to 8.0 and a temperature of 4 to 42° C., preferably pH 7.0 to 7.5 and a temperature of 4 to 37° C.

The present embodiment is characterized in that when the protein-binding motif sequence **21** on the shRNA specifically bind the protein 4 to form an RNP by specific binding, Dicer fails to recognize the shRNA in this RNP form. As a result, the Dicer fails to cleave the shRNA. Then, it cannot proceed to the next step of RNAi such that mRNA to be controlled is made insusceptible to cleavage. In other words, the coexistence of the shRNA according to the present embodiment with the protein 4 can inhibit RNAi. Moreover, according to the embodiment the protein 4 as input information is transformed to RNAi inhibition as output.

In this context, the system in which the shRNA and the protein 4 coexist may be a mixture of separately prepared shRNA and protein 4 molecules in a medium. Alternatively, a vector for expression of the shRNA molecule may be designed, and a vector for expression of the molecule of the protein 4 may be designed. These vectors may be introduced into the same cell, and their expressions can also be caused to achieve the system in which the shRNA and the protein 4 coexist. The vector design will be described in detail in Examples described later.

The RNAi control system according to the second embodiment of the present invention can achieve the inhibition of shRNA-mediated RNAi in a protein-specific manner.

Next, according to the third embodiment, the present invention relates to an RNAi control method comprising the steps of: contacting the sensor shRNA according to the first embodiment with an RNP-derived protein which specifically binds to a protein-binding motif sequence in the shRNA, in a solution; and introducing the solution containing the shRNA and the protein into a cell. The combination of the sensor shRNA and the protein used in the present embodiment can be selected based on the first and second embodiments to determine their sequences.

[In-Vitro Synthesis of a Sensor ShRNA]

In this context, the sensor shRNA can be obtained by an in-vitro synthesis method called in-vitro transcription. Single-stranded DNA in which a 19-base antisense sequence (TATAGTGAGTCGTATTAGC; SEQ ID NO: 3) of a T7 promoter sequence may be bound to the 3' end of a sequence serving as a template of shRNA is artificially synthesized (Hokkaido System Science Co., Ltd.), and this may be associated with a 19-base T7 promoter sequence (GCTAATACGACTCACTATA (SEQ ID NO: 4)) artificially synthesized in the same way. This associate may be mixed with ribonucleic acids, salts, and T7 RNA polymerase and reacted at 37° C., as specifically described in detail in Examples to obtain the sensor shRNA.

[Production of Protein]

On the other hand, the protein can be obtained by preparing a vector for expression of the protein and causing its expression using *E. coli*, followed by purification. For example, a vector for expression of L7Ae (SEQ ID NO: 2), which is a protein specifically binding to a Box-C/D motif represented by SEQ ID NO: 1, can be prepared with reference to Nucleic Acid Research, 2003, Vol. 31, No. 3 869-877. One example of the vector for expression of L7Ae (SEQ ID NO: 2) in *E. coli* is shown in SEQ ID NO: 5.

In the present embodiment, the contacting step may be performed by mixing the thus-prepared sensor shRNA and protein in the same solution system. The mixing of the shRNA and the protein may be performed under physiological conditions involving pH 6.5 to 8.0 and a temperature of 4 to 42° C., preferably pH 7.3 to 7.5 and a temperature of 4 to 37° C. As a result, the shRNA and the protein may specifically interact with each other to form an RNP complex.

RNAi may be inhibited in the presence of Dicer, ATP, and Mg ions and under appropriate physiological conditions, in addition to the conditions described above. Accordingly, in the contacting step, the sensor shRNA and the protein may be mixed to form an RNP complex, and then, the step of introduction into a cell can be carried out. The concentration of the RNP complex introduced into a cell is, for example, 1 nM to 40 nM shRNA, preferably 1 nM to 20 nM shRNA, and a protein concentration of preferably, but not limited to, that about 1 to 10 times the shRNA concentration. The introduction of the RNP complex into a cell can be performed by, but not limited to, transfection using liposomes and can be performed by those skilled in the art using general methods for introduction of an RNP into cells known in the art.

After the introduction of the RNP complex into a cell, the phenomenon described in the first embodiment with reference to FIG. **2** may take place in the cell containing Dicer. Specifically, Dicer neither recognizes the RNA cleavage site in the prepared complex of the sensor shRNA and the protein nor cleaves the shRNA. Then, mRNA complementary to the guide strand may not undergo RNAi. Thus, the expression of a protein encoded by this mRNA may be inhibited.

According to the third embodiment of the present invention, RNAi can be inhibited in vitro.

In an modification of the third embodiment, the protein alone can be administered directly to a cell. This modification involves, after the introduction of the protein into a cell, forming an RNP complex of the sensor shRNA and the protein in the cell, and differs in this point from the third embodiment which involves forming an RNP complex and then introducing it into a cell. In this modification, the sensor shRNA can be introduced into the cell before or after the introduction of the protein into the cell. When the sensor shRNA is introduced into the cell before the protein introduction, a vector for expression of the shRNA may be designed such that the expression of the shRNA in this vector introduced into the cell can be controlled using a small molecule such as tetracycline. In this method, the shRNA can be expressed using tetracycline after the protein introduction. When the sensor shRNA is introduced into the cell after the protein introduction, the shRNA may be introduced directly into the cell or a vector for expression of the shRNA may be introduced into the cell. The protein introduced in the cell forms an RNP complex with the sensor shRNA and enables the expression of a gene of interest to be controlled by RNAi in the cell. Thus, the present embodiment seems to be effective in the development of protein drugs or the treatment of diseases such as cancer.

According to the fourth embodiment, the present invention relates to an intracellular RNAi control method comprising the steps of: introducing a vector for expression of the shRNA according to the first embodiment into a cell; introducing a vector for expression of an RNP-derived protein which specifically binds to a protein-binding motif sequence in the shRNA, into the cell; and causing their expressions from the vector for expression of the shRNA and the vector for expression of the protein.

The vector for expression of the sensor shRNA can be prepared based on the primary structure sequence of the sensor shRNA determined as described in the first embodiment. One example of the vector preparation method is shown in FIG. **3**. FIG. **3**(A) shows a pENTR (trademark)/H1/TO vector (SEQ ID NO: 8) sold by Invitrogen Corp. A DNA sequence encoding the shRNA of the present invention may be inserted to the site shown by the arrow of FIG. **3**(A). The inserted DNA duplex is schematically shown in FIG. **3**(B). The upper strand shown in FIG. **3**(B) comprises overhang CACC, a guide strand-encoding DNA sequence **1***d*, a linker strand-encoding DNA sequence **2***d*, and a passenger strand-encoding DNA sequence **3***d* located in this order from the 5' end. The lower DNA strand shown in the drawing is a strand complementary to the upper strand and has overhang AAAA located at the 5' end. Those skilled in the art can obtain the desired sensor shRNA by inserting the desired sequence into commercially available plasmids to prepare plasmids producing the desired sensor shRNA.

The protein expression vector can be prepared by incorporating a DNA sequence encoding a protein to be expressed, into vectors for mammalian expression. A vector preparation example is shown in FIG. **4**. The vector shown in this drawing is a vector for expression of L7Ae (SEQ ID NO: 2), which is a protein specifically binding to a Box-C/D motif represented by SEQ ID NO: 1. The sequence of the vector is shown in SEQ ID NO: 6. Such a vector can be prepared with reference to Nucleic Acid Research, 2003, Vol. 31, No. 3 869-877, and a gene can be extracted therefrom and recombined to mammalian expression vector. This vector can be prepared using a pcDNA3.1 (+) myc H is A vector (SEQ ID NO: 7) commercially available from Invitrogen Corp.

The present embodiment is characterized by introducing the vector for expression of the shRNA and the vector for

expression of the protein into the same cell and causing their expressions. The introducing step can be carried out by transfection. Examples of the transfection include, but not limited to, transfection using liposomes, direct injection, electroporation, and a lentiviral transfection method. The amounts of the shRNA expression vector and the protein expression vector introduced into a cell may differ depending on the purpose and are, for example, the amount of the L7Ae protein expression vector ¼ times to 10 times, preferably 1 time to 4 times that of the shRNA expression vector.

According to the fourth embodiment, RNAi can be inhibited by causing the intracellular expressions of the shRNA and the protein. Examples of a practical application of the embodiment include the treatment of cancer. Moreover, an RNAi control system can also be prepared by combining the vector for expression of the sensor shRNA and the protein expression vector used in the present embodiment.

In a modification, shRNA can be designed such that the protein specifically binding to the protein-binding motif sequence shown in FIGS. **1** and **2** is, for example, a biological molecule expressed only in cancer cells, and the target sequence of the guide strand is an apoptosis-promoting gene. In this case, the shRNA may be introduced directly or via a vector into a cancer cell to form an RNP complex with a biological molecule expressed only in cancer, in the cancer cell. As a result, the shRNA may be made insusceptible to recognition by Dicer. Then, in the cancer cell, the RNAi of the apoptosis-promoting gene may be inhibited, resulting in the selective expression of the apoptosis-promoting gene to promote the apoptosis of the cancer cell. On the other hand, in a normal cell, which may be free from the biological molecule expressed only in cancer, RNAi may not be inhibited even if the same shRNA is introduced thereinto directly or via a vector. Therefore, the expression of the apoptosis-promoting gene may be inhibited by RNAi.

Moreover, in a further modification of this embodiment, shRNA can be designed such that the protein specifically binding to the protein-binding motif shown in FIGS. **1** and **2** is, for example, a biological molecule that becomes no longer expressed by carcinogenesis, and the target sequence of the guide strand may be an apoptosis-suppressing gene. In this case, the shRNA is introduced directly or via a vector into a normal cell to form an RNP complex with a biological molecule that becomes no longer expressed by carcinogenesis, in the normal cell. As a result, the shRNA may be made insusceptible to recognition by Dicer. Then, in the normal cell, the RNAi of the apoptosis-suppressing gene may be inhibited, resulting in the selective expression of the apoptosis-suppressing gene to suppress the apoptosis of the normal cell. On the other hand, in a cancer cell, which may be free from the biological molecule that becomes no longer expressed by carcinogenesis, RNAi may not be inhibited even if the same shRNA is introduced thereinto directly or via a vector. Therefore, the expression of the apoptosis-suppressing gene may be inhibited by RNAi.

Furthermore, a cancer control system using L7Ae may be constructed. The same promoter as that expressing such a cancer-related protein is incorporated upstream of the L7Ae protein expression vector. As a result, the RNAi function of apoptosis-controlling sensor shRNA comprising Box C/D incorporated in the linker strand can be regulated freely by regulating the expression of L7 in response to the expression of the cancer-related protein.

EXAMPLE 1

[Design of Protein Molecule-Responsive shRNA]

An shRNA-GFP sequence for EGFP knockdown (shRNA-GFP (59 mer) GGCAUCAAGGUGAACUUCAAGAUCCAGCAUAGGGAUCUUGAAGUUCACCUUGA UGCCAG; FIG. **5**A (SEQ ID NO: 10)) was kindly provided by Dr. Tsutomu Suzuki and Dr. Takayuki Kato, the University of Tokyo. For shRNA-GFP-mut (shRNA-GFP-mut (59 mer) GCACUAGCGUAUGAAUGAAAGAUCCAGCAUAGGGAUCUUUCAUUCAUACGCUA GUGCAG; FIG. **5**B (SEQ ID NO: 12)), first, a guide strand in which three sequences complementary to the stop codons were inserted in the reading frame of three codons one for each was designed, and this was replaced for the guide strand of shRNA-GFP to design the shRNA-GFP-mut sequence. This shRNA-GFP-mut was used as a negative control that did not cause EGFP knockdown. Next, from the structure of an RNP motif L7Ae-Box C/D, the RNA sequence of Box C/D was obtained and inserted such that it was located as close to a Dicer protein cleavage site in shRNA as possible and the duplex structure of guide and passenger strands was maintained to design shRNA-Box C/D-GFP (shRNA-Box C/D-GFP (63 mer) GGCAUCAAGGUGAACUUCAGCUGACCCGAAAGGGCGUGAUGCUGAAGUUCACC UUGAUGCCAG; FIG. **5**C (SEQ ID NO: 9)) that was expected to specifically bind at the Box C/D sequence to an L7Ae protein. In its guide strand, the 3'-terminal 21 bases of the guide strand of shRNA-GFP were used. Furthermore, shRNA in which adenine at base 24 from the 5' end of this shRNA-Box C/D-GFP was deleted and guanine at base 38 was replaced by cytosine was designed as shRNA-Box C/D-mut-GFP (shRNA-Box C/D-mut-GFP (62 mer) GGCAUCAAGGUGAACUUCAGCUGCCCGAAAGGGCGUCAUGCUGAAGUUCACCU UGAUGCCAG; FIG. **5**(D) (SEQ ID NO: 11)) that did not bind to L7Ae.

[In-Vitro Synthesis of shRNA]

[shRNA-Box C/D-GFP]

5.25 μL of L7Aer template (100 μM, 5'-CTGGCATCAAGGTGAACTTCAGCATCACGCCCTTTCGGGTCAGCTGAAGTTCACC TTGATGCCTATAGTGAGTCGTATTAGC-3; SEQ ID NO: 13) as template DNA of shRNA, 5.25 μL of T7 sense primer (100 μM, 5'-GCTAATACGACTCACTATA-3; SEQ ID NO: 4), 30 μL of T7 RNA polymerase, 5 μL of 1 mg/mL pyrophosphatase (ROCHE), 1.75 μL of 20 mg/mL BSA, 28 μL of 1 M Hepes-KOH, 14 μL of 1 M $MgCl_2$, 3.5 μL of 1 M DTT, 14 μL of 0.1 M spermidine, 33.6 μL of 0.1 M ATP (the same holds true for CTP and UTP), 8.96 μL of 0.1 M GTP, 89.6 μL of 0.1 M GMP, and 385 μL of ultrapure water were mixed and reacted overnight at 37° C. After the reaction, 10 μL of TURBO DNase was added thereto and reacted at 37° C. for 30 minutes to degrade the template DNA. After the reaction, phenol extraction and chloroform extraction were performed, and the supernatant was charged into a PD-10 column (GE Healthcare) equilibrated with a PD-10 buffer (0.3 M potassium acetate, 15% (v/v) ethanol, pH 6.0), and washed with 3 mL of PD-10 buffer, followed by elution with 500 μL of PD-10 buffer twice. Then, to the eluate, an equal amount of ethanol was added to perform ethanol precipitation. The supernatant was discarded, and the pellet was dried and then dissolved in 20 μL of 5× dye solution (0.25% BPB, 30% glycerol). The solution was layered on a nondenaturing 15% polyacrylamide (1/30 bisacrylamide) gel and electrophoresed at room temperature for 50 minutes for separation. A band with the size of interest was excised, and 500 μL of elution

buffer (0.5 M NaCl, 0.1% SDS, 1 mM EDTA) was added thereto, followed by elution overnight at 37° C. Then, a microfilter (22 μm Millex GP) was attached to a 5-mL syringe (TERUMO CORP.), and the eluate was added to the syringe and filtered through the filter. To this filtrate, a 2.5-fold volume of ethanol was added to perform ethanol precipitation. The supernatant was discarded, and the pellet was dried and then dissolved in 22 μL of ultrapure water. After concentration measurement, this solution was used in subsequent experiments.

[shRNA-Box C/D mut-GFP]

5.25 μL of L7AerN template (100 μM, 5'-CTGGCATCAAGGTGAACTTCAGCATGACGCCCTTTCGGGCAGCTGAAGTTCACCT TGATGCCTATAGTGAGTCGTATTAGC-3; SEQ ID NO: 15) as template DNA of shRNA and 5.25 μL of T7 sense primer (100 μM, SEQ ID NO: 4) were used to perform transcription/synthesis and purification in the same way as in shRNA-Box C/D-GFP. The purification product was dissolved in 22 μL of ultrapure water. After concentration measurement, this solution was used in subsequent experiments.

[shRNA-GFP]

5.25 μL of 481P template (100 μM, 5'-CTGGCATCAAGGTGAACTTCAAGATCCCTATGCTGGATCTTGAAGTTCACCTTGA TGCCTATAGTGAGTCGTATTAGC-3; SEQ ID NO: 16) as template DNA of shRNA and 5.25 μL of T7 sense primer (100 μM, SEQ ID NO: 4) were used to perform transcription/synthesis and purification in the same way as in shRNA-Box C/D-GFP. The purification product was dissolved in 22 μL of ultrapure water. After concentration measurement, this solution was used in subsequent experiments.

[Expression and Purification of L7Ae Protein]

Plasmids comprising a gene of the L7Ae protein incorporated in pET-28b+, kindly provided by Dr. Huttenhofer were amplified. From a −80° C. glycerol bacterial stock of *E. coli* BL21 (DE3) pLysS transformed with the pET-28b+L7Ae plasmid (SEQ ID NO: 5), the bacterial cells were inoculated to 5 mL of medium and shaken-cultured overnight at 37° C. Subsequently, the whole amount of the culture solution was inoculated to 500 mL of LB medium containing 50 μg/mL kanamycin and 100 μg/mL chloramphenicol. The bacterial cells were shake-cultured at 37° C. until $O.D._{600}$ became 0.6 to 0.7. Then, 500 μL of 1 M IPTG was added (final concentration: 1 mM) thereto for inducing expression, and the cells were shake-cultured overnight at 30° C. The bacterial cells were collected by centrifugation (4° C., 6000 rpm, 20 minutes). After addition of 5 mL of sonication buffer (50 mM Na phosphate, 0.3 M NaCl, 2.5 mM imidazole, pH 8.0), sonication was performed to disrupt the bacterial cells. In this sonication, the procedure of cooling on ice and then irradiation with ultrasonic waves for 15 seconds was repeated 6 times. Then, impurity proteins were denatured at 80° C. for 15 minutes. The supernatant was collected by centrifugation (4° C., 6000 rpm, 20 minutes), and histidine-tagged proteins were purified by the batch method using an Ni-NTA column (Qiagen). Specifically, first, the supernatant and 1 mL of Ni-NTA were mixed and stirred at 4° C. for 1 hour. Then, the mixture was charged into the column and washed twice with 4 mL of wash buffer (50 mM Na phosphate, 0.3 M NaCl, 20 mM imidazole, pH 8.0). Stepwise elution was performed with 1 mL each of 50 mM, 100 mM, 200 mM, and 300 mM imidazole elution buffers (prepared by adding imidazole to 50 mM Na phosphate, 0.3 M NaCl, pH 8.0) in each of two runs. The proteins of interest were confirmed by 15% SDS-PAGE. Subsequently, Microcon YM-3 (Millipore Corp.) was used to concentrate the proteins, followed by buffer replacement by a dialysis buffer (20 mM Hepes-KOH, 1.5 mM $MgCl_2$, 150 mM KCl, 5% glycerol, pH 7.5). Moreover, the protein concentration was determined by the Bradford method using Protein Assay (Bio-Rad Laboratories, Inc.).

[Confirmation of RNP Complex by Gel Shift Assay]

The binding between each shRNA and the protein was confirmed as follows: after dilution with a dialysis buffer to bring the protein concentration to concentrations 25 times the final concentrations of 80 to 640 nM, 2 μL of the protein solution with each concentration, 2 μL of 1 μM shRNA-Box C/D-GFP, 6 μL of ultrapure water, and 40 μL of Opti-MEM I (trademark, Invitrogen Corp.) were mixed and left standing at room temperature for 30 minutes to bind shRNA (40 nM) to L7Ae (80 nM, 160 nM, 320 nM, or 640 nM). shRNA-GFP and shRNA-Box C/D mut-GFP were bound to L7Ae in the same way. To each solution, 13 μL of 5× dye solution (0.25% BPB, 30% glycerol) was added and mixed, and 15 μL of this mixed solution was layered on a nondenaturing 15% polyacrylamide (1/30 bisacrylamide) gel and electrophoresed at 250 V at 4° C. for 50 minutes. After the electrophoresis, the gel was stained with SYBR Green for 15 minutes, and bands were confirmed using FLA-7000 (FUJI FILM). FIG. **6** shows the binding of shRNA-GFP, shRNA-Box C/D-GFP, and shRNA-Box C/D mut-GFP to L7Ae by gel shift assay. The results suggested that shRNA-Box C/D-GFP is bound to L7Ae in a sequence-specific manner.

[Confirmation of Dicer Cleavage Inhibition by In-Vitro Dicer Cleavage Assay]

The inhibition of Dicer cleavage of shRNA-Box C/D-GFP and shRNA-Box C/D mut-GFP by L7Ae was confirmed as follows using GTS, Inc. Recombinant Human Dicer Enzyme Kit according to the protocol: first, 0.4 μL of 4 μM shRNA, 2 μL of 4 μM or 8 μM L7Ae, 1 μL of 10 mM ATP, 0.5 μL of 50 mM $MgCl_2$, 4 μL of Dicer Reaction Buffer (GTS, Inc.), 2 μL of 0.5 unit/μL Recombinant Dicer Enzyme, and 0.1 μL of ultrapure water were mixed and reacted at 37° C. for 15 hours. Then, 2 μL of Dicer Stop Solution was added thereto and mixed. To 8 μL of this mixed solution, 2 μL of 5× dye solution was added, and the mixture was layered on a nondenaturing 15% polyacrylamide (1/30 bisacrylamide) gel and electrophoresed at 4° C. for 50 minutes. After the electrophoresis, the gel was stained with SYBR Green, and bands were confirmed using FLA-7000 (FUJI FILM). FIG. **7** shows results of the inhibition of Dicer cleavage of shRNA-Box C/D-GFP and shRNA-Box C/D mut-GFP by L7Ae using an in-vitro reconstituted Dicer system. In FIG. **7**, the mark + in L7Ae represents a sample prepared from 4 μM L7Ae, the mark ++ represents a sample prepared from 8 μM L7Ae, and the mark − represents that L7Ae was not used. Likewise, the mark + in Dicer represents that Dicer was used, and the mark − represents that Dicer was not used. The results suggested that upon sequence-specific binding to L7Ae, shRNA-Box C/D-GFP is made insusceptible to cleavage by Dicer.

[Construction/Synthesis of shRNA Expression Plasmid]

[Synthesis of pENTR/H1/TO-shRNA-Box C/D-GFP (SEQ ID NO: 17)]

5 μL of pENTR L7Aer Top strand (200 μM, 5'-CACCGGCATCAAGGTGAACTTCAGCTGACCCGAAAGGGCGTGATGCTGAAGTTC ACCTTGATGCC-3; SEQ ID NO: 18) as single-stranded DNA containing an shRNA-encoding sequence for insertion to pENTR/H1/TO vectors (Invitrogen Corp.), 5 μL of pENTR L7Aer Bottom strand (200 μM, 5'-AAAAGGCATCAAGGTGAACTTCAGCATCACGCCCTTTCGGGTCAGCTGAAGTTCA CCTTGATGCC-3; SEQ ID NO: 19) as single-stranded DNA containing a complementary strand thereof, 2 μL of 10× Oligo Annealing Buffer (Invitrogen Corp.), and 2 μL of ultra-

pure water were mixed, incubated at 95° C. for 4 minutes, and then left standing at room temperature for 5 minutes to form a DNA duplex. This duplex is a duplex shown in FIG. **3**(B). This DNA duplex solution was diluted 100-fold with ultrapure water, and then, 1 μL of the diluted solution was diluted 100-fold by mixing with 10 μL of 10×Oligo Annealing Buffer and 89 μL of ultrapure water. Then, 4 μL of 5× Ligation Buffer, 2 μL of 0.75 ng/μL pENTR/H1/TO vectors, 5 μL of the 10,000-fold diluted DNA solution, 8 μL of ultrapure water, and 1 μL of 1 U/μL T4 DNA Ligase were mixed and left standing at room temperature for 5 minutes to incorporate the shRNA-encoding sequence into the pENTR/H1/TO vectors. 4 μL of this reaction solution was added to TOP 10 Competent *E. coli* for transformation. After addition of 250 μL of S.O.C. medium, the bacterial cells were shake-cultured for 1 hour, then seeded over an LB plate containing 50 μg/mL kanamycin, and cultured overnight at 37° C. The formed colonies were confirmed, and the insert in the plasmid vectors was confirmed by colony PCR using H1 Forward Primer (10 μM, 5'-TGTTCTGGGAAATCACCATA-3; SEQ ID NO: 20), M13 Reverse Primer (10 μM, 5'-CAGGAAACAGCTATGAC-3; SEQ ID NO: 21), and KOD-Plus-ver. 2 (TOYOBO CO., LTD.). This colony was inoculated to 50 mL of LB medium containing 50 μg/mL kanamycin and shake-cultured at 37° C. for 16 hours. The bacterial cells were collected by centrifugation (4° C., 6000 rpm, 15 minutes) and purified according to the protocol of Plasmid Purification Kit (Qiagen), followed by isopropanol precipitation. The supernatant was discarded, and the pellet was dried and then dissolved by the addition of 55 μL of ultrapure water. After plasmid vector concentration measurement, this was used in subsequent experiments.

[Synthesis of pENTR/H1/TO-shRNA-Box C/D-mut-GFP (SEQ ID NO: 22)]

pENTR/H1/TO-shRNA-Box C/D-mut-GFP was synthesized and purified in the same way as above using 5 μL of pENTR L7AerN Top strand (200 μM, 5'-CACCGGCATCAAGGTGAACTTCAGCTGCCCGAAAGGGCGTCATGCTGAAGTTCAC CTTGATGCC-3; SEQ ID NO: 23) as single-stranded DNA containing an shRNA-encoding sequence for insertion to pENTR/H1/TO vectors (Invitrogen Corp.) and pENTR L7AerN Bottom strand (200 μM, 5'-AAAAGGCATCAAGGTGAACTTCAGCATGACGCCCTTTCGGGCAGCTGAAGTTCAC CTTGATGCC-3; SEQ ID NO: 24) as single-stranded DNA containing a complementary strand thereof, and dissolved by the addition of 55 μL of ultrapure water. After plasmid vector concentration measurement, this was used in subsequent experiments.

[Synthesis of pENTR/H1/TO-shRNA-GFP (SEQ ID NO: 25)]

pENTR/H1/TO-shRNA-GFP was synthesized and purified in the same way as above using 5 μL of pENTR 481P Top strand (200 μM, 5'-CACCGGCATCAAGGTGAACTTCAAGATCCAGCATAGGGATCTTGAAGTTCACCTT GATGCC-3; SEQ ID NO: 26) as single-stranded DNA containing an shRNA-encoding sequence for insertion to pENTR/H1/TO vectors (Invitrogen Corp.) and pENTR 481P Bottom strand (200 μM, 5'-AAAAGGCATCAAGGTGAACTTCAAGATCCCTATGCTGGATCTTGAAGTTCACCTT GATGCC-3; SEQ ID NO: 27) as single-stranded DNA containing a complementary strand thereof, and dissolved by the addition of 55 μL of ultrapure water. After plasmid vector concentration measurement, this was used in subsequent experiments.

[Synthesis of pENTR/H1/TO-shRNA-GFP-mut (SEQ ID NO: 28)]

pENTR/H1/TO-shRNA-GFP-mut was synthesized and purified in the same way as above using 5 μL of pENTR Sk-7N Top strand (200 μM, 5'-CACCGCACTAGCGTATGAATGAAAGATCCAGCATAGG-GATCTTTCATTCATACGC TAGTGC-3; SEQ ID NO: 29) as single-stranded DNA containing an shRNA-encoding sequence for insertion to pENTR/H1/TO vectors (Invitrogen Corp.) and pENTR Sk-7N Bottom strand (200 μM, 5'-AAAAGCACTAGCGTATGAATGAAAGATC-CCTATGCTGGATCTTTCATTCATACGC TAGTGC-3; SEQ ID NO: 30) as single-stranded DNA containing a complementary strand thereof, and dissolved by the addition of 55 μL of ultrapure water. After plasmid vector concentration measurement, this was used in subsequent experiments.

[Synthesis of pcDNA3.1-L7Ae-myc-His6 (SEQ ID NO: 6)]

PCR was performed using pET-28b+L7Ae (SEQ ID NO: 5) as template DNA and BamHI-NdeI-NotI-L7Ae-primer (5'-AAGGATCCATCATATGCGGCCGCTTATG-TACGTGAGATTTGAGG-3') (SEQ ID NO: 32) and L7Ae-EcoRI-XhoI-primer (5'-CACTCGAGTTGAATTCTCTTCTGAAGGCCTTTAATC-3') (SEQ ID NO: 33) as primers. 50 μL of this reaction solution contained a mixture of 2.5 μL of 10 ng/μL template DNA, 1.5 μL of each 10 μM DNA primer, 5 μL of 2 mM dNTPs, 5 μL of 10×KOD-PLUS-buffer ver. 2, 2 μL of 25 mM $MgSO_4$, 1 μL of KOD-PLUS-DNA Polymerase, and 31.5 μL of ultrapure water. The reaction was performed by initial incubation at 94° C. for 2 minutes, followed by 36 cycles each involving 98° C. for 10 seconds, 55° C. for 30 seconds, and 68° C. for 1 minute. From the resulting PCR product, DNA was purified using PCR Purification Kit (QIAGEN). However, elution was performed with 30 μL of ultrapure water, and this DNA was used as a template in restriction enzyme treatment. 27 μL of the template, 5 μL of B Buffer (ROCHE), 1 μL of 10 U/μL BamH1 (ROCHE), 1 μL of 10 U/μL XhoI (ROCHE), and 16 μL of ultrapure water were mixed and reacted at 37° C. for 2 hours for restriction enzyme treatment. Also for pcDNA3.1 (+) myc H is A vectors (Invitrogen Corp.), 1.88 μL of 1.6 μg/μL pcDNA vectors, 5 μL of B Buffer, 1 μL of 10 U/μL BamH1, 1 μL of 10 U/μL XhoI, and 41.12 μL of ultrapure water were mixed, and restriction enzyme treatment was performed in the same way. These treatment products were purified using PCR Purification Kit (QIAGEN). However, for elution, DNA was eluted into 10 μL of ultrapure water.

1.75 μL of the PCR product thus subjected to restriction enzyme treatment, 0.25 μL of the vectors thus subjected to restriction enzyme treatment, and 2 μL of Ligation High were mixed and incubated at 16° C. for 30 minutes. To the whole amount of this ligation reaction solution, 20 μL of Top10 Chemically competent cells (Invitrogen Corp.) was added. The cells were left standing on ice for 45 minutes, then placed in water bath at 42° C. for 1 minute, and left standing again on ice for 2 minutes for transformation. After further addition of 160 μL of LB medium, the cells were seeded over an LB plate containing 50 μg/mL ampicillin and incubated overnight at 37° C. The formed colonies were subjected to colony PCR using Extaq (TAKARA BIO INC.) and the DNA primers described above to check the insert. A colony in which the insert was confirmed was inoculated to 50 mL of LB medium containing 50 μg/mL ampicillin and shake-cultured overnight at 37° C. The bacterial cells were collected by centrifugation (4° C., 6000 rpm, 15 minutes) and purified according to the protocol of Plasmid Purification Kit (Qiagen), followed by isopropanol precipitation. The supernatant was discarded,

and the pellet was dried and then dissolved by the addition of 55 μL of ultrapure water. After plasmid vector concentration measurement, this plasmid vector was sequenced using T7 promoter primer (5'-TAATACGACTCACTATAGGG-3; SEQ ID NO: 34) and BGH rev primer (5'-GCTGGCAACTAGAAGGCACAG-3; SEQ ID NO: 35) and used in subsequent experiments.

[Confirmation of Knockdown Function Control by Fluorescence Microscope Image]

On the day before transfection, HeLa cells were seeded over a 24-well plate at a concentration of 0.8×10^5 cells/well and cultured in a CO_2 incubator at 37° C. On the next day, the cells were cotransfected with each pENTR/H1/TO shRNA expression vector, pcDNA3.1-AsRed2-L7Ae-myc-His6 (SEQ ID NO: 40), and pcDNA3.1-EGFP-myc-His6 (SEQ ID NO: 41) using Lipofectamine 2000 (Invitrogen Corp.) (trademark). To 0.3 μg each of pENTR-shRNA-GFP, pENTR-shRNA-GFP mut, pENTR-shRNA-Box C/D-GFP, and pENTR-shRNA-Box C/D mut-GFP, 0.3 μg of pcDNA3.1-AsRed2-L7Ae-myc-His6 or pcDNA3.1-AsRed2-myc-His6 (SEQ ID NO: 42) and 0.2 μg of pcDNA3.1-EGFP-myc-His6 were added, and 1.25 μl of Lipofectamine 2000 was added per sample. These DNA-lipid complexes were incubated at room temperature for 20 minutes and added dropwise to the medium for HeLa cells. 4 hours later, medium replacement was performed. 24 hours later, the fluorescence microscope image of the cells was obtained using a fluorescence microscope (OLYMPUS IX-81) to observe the inhibition of shRNA-Box C/D-GFP function by AsRed2-L7Ae expression.

FIG. **12** is an EGFP fluorescence image obtained through a 510-550 nm wavelength filter by irradiation with excitation light having a wavelength around 488 nm. In FIG. **12**, the mark "+" below each panel represents high fluorescence intensity, and the mark "−" represents low fluorescence intensity. This image demonstrated the inhibitory effect of AsRed2-L7Ae on the knockdown function of shRNA-Box C/D-GFP.

[Confirmation of RNAi Control by RT-PCR Analysis]

Change in GFP mRNA level caused by RNAi control by L7Ae was determined by real-time PCR.

On the previous day, HeLa-GFP cells were seeded over a 6-well plate at a concentration of 0.5×10^6 cells/well and cultured in a CO_2 incubator at 37° C. Then, the cells were cotransfected with each pENTR/H1/TO shRNA expression vector and pcDNA3.1-L7Ae-myc-His6. To 4 μg each of pENTR-shRNA-Box C/D-GFP and pENTR-shRNA-Box C/D mut-GFP, 0, 2, or 4 μg of pcDNA3.1-L7Ae-myc-His6 was added, and 5 μl of Lipofectamine 2000 was added per sample. These DNA-lipid complexes were incubated at room temperature for 20 minutes and added dropwise to the cells. 4 hours later, medium replacement was performed. 24 hours after the transfection, the cells were collected, and RNA extraction and DNA removal were performed using RNAqueous 4PCR Kit (Ambion, Inc. (trademark)).

1.5 μg (or 0.5 μg) of the extracted RNA was used as a template to synthesize cDNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems Inc. (trademark)), random primers, and reverse transcriptase. Real-time PCR was performed using 1/20-diluted cDNA as a template and LightCycler 480 Taqman probe (Roche) (trademark). PCR reaction and real-time fluorescence detection were carried out using LightCycler 480 (Roche) (trademark). The reaction conditions involved an initial denaturation step at 95° C. for 5 minutes and an amplification step of 45 cycles each involving denaturation at 95° C. for 10 seconds and annealing/elongation at 60° C. for 25 seconds. Finally, the reaction solution was cooled at 50° C. for 15 seconds to terminate the measurement. The Ct value was determined by the Abs Quant/fit point method. The GFP gene of interest was amplified using 481P Fwd (5'-CAAGGAGGACGGCAACA-3') (SEQ ID NO: 36) and Rev (5'-CCTTGATGCCGTTCTTCTGC-3') (SEQ ID NO: 37). A reference gene GAPDH was amplified using GAPDH Fwd (5'-AGCCACATCGCTCAGACAC-3') (SEQ ID NO: 38) and GAPDH Rev (5'-GCCCAATACGACCAAATCC-3') (SEQ ID NO: 39). The amplification efficiency of GFP mRNA and GAPDH mRNA was determined using Universal probe Library probe #148 (ROCHE) and Universal probe Library probe #60 (ROCHE), respectively. The amplification product was confirmed by electrophoresis to be the single product of interest, and the results were evaluated by relative quantification. The EGFP level was normalized with GAPDH, and this normalized value was used in comparison among samples with the GFP mRNA relative level of a sample supplemented only with pENTR-shRNA-GFP mut as 1. FIG. **8** shows results of RT-PCR-analyzing RNAi inhibition by L7Ae. The difference in expression level among the samples suggested that the GFP mRNA level recovers at the time of cotransfection with pENTR-shRNA-Box C/D-GFP and pcDNA3.1-L7Ae-myc-His6 and that RNAi is inhibited in a Box C/D sequence-specific manner in the presence of L7Ae.

[Confirmation of RNAi Control of L7Ae by FACS Analysis]

On the day before transfection, HeLa-GFP cells were seeded over a 24-well plate at a concentration of 0.5×10^5 cells/well and cultured in a CO_2 incubator at 37° C. On the next day, the cells were cotransfected with each pENTR/H1/TO shRNA expression vector and pcDNA3.1-L7Ae-myc-His6 using Lipofectamine 2000 (Invitrogen Corp.) (trademark). To 0.8 μg each of pENTR-shRNA-Box C/D-GFP and pENTR-shRNA-Box C/D mut-GFP, 0, 0.40, or 0.80 μg of pcDNA3.1-L7Ae-myc-His6 was added, and 2 μl of Lipofectamine 2000 was added per sample. These DNA-lipid complexes were incubated at room temperature for 20 minutes and added dropwise to the cells. 4 hours later, medium replacement was performed.

24 hours after the transfection, the medium in each well was removed, and the cells were dissociated using 200 μl of Trypsin-EDTA and suspended by the addition of 200 μl of DMEM/F12. The cell suspension was transferred to a FACS tube and analyzed using FACSAria (BD). In this context, live cells were gated, and FITC was determined for 20000 cells. The analysis was conducted using a general method comprising calculating a mean value of the fluorescence intensities of all the measured cells. FIG. **9** shows the results of FACS analysis (results showing a mean value of GFP intensities of 20000 cells per sample). The results demonstrated that recovery in GFP expression is seen in a manner specific for cells cotransfected with pcDNA3.1-L7Ae-myc-His6 and pENTR-shRNA-Box C/D-GFP. This suggested that as in the results of RT-PCR analysis, RNAi is inhibited in a Box C/D sequence-specific manner in the presence of L7Ae.

EXAMPLE 2

[Design of Protein Molecule-Responsive shRNA]

shRNA-U1A-4 for EGFP knockdown (5'-GGCAUCAAGGUGAACUUCAGGGCGAAAGCCCUGAAGUUCACCUUGAUGCCAG-3; SEQ ID NO: 14) was designed. The shRNA-U1A-4 comprised: 5'-terminal 24 bases which were the same as those in the passenger strand of shRNA-GFP shown in FIG. **5**(A) used in Example 1; a guide strand which formed a duplex therewith; and a nonhybridized loop structure GAAA. This shRNA-U1A-4 was functionally

similar to shRNA-GFP and was used as a negative control. Moreover, shRNA-Box C/D-GFP (SEQ ID NO: 9) of FIG. **5**(C) designed in Example 1 was used.

[In-Vitro Synthesis of shRNA]

[shRNA-U1A-4]

5.25 μL of template single-stranded DNA of shRNA-U1A-4 (100 μM, 5'-CTGGCATCAAGGTGAACT-TCAGGGCTTTCGCCCTGAAGTTCACCT-TGATGCCTAT AGTGAGTCGTATTAGC-3' SEQ ID NO: 31) and 5.25 μL of T7 sense primer (SEQ ID NO: 4) were used to perform transcription/synthesis and purification in the same way as in shRNA-Box C/D-GFP. The purification product was dissolved in 22 μL of ultrapure water. After concentration measurement, this solution was used in subsequent experiments.

[shRNA-Box C/D-GFP]

It was produced according to the in-vitro synthesis method described in Example 1.

[Expression and Purification of L7Ae Protein]

It was produced according to the L7Ae protein expression and purification using *E. coli* described in Example 1.

[Evaluation in Cultured Cell System and RNP]

[Confirmation of RNAi Inhibition of L7Ae-shRNA Complex by Observation Under Fluorescence Microscope]

On the day before transfection, HeLa-GFP cells were seeded over a 24-well plate at a concentration of 0.5×10^5 cells/well and cultured in a CO_2 incubator at 37° C. In this context, the HeLa-GFP cell strain was HeLa cells in which GFP was stably expressed in a hygromycin-resistant manner, and was kindly provided by Dr. T. Suzuki. On the next day, the medium in the 24 wells was replaced by 500 μl of Opti-MEM (Invitrogen Corp.). At the same time, complexes of shRNA-Box C/D-GFP and the L7Ae protein were introduced into the cells by transfection using Lipofectamine 2000 (Invitrogen Corp.) (trademark). 0.6 μl of 10 μM shRNA-Box C/D-GFP, 0 μl, 0.6 μl, 1.2 μl, 2.4 μl, 4.8 μl, or 9.6 μl of 20 μM L7Ae protein, and 2 μl of 5× binding buffer were mixed, and ultrapure water was added thereto to bring the whole amount to 10 μl (for 9.6 μl of the L7Ae protein, the whole amount was brought to 12.2 μl without the addition of ultrapure water). 40 μl (for 9.6 μl of the L7Ae protein, 37.8 μl) of Opti-MEM was further added thereto and mixed, and the mixture was left standing at 4° C. for 30 minutes to form RNA-protein complexes. 48 μl of Opti-MEM and 2 μl of Lipofectamine 2000 were mixed and left standing at room temperature for 5 minutes. To this mixture, 50 μl of the RNA-protein complex solution was added and mixed, and the mixture was left standing at room temperature for 20 minutes to form RNA-protein-lipid complexes, which were in turn added dropwise to the cells. 4 hours later, the medium was replaced by 500 μl of DMEM/F12. An shRNA-free molecule (Mock) and shRNA-U1A-4 were mixed with the L7Ae protein in the same way to form RNA-protein complexes, which were in turn introduced into the cells by transfection.

45 hours after the transfection, the cells were observed under a fluorescence microscope. The intracellular GFP fluorescence was photographed with settings of 20× magnification and 488 nM excitation wavelength on a fluorescence microscope (OLYMPUS) per sample. At the same time, a phase-contrast image of the cells was also taken using transmitted light. FIG. **10** shows a superimposed image of the fluorescence and phase-contrast images.

[Confirmation of RNAi Inhibition of L7Ae-shRNA Complex by FACS Analysis]

47 hours after the transfection, the medium in each well was removed, and the cells were dissociated using 200 μl of Trypsin-EDTA and suspended by the addition of 200 μl of DMEM/F12. The cell suspension was transferred to a FACS tube and analyzed using FACSAria (BD). The FACS is a method comprising irradiating, with laser light, free cells passing through a thin tube and analyzing the intensity of fluorescence emitted from the cells. In this context, live cells were gated, and FITC was determined for 10000 cells. FIG. **11** shows results of FACS-analyzing fluorescence intensity distribution. The results demonstrated that recovery in GFP expression was observed in a manner specific for the cells transfected with the complex of L7Ae and shRNA-Box C/D-GFP. This suggested that RNAi is inhibited in a Box C/D sequence-specific manner in the presence of L7Ae. For Mock, RNAi did not occur, and thus, GFP expression is seen in an L7Ae concentration-independent manner. On the other hand, for shRNA-U1A-4, RNAi was not inhibited, and thus, GFP expression was inhibited in an L7Ae concentration-independent manner.

EXAMPLE 3

[Design of Protein Molecule-Responsive shRNA]

shRNA-Box C/D-Bc1-xL (FIG. **13**(B)) and shRNA-Box C/D mut-Bc1-xL (FIG. **13**(C)) were designed by replacing a duplex site comprising the 5'-terminal 21 bases of shRNA-Box C/D-GFP or shRNA-Box C/D mut-GFP, by a duplex comprising a sequence of bases 365 to 385 of the Bc1-xL gene.

[Synthesis of shRNA]

[shRNA-Box C/D-Bc1-xL (FIG. **13**(B) (SEQ ID NO: 43))]

5.25 μL of shRNA-Box C/D-Bc1xL template (100 μM, 5'-CTGCTTTGAACAGGTAGTGAATGAT-CACGCCCTTTCGGGTCACATTCACTACCTGT TCAAAGCTATAGTGAGTCGTATTAGC-3' (SEQ ID NO: 44)) as template DNA of shRNA and 5.25 μL of T7 sense primer (100 μM, 5'-GCTAATACGACTCACTATA-3' (SEQ ID NO: 4)) were used to perform transcription/synthesis and purification in the same way as in shRNA-Box C/D-GFP. The purification product was dissolved in 22 μL of ultrapure water. After concentration measurement, this solution was used in subsequent experiments.

[shRNA-Box C/D mut-Bc1-xL (FIG. **13**(C) (SEQ ID NO: 45))]

5.25 μL of shRNA-Box C/D mut-Bc1xL template (100 μM, 5'-CTGCTTTGAACAGGTAGTGAATGAT-GACGCCCTTTCGGGCACATTCACTACCTGTT CAAAGCTATAGTGAGTCGTATTAGC-3' (SEQ ID NO: 46)) as template DNA of shRNA and 5.25 μL of T7 sense primer (100 μM, 5'-GCTAATACGACTCACTATA-3' (SEQ ID NO: 4)) were used to perform transcription/synthesis and purification in the same way as in shRNA-Box C/D-GFP. The purification product was dissolved in 22 μL of ultrapure water. After concentration measurement, this solution was used in subsequent experiments.

[shRNA-Bc1-xL (FIG. **13**(A) (SEQ ID NO: 47))]

5.25 μL of shRNA-Bc1-xL template (100 μM, 5'-CT-GCTTTGAACAGGTAGTGAATGAACTC-TATGCTAGTTCATTCACTACCTGTTCAA AGCTAT-AGTGAGTCGTATTAGC-3' (SEQ ID NO: 48)) as template DNA of shRNA and 5.25 μL of T7 sense primer (100 μM, 5'-GCTAATACGACTCACTATA-3' (SEQ ID NO: 4)) were used to perform transcription/synthesis and purification in the same way as in shRNA-Box C/D-GFP. The purification product was dissolved in 22 μL of ultrapure water. After concentration measurement, this solution was used in subsequent experiments.

[Confirmation of knockdown of Bc1-xL by shRNA-Bc1-xL]

On the day before transfection, HeLa-GFP cells were seeded over a 24-well plate at a concentration of 0.5×10^5 cells/well and cultured in a CO2 incubator at 37° C. On the next day, the cells were cotransfected with Bc1-xL, expression vectors, and shRNA using Lipofectamine 2000 (Invitrogen Corp.). 0 or 0.4 μg of pBc1-xL and 10 μM shRNA-Bc1-xL were mixed and brought to 50 μl with Opti-MEM I medium (Invitrogen Corp.). Then, a mixture of 1 μl of Lipofectamine 2000 supplemented with 49 μl of Opti-MEM I medium was added per sample. These DNA-lipid complexes were incubated at room temperature for 20 minutes. After addition of 400 μl of Opti-MEM I medium, the mixture was added dropwise to the cells. 4 hours later, medium replacement was performed.

24 hours after the transfection, the medium in each well was collected. Then, the cells were dissociated using 200 μl of Trypsin-EDTA and suspended by the addition of each medium collected in the previous step. The cell suspension was subjected to centrifugal sedimentation at 500×g at 4° C. for 5 minutes and washed with 500 μl of PBS. Then, to the cell pellet, 30 μl of RIPA buffer (1×PBS, 1% NP40, 0.5% Sodium deoxycholate, 0.1% SDS, 0.3 mg/ml PMSF+2 μg/ml Aprotinin) was added, and the mixture was left standing on ice for 30 minutes. The supernatant was collected by centrifugation (4° C., 15000 g, 20 minutes). The protein concentration was determined by the Lowry method using DC-Protein Assay (Bio-Rad Laboratories, Inc.).

Bc1-xL was detected by western blotting. Proteins extracted from the cells were developed by SDS-PAGE and subjected to western blotting. A primary antibody Anti-Bc1-xL (SC-634) (Santa Cruz Biotechnology, Inc.) (1/500) and a secondary antibody Goat Anti-Rabbit IgG (H+L)-HRP conjugate (Bio-Rad Laboratories, Inc.) (1/2000) were used. Color was developed using ECL Plus (GE Healthcare) (trademark) and detected using LAS3000 (FUJI FILM). By virtue of the cotransfection of shRNA-Bc1-xL with pBc1-xL, a band showing the expression of Bc1-xL did not appear. The results demonstrated Bc1-xL knockdown by shRNA-Bc1-xL in the HeLa cells.

[Confirmation of Dicer Cleavage Inhibition by In-Vitro Dicer Cleavage Assay]

The inhibition of Dicer cleavage of shRNA-Box C/D-Bc1-xL and shRNA-Box C/D mut-Bc1-xL by L7Ae was confirmed as follows using GTS, Inc. Recombinant Human Dicer Enzyme Kit according to the protocol: first, 0.4 μL of 1 μM shRNA, 2 μL of 4 μM or 8 μM L7Ae, 1 μL of 10 mM ATP, 0.5 μL of 50 mM $MgCl_2$, 4 μL of Dicer Reaction Buffer (GTS, Inc.), 2 μL of 0.5 unit/μL Recombinant Dicer Enzyme, and 0.1 μL of ultrapure water were mixed and reacted at 37° C. for 14 hours. Then, 2 μL of Dicer Stop Solution was added thereto and mixed. To 8 μL of this mixed solution, 2 μL of 5× dye solution was added, and the mixture was layered on a nondenaturing 15% polyacrylamide (1/30 bisacrylamide) gel and electrophoresed at 4° C. for 50 minutes. After the electrophoresis, the gel was stained with SYBR Green, and bands were confirmed using FLA-7000 (FUJI FILM). FIG. **14** shows results of the inhibition of Dicer cleavage of shRNA-Box C/D-Bc1-xL and shRNA-Box C/D mut-Bc1-xL by L7Ae using an in-vitro reconstituted Dicer system. In FIG. **14**, the mark "−" in L7Ae represents that L7Ae was not used. Likewise, the mark "+" in Dicer represents that Dicer was used, and the mark "−" represents that Dicer was not used. The results suggested that upon sequence-specific binding to L7Ae, shRNA-Box C/D-Bc1-xL is made insusceptible to cleavage by Dicer.

Construction/Synthesis of shRNA Expression Plasmid

[Synthesis of pENTR/H1/TO-shRNA-Box C/D-Bc1-xL (SEQ ID NO: 49)]

pENTR/H1/TO-shRNA-Box C/D-Bc1-xL was synthesized and purified in the same way as above using 5 μL of Box C/D Bc1-xL Top strand (200 μM, 5'-CACCGCTTTGAACAGGTAGTGAATGTGAC-CCGAAAGGGCGTGATCATTCACTACC TGTTCAAAGC-3' (SEQ ID NO: 50)) as single-stranded DNA containing an shRNA-encoding sequence for insertion to pENTR/H1/TO vectors (Invitrogen Corp.) and Box C/D Bc1-xL Bottom strand (200 μM, 5'-AAAAGCTTTGAACAGGTAGTGAATGATCACGCCCTTTCGGGTCA-CATTCACTACC TGTTCAAAGC-3' (SEQ ID NO: 51)) as single-stranded DNA containing a complementary strand thereof, and dissolved by the addition of 55 μL of ultrapure water. After plasmid vector concentration measurement, this was used in subsequent experiments.

[Synthesis of pENTR H1 TO-shRNA-Box C/D mut-Bc1-xL (SEQ ID NO: 52)]

pENTR/H1/TO-Box C/D mut-Bc1-xL was synthesized and purified in the same way as above using 5 μL of Box C/D mut Bc1-xL Top strand (200 μM, 5'-CACCGCTTTGAACAGGTAGTGAATGTGCCCGAAAGGGCGT-CATCATTCACTACCT GTTCAAAGC-3' (SEQ ID NO: 53)) as single-stranded DNA containing an shRNA-encoding sequence for insertion to pENTR/H1/TO vectors (Invitrogen Corp.) and Box C/D mut Bc1-xL Bottom strand (200 μM, 5'-AAAAGCTTTGAACAGGTAGTGAATGATGACGCCCTTTCGGGCACATTCACTACCT GTTCAAAGC-3' (SEQ ID NO: 54)) as single-stranded DNA containing a complementary strand thereof, and dissolved by the addition of 55 μL of ultrapure water. After plasmid vector concentration measurement, this was used in subsequent experiments.

[Synthesis of pENTR/H1/TO-shRNA-Bc1-xL (SEQ ID NO: 55)]

pENTR/H1/TO-shRNA-Bc1-xL was synthesized and purified in the same way as above using 5 μL of Bc1-xL Top strand (200 μM, 5'-CACCGCTTTGAACAGGTAGTGAATGAACTAGCATAGAGTTCATTCACTACCTGTT CAAAGC-3' (SEQ ID NO: 56)) as single-stranded DNA containing an shRNA-encoding sequence for insertion to pENTR/H1/TO vectors (Invitrogen Corp.) and Bc1-xL Bottom strand (200 μM, 5'-AAAAGCTTTGAACAGGTAGTGAATGAACTCTATGCTAGTTCATTCACTACCTGTT CAAAGC-3' (SEQ ID NO: 57)) as single-stranded DNA containing a complementary strand thereof, and dissolved by the addition of 55 μL of ultrapure water. After plasmid vector concentration measurement, this was used in subsequent experiments.

[Experiment of RNA-Protein Complex Introduction]

To confirm the control of Bc1-xL knockdown by the binding between the L7Ae protein and shRNA-Box C/D-Bc1-xL in cultured human cancer cells, RNA-protein complexes were introduced into the cells, and the expression of Bc1-xL was detected by western blotting.

On the previous day, uterine cervix cancer-derived HeLa cells were seeded over a 24-well plate at a concentration of 1.0×10^5 cells/well and cultured in a CO_2 incubator at 37° C. On the next day, transfection was performed twice using Lipofectamine 2000 (Invitrogen Corp.) (trademark). To 0.4 μg of pBc1-xL or pBimEL, 1 μl of Lipofectamine 2000 was added. These DNA-lipid complexes were incubated at room temperature for 20 minutes and added dropwise to the medium for HeLa cells. 4.5 hours later, medium replacement was performed. Immediately thereafter, the second transfec-

tion was performed. 2.5 pmol (concentration in the medium: 5 nM) of shRNA-Box C/D-Bc1-xL or shRNA-Box C/D mut-Bc1-xL and 0 or 200 pmol (concentration in the medium: 400 nM) of the purified L7Ae protein were mixed to form complexes. To the complexes, 1 μl of Lipofectamine 2000 was added, and the complexes were incubated at room temperature for 20 minutes and added dropwise to the medium for HeLa cells. 5 hours later, medium replacement was performed.

22 hours after the second transfection, the medium in each well was collected. Then, the cells were dissociated using 200 μl of Trypsin-EDTA and suspended by the addition of each medium collected in the previous step. The cell suspension was subjected to centrifugal sedimentation at 500× g at 4° C. for 5 minutes and washed with 500 μl of PBS. Then, to the cell pellet, 30 μl of RIPA buffer (1×PBS, 1% NP40, 0.5% Sodium deoxycholate, 0.1% SDS, 0.3 mg/ml PMSF+2 g/ml Aprotinin) was added, and the mixture was left standing on ice for 30 minutes. The supernatant was collected by centrifugation (4° C., 15000× g, 20 minutes). The protein concentration was determined by the Lowry method using DC-Protein Assay (Bio-Rad Laboratories, Inc.).

Bc1-xL or GAPDH was detected by western blotting. Proteins extracted from the cells were developed by SDS-PAGE and subjected to western blotting. A primary antibody Anti-Bc1-xL (SC-634) (Santa Cruz Biotechnology, Inc.) (1/500) and a secondary antibody Goat Anti-Rabbit IgG (H+L)-HRP conjugate (Bio-Rad Laboratories, Inc.) (1/2000) were used. Color was developed using ECL Plus (GE Healthcare) (trademark) and detected using LAS3000 (FUJI FILM). Likewise, GAPDH was subjected to western blotting using a primary antibody Anti-GAPDH (MAB374) (Chemicon International, Inc.) (1/2000) and a secondary antibody Goat Anti-Mouse IgG (H+L)-HRP conjugate (Bio-Rad Laboratories, Inc.) (1/2000). These results demonstrated that Bc1-xL knockdown was inhibited (lane 3) by the binding between the L7Ae protein and shRNA-Box C/D-Bc1-xL in the HeLa cells. Protein extraction from cells and L7Ae detection shown below were performed in the same way. FIG. **15** shows intracellular Bc1-xL expression. It was also confirmed that the protein expression level of GAPDH used as a standard control did not change. The intensities of detected Bc1-xL bands were added up. The results are shown in FIG. **16**. In FIGS. **15** and **16**, sh is an abbreviation of "shRNA".

[Experiment of Plasmid Introduction]

To confirm the control of Bc1-xL knockdown by the binding between the L7Ae protein and shRNA-Box C/D-Bc1-xL in cultured human cancer cells, the cells were cotransfected with plasmids for expressions of Bc1-xL, L7Ae, and shRNA, and the expression of Bc1-xL was detected by western blotting.

On the previous day, uterine cervix cancer-derived HeLa cells were seeded over a 12-well plate at a concentration of 3.0×10^5 cells/well and cultured in a CO_2 incubator at 37° C. On the next day, transfection was performed using Lipofectamine 2000 (Invitrogen Corp.) (trademark). 0.2 μg of pBc1-xL, 0.4 μg of pcDNA3.1-L7Ae, and 0.6 μg of pENTR/H1/TO-shRNA-Box C/D-Bc1-xL were mixed, and 2.5 μl of Lipofectamine 2000 was added thereto. These DNA-lipid complexes were incubated at room temperature for 20 minutes and added dropwise to the medium for HeLa cells. For pENTR/H1/TO-shRNA-Box C/D-mut Bc1-xL, pENTR/H1/TO-shRNA-Bc1-xL, and pENTR/H1/TO-shRNA-GFP mut (negative control), the same procedures were also performed. 0.25 μg of pcDNA-AmCyan-myc-His6 ((SEQ ID NO: 58) or 0.2 μg of pBc1-xL were introduced alone as a control plasmid into the cells. 4 hours later, medium replacement was performed.

24 hours after the transfection, the medium in each well was collected. Then, the cells were dissociated using 200 μl of Trypsin-EDTA and suspended by the addition of each medium collected in the previous step. The cell suspension was subjected to centrifugal sedimentation at 500× g at 4° C. for 5 minutes and washed with 500 μl of PBS. Then, to the cell pellet, 100 μl of RIPA buffer (1×PBS, 1% NP40, 0.5% Sodium deoxycholate, 0.1% SDS, 0.3 mg/ml PMSF+2 μg/ml Aprotinin) was added, and the mixture was left standing on ice for 30 minutes. The supernatant was collected by centrifugation (4° C., 15000 g, 20 minutes). The protein concentration was determined by the Lowry method using DC-Protein Assay (Bio-Rad Laboratories, Inc.).

Bc1-xL or GAPDH was detected by western blotting. Proteins extracted from the cells were developed by SDS-PAGE and subjected to western blotting. A primary antibody Anti-Bc1-xL (SC-634) (Santa Cruz Biotechnology, Inc.) (1/500) and a secondary antibody Goat Anti-Rabbit IgG (H+L)-HRP conjugate (Bio-Rad Laboratories, Inc.) (1/2000) were used. Color was developed using ECL Plus (GE Healthcare) (trademark) and detected using LAS3000 (FUJI FILM). Likewise, GAPDH was subjected to western blotting using a primary antibody Anti-GAPDH (MAB374) (Chemicon International, Inc.) (1/2000) and a secondary antibody Goat Anti-Mouse IgG (H+L)-HRP conjugate (Bio-Rad Laboratories, Inc.) (1/2000). These results demonstrated that Bc1-xL knockdown was inhibited (lane 5) by the binding between the L7Ae protein and shRNA-Box C/D-Bc1-xL in the HeLa cells. FIG. **17** shows intracellular Bc1-xL expression. It was also confirmed that the protein expression level of GAPDH used as a standard control did not change. The intensities of detected Bc1-xL bands were added up. The results are shown in FIG. **18**. In FIG. **18**, sh in the lanes 3 to 6 is an abbreviation of "shRNA".

[Experiment of Cell Death Control by Bc1-xL Expression Level Control Responsive to L7Ae Protein]

An apoptosis-promoting protein Bim-EL and an apoptosis-suppressing protein Bc1-xL have antagonistic effect on each other, and a relatively larger amount of the protein affects the fate of cells. Thus, an experiment was conducted which involved controlling Bc1-xL expression levels using the control of Bc1-xL knockdown by the binding between the L7Ae protein and shRNA-Box C/D-Bc1-xL in cultured human cancer cells, and controlling cell death by changing the relative amount of Bc1-xL to Bim-EL.

On the previous day, uterine cervix cancer-derived HeLa cells were seeded over a 24-well plate at a concentration of 0.5×10^5 cells/well and cultured in a CO_2 incubator at 37° C. On the next day, transfection was performed using Lipofectamine 2000 (Invitrogen Corp.) (trademark). To 0.3 μg of pENTR/H1/TO-shRNA-Box C/D-Bc1-xL, 0.2 μg of pBc1-xL, 0.2 μg of pBimEL, and 0.2 μg of pcDNA3.1-AsRed2-L7Ae were added and mixed with a medium, and 1.25 μl of Lipofectamine 2000 was added thereto. These DNA-lipid complexes were incubated at room temperature for 20 minutes and added dropwise to the medium for HeLa cells. Approximately 4 hours later, medium replacement was performed. For pcDNA3.1 (+) myc H is A vectors (Invitrogen Corp., control vector), pENTR/H1/TO-shRNA-Box C/D-mut Bc1-xL, pENTR/H1/TO-shRNA-Bc1-xL, and pENTR/H1/TO-shRNA-GFP mut (negative control), the same procedures were also performed.

24 hours after the transfection, the medium in each well was collected. Then, the cells were dissociated using 200 μl of

Trypsin-EDTA and suspended by the addition of each medium collected in the previous step. The cell suspension was subjected to centrifugal sedimentation at 500× g at 4° C. for 3 minutes and washed with 300 μl of PBS. Then, to the cell pellet, a mixed solution of 3 μl of annexin V, Pacific Blue conjugate for flow cytometry (Invitrogen Corp.) and 50 μl of annexin-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) was added. After tapping, the mixture was left standing at room temperature for 30 minutes for staining. Then, each sample was suspended by the addition of 200 μl of annexin-binding buffer. The cell suspension was transferred to a FACS tube and analyzed using FACSAria (BD). In this context, measurement was conducted for 30000 cells. For the analysis, first, cells emitting red fluorescence attributed to AsRed2-L7 were gated, and blue fluorescence intensity derived from Pacific Blue was measured for the cells within the gate using a filter at an excitation wavelength of 405 nm and a fluorescence wavelength of 430-470 nm. The ratio of the number of cells having this fluorescence intensity larger than the reference was measured. As the reference for determining cell death, phosphatidylserine, which is a lipid present in a manner specific for the outer membranes of dead cells, was stained with annexin V, Pacific Blue conjugate for flow cytometry (Invitrogen Corp.), and cells in which blue fluorescence intensity was larger than the upper limit of intensities of untreated cell samples stained in the same way were counted as dead cells.

As a result, the sample supplemented with the control vector (pcDNA3.1 (+) myc H is A vector) had a dead cell ratio of 8.3%, the sample supplemented with pENTR/H1/TO-shRNA-GFP mut (negative control) had a dead cell ratio of 10.1%, and the sample supplemented with pENTR/H1/TO-shRNA-Box C/D-Bc1-xL had a dead cell ratio of 13.4%. By contrast, the sample supplemented with pENTR/H1/TO-shRNA-Bc1-xL had a dead cell ratio of 34.5%, and the sample supplemented with pENTR/H1/TO-shRNA-Box C/D-mut Bc1-xL had a dead cell ratio of 42.5%. This experimental result and the Bc1-xL detection results shown in FIGS. **17** and **18** demonstrated that cells in which Bc1-xL expression level was suppressed by knockdown died approximately 3 to 4 times more than cells maintaining the expression of Bc1-xL. From the results showing that the sample supplemented with pENTR/H1/TO-shRNA-Box C/D-Bc1-xL had a dead cell ratio of 13.4%, whereas the sample supplemented with pENTR/H1/TO-shRNA-Box C/D-mut Bc1-xL had a dead cell ratio of 42.5%, it was further confirmed that cell death could be controlled suppressively by inhibiting Bc1-xL protein knockdown by L7Ae in a Box C/D sequence-specific manner.

Industrial Applicability

According to a protein-responsive shRNA and an RNAi control system using an RNP motif according to the present invention, a sensor shRNA and a protein specifically binding to it can control RNAi and are thus useful in the construction of biosensors for quantifying the expression of intracellular marker proteins without destroying cells or artificial gene circuits capable of activating the translation of proteins of interest in response to the expression level of marker proteins. For example, the present invention produces significant effect that leads to the treatment of diseases such as cancer or Alzheimer's disease by activating apoptosis-inducing proteins in response to the expression of cancer marker proteins or as a basic technique for developing protein drugs.

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<212> TYPE: RNA
<213> ORGANISM: Artificial
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<223> OTHER INFORMATION: shRNA-U1A-4

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<210> SEQ ID NO 15
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<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: L7AerN template

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<210> SEQ ID NO 16
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<212> TYPE: DNA

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<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: 481P template

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<210> SEQ ID NO 17
<211> LENGTH: 3930
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: pENTR/H1/TO-shRNA-BoxC/D-GFP

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<223> OTHER INFORMATION: pENTR L7Aer Top strand

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<210> SEQ ID NO 19
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<212> TYPE: DNA
<213> ORGANISM: Artificial
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<223> OTHER INFORMATION: pENTR L7Aer Bottom strand

<400> SEQUENCE: 19

aaaaggcatc aaggtgaact tcagcatcac gccctttcgg gtcagctgaa gttcaccttg 60

atgcc 65

<210> SEQ ID NO 20
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: H1 Forward Primer

<400> SEQUENCE: 20

tgttctggga aatcaccata 20

<210> SEQ ID NO 21
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: M13 Reverse Primer

<400> SEQUENCE: 21

caggaaacag ctatgac 17

<210> SEQ ID NO 22
<211> LENGTH: 3929
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: pENTR/H1/TO-shRNA-BoxC/D-mut-GFP

<400> SEQUENCE: 22

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gcgcccaata cgcaaaccgc ctctccccgc gcgttggccg attcattaat gcagctggca 180

cgacaggttt cccgactgga aagcgggcag tgagcgcaac gcaattaata cgcgtaccgc 240

tagccaggaa gagtttgtag aaacgcaaaa aggccatccg tcaggatggc cttctgctta 300

gtttgatgcc tggcagttta tggcgggcgt cctgcccgcc accctccggg ccgttgcttc 360

acaacgttca aatccgctcc cggcggattt gtcctactca ggagagcgtt caccgacaaa 420

caacagataa aacgaaaggc ccagtcttcc gactgagcct ttcgttttat ttgatgcctg 480

gcagttccct actctcgcgt taattcgccc ttagacatga taagatacat tgatgagttt 540

ggacaaacca caactagaat gcagtgaaaa aaatgcttta tttgtgaaat ttgtgatgct 600

attgctttat ttgtaaccat tataagctgc aataaacaag ttggggtggg cgaagaactc 660

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<210> SEQ ID NO 23
<211> LENGTH: 64
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: pENTR L7AerN Top strand

<400> SEQUENCE: 23

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tgcc 64

<210> SEQ ID NO 24
<211> LENGTH: 64
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: pENTR L7AerN Bottom strand

<400> SEQUENCE: 24

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tgcc 64

<210> SEQ ID NO 25
<211> LENGTH: 3926
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: pENTR/H1/TO-shRNA-GFP

<400> SEQUENCE: 25

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gcgcccaata cgcaaaccgc ctctccccgc gcgttggccg attcattaat gcagctggca 180

cgacaggttt cccgactgga aagcgggcag tgagcgcaac gcaattaata cgcgtaccgc 240

tagccaggaa gagtttgtag aaacgcaaaa aggccatccg tcaggatggc cttctgctta 300

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<210> SEQ ID NO 26
<211> LENGTH: 61
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: pENTR 481P Top strand

<400> SEQUENCE: 26

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c 61

<210> SEQ ID NO 27
<211> LENGTH: 61
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: pENTR 481P Bottom strand

<400> SEQUENCE: 27

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c 61

<210> SEQ ID NO 28
<211> LENGTH: 3926
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: pENTR/H1/TO-shRNA-GFP-mut

<400> SEQUENCE: 28

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<210> SEQ ID NO 29
<211> LENGTH: 61
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: pENTR Sk-7N Top strand

<400> SEQUENCE: 29

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<210> SEQ ID NO 37

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<211> LENGTH: 20
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<400> SEQUENCE: 37

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<400> SEQUENCE: 39

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<210> SEQ ID NO 40
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<220> FEATURE:
<223> OTHER INFORMATION: pcDNA3.1-AsRed2-myc-His6

<400> SEQUENCE: 42

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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: shRNA-BoxC/D- Bcl-xL

<400> SEQUENCE: 43

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<210> SEQ ID NO 44
<211> LENGTH: 82
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: shRNA-BoxC/D-BclxL template

<400> SEQUENCE: 44

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<210> SEQ ID NO 45
<211> LENGTH: 62
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: shRNA-BoxC/D mut-Bcl-xL

<400> SEQUENCE: 45

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<210> SEQ ID NO 46
<211> LENGTH: 81
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: shRNA-BoxC/D mut-BclxL template

<400> SEQUENCE: 46

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<210> SEQ ID NO 47
<211> LENGTH: 59
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: shRNA-Bcl-xL

<400> SEQUENCE: 47

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<210> SEQ ID NO 48
<211> LENGTH: 78
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: shRNA-Bcl-xL template

<400> SEQUENCE: 48

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atagtgagtc gtattagc 78

<210> SEQ ID NO 49
<211> LENGTH: 3930
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: pENTR/H1/TO-shRNA-BoxC/D-Bcl-xL

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<400> SEQUENCE: 49

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<210> SEQ ID NO 50
<211> LENGTH: 65
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: BoxC/D Bcl-xL Top strand

<400> SEQUENCE: 50

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aaagc 65

<210> SEQ ID NO 51
<211> LENGTH: 65
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: BoxC/D Bcl-xL Bottom strand

<400> SEQUENCE: 51

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aaagc 65

<210> SEQ ID NO 52
<211> LENGTH: 3929
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: pENTR H1 TO-shRNA-BoxC/D mut-Bcl-xL

<400> SEQUENCE: 52

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<210> SEQ ID NO 53
<211> LENGTH: 64
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: BoxC/D mut Bcl-xL Top strand

<400> SEQUENCE: 53

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<210> SEQ ID NO 54
<211> LENGTH: 64
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: BoxC/D mut Bcl-xL Bottom strand

<400> SEQUENCE: 54

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<210> SEQ ID NO 55
<211> LENGTH: 3926
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: pENTR/H1/TO-shRNA-Bcl-xL

<400> SEQUENCE: 55

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<210> SEQ ID NO 56
<211> LENGTH: 61
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Bcl-xL Top strand

<400> SEQUENCE: 56

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c 61

<210> SEQ ID NO 57
<211> LENGTH: 61
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Bcl-xL Bottom strand

<400> SEQUENCE: 57

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c 61

<210> SEQ ID NO 58
<211> LENGTH: 6173
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: pcDNA-AmCyan-myc-His6

<400> SEQUENCE: 58

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The invention claimed is:

1. An RNAi control system responsive to a protein expressed in a cell, the system comprising:

a vector for expression of an shRNA comprising: a guide strand having a sequence complementary to an mRNA of a target sequence; a passenger strand which forms a duplex with the guide strand; and a linker strand which links the guide strand and the passenger strand, the linker strand comprising a Box CD sequence,

wherein the binding of an L7Ae protein or L7Ae protein-containing fusion protein expressed in the cell to the shRNA inhibits the cleavage of the shRNA by Dicer.

2. An RNAi control method responsive to a protein expressed in a cell, the method comprising the step of:

introducing into the cell a vector for expression of an shRNA comprising a guide strand having a sequence complementary to an mRNA of a target sequence; a passenger strand which forms a duplex with the guide strand; and a linker strand which links the guide strand and the passenger strand, the linker strand comprising a Box CD sequence,

wherein the binding of an L7Ae protein or L7Ae protein-containing fusion protein expressed in the cell to the shRNA inhibits the cleavage of the shRNA by Dicer.

3. The RNAi control system according to claim **1**, wherein the target sequence of the shRNA is Bc1-xL mRNA, and the RNAi control system controls the expression of an apoptosis regulatory protein.

4. An shRNA comprising: a guide strand having a sequence complementary to an mRNA of a target sequence; a passenger strand which forms a duplex with the guide strand; and a linker strand which links the guide strand and the passenger strand, the linker strand comprising a Box CD sequence,

wherein in response to an L7Ae protein or L7Ae protein-containing fusion protein expressed in the cell, the cleavage of the shRNA by Dicer is inhibited to control the expression of a protein encoded by the mRNA of the target sequence.

5. The RNAi control system according to claim **1**, further comprising a vector for intracellular expression of the L7Ae protein or L7Ae protein-containing fusion protein.

6. The RNAi control system according to claim **1**, wherein the target sequence of the shRNA is GFP mRNA.

7. The RNAi control method according to claim **2**, wherein the target sequence of the shRNA is Bc1-xL mRNA, and the RNAi control method controls the expression of an apoptosis regulatory protein.

8. The RNAi control method according to claim **2**, further comprising the step of introducing a vector for intracellular expression of the L7Ae protein or L7Ae protein-containing fusion protein into the cell.

9. A method for quantifying the expression of an intracellular marker protein without destroying a cell, comprising the steps of:

introducing to the cell the shRNA of claim **4** wherein the target sequence is a GFP mRNA; and

measuring the fluorescence intensity of the GFP.

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