

RESEARCH ARTICLE

A novel expression system for artificial miRNAs containing no endogenous miRNA precursor sequences

Atsushi Shibata, Akiko Iwaki and Yasuyuki Fukumaki

Division of Disease Genes, Research Center for Genetic Information, Medical Institute of Bioregulation, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

*Correspondence to: Atsushi Shibata, Email: ash@gen.kyushu-u.ac.jp, Tel: +81 92 642 6168, Fax: +81 92 632 2375

Journal of RNAi and Gene Silencing (2007), 3(1), 237-247

© Copyright The Authors

(Received 19 February 2007; Revised 13 April 2007; Accepted 17 April 2007; Published online 27 May 2007)

ABSTRACT

Recently, artificial microRNA (miRNA)-mediated RNA interference (RNAi) systems have been developed as useful tools to study gene functions. We report an artificial miRNA precursor motif (AMPM) containing several restriction sites in the loop and in the extended stem structures, and generated a vector-based expression system for AMPM under the control of an RNA polymerase II promoter. The AMPM located in the intron or the exon of the selection marker gene mediated silencing of the reporter luciferase gene expression in HeLa cells. Northern blotting and colony formation assays revealed that the AMPM was efficiently and appropriately processed into mature miRNA. The AMPA system also down-regulated endogenous p53 and laminA/C proteins in stable transfectants. Moreover, clustered AMPMs separated by an appropriate spacer, targeting different sites within a single mRNA mediated gene silencing, even if the sequences of the target and the AMPM were partially complementary. This indicates the potential therapeutic utility of clustered AMPMs for highly-mutable targets, such as human immunodeficiency virus type 1.

KEYWORDS: *microRNA, miRNA, cluster, RNA polymerase II, HIV-1, gene therapy*

INTRODUCTION

RNA interference (RNAi) is now a powerful tool to suppress gene expression in various eukaryotic systems (Murchison and Hannon, 2004; Filipowicz, 2005; Sandy et al, 2005). In mammalian cells, chemically synthesized small interfering RNAs (siRNAs) and vector-based short hairpin RNAs (shRNAs), which are processed by a cytoplasmic endonuclease, Dicer, into siRNAs, have been used to mediate RNAi. Cells express small single-stranded RNAs 20-24 nucleotides (nt) in length, called microRNAs (miRNAs) (Lagos-Quintana et al, 2001; Lau et al, 2001; Lee and Ambros, 2001), which mediate gene silencing similarly to shRNAs (Bartel, 2004). miRNAs are encoded within intergenic regions and intronic regions of the eukaryotic genome, and are sometimes clustered (Altuvia et al, 2005; He et al, 2005). Initially, miRNAs are transcribed as a part of long precursor RNA (primary-miRNA or pri-miRNA) (Lee et al, 2002; Suh et al, 2004; He et al, 2005), and form a hairpin-like structure. A nuclear endonuclease, Drosha, processes this stem-loop

structure into miRNA precursors (pre-miRNAs) ~70 nt long (Zeng et al, 2005b). The nuclear pre-miRNAs are exported to the cytoplasm by a nuclear transport receptor, Exportin-5 (Yi et al, 2003; Bohnsack et al, 2004), and processed by Dicer into mature miRNAs ~22 nt long. After incorporation into miRNA-containing RNA-induced silencing complexes, miRNAs bind to their target mRNAs with partial complementarity, and direct degradation of mRNA and/or translational suppression (Yekta et al, 2004; Bagga et al, 2005; Petersen et al, 2006).

Effective gene silencing in cultured cells (Zeng et al, 2002; Boden et al, 2004; Stegmeier et al, 2005; Zeng et al, 2005a; Zhou et al, 2005; Chung et al, 2006) and mice (Li et al, 2006; Rao et al, 2006; Xia et al, 2006) using expression vectors for artificially designed miRNA precursors is well documented. In these reports, expression vectors for synthetic miRNA precursors were constructed by substitution of the stem regions of natural miRNA precursors, such as miR-30 or miR-155, to fully match arbitrary target mRNAs, but the loop sequences remained

intact. Here we designed a novel artificial miRNA, named AMPM, which incorporates restriction enzyme recognition sequences in the loop and stem region of the miRNA precursor structure. Despite the absence of any sequence of natural miRNA precursors, AMPMs were efficiently processed into mature miRNAs and suppressed the target gene expression. We also assessed the ability of dicistronic AMPM clusters targeting multiple sites within a single mRNA to suppress highly-mutable targets. The usefulness of AMPM as a gene silencing tool is discussed.

MATERIALS AND METHODS

Target site selection and AMPM design

The sequences of the GL3 firefly luciferase gene, the human p53 mRNA, the human lamin A/C mRNA and the HIV-1 genome were obtained from GenBank (GenBank accession Nos. U47296, NM000546, NM170707 and AF033819, respectively). The target site of the GL3 firefly luciferase included the target site of shRNA used in a previous report (Elbashir et al, 2001). The target sites of other genes were selected using the siRNA design tool freely provided by QIAGEN on the following website: (<http://www1.qiagen.com/Products/GeneSilencing/CustomSiRna/SiRnaDesigner.aspx>). This design tool makes choices of 21 nt long target sites of siRNAs based on an original score. Endogenous miRNA precursors are processed at positions 22–26 nt from their stem-loop junctions by Droscha (Weber, 2005). We therefore selected 26-nt target sequences. The antisense regions of all AMPMs used in this study were designed to be fully complementary to their target mRNAs. The sense regions were modified to form a mismatch and a bulge structure in their stem regions and G-U wobble pairs in the 3' half of the stem regions. Secondary structure of AMPMs was predicted using the program mfold (ver 3.2) (Zuker, 2003). (<http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi>).

Plasmid construction

Oligonucleotides used in plasmid construction are given in Table 1. To construct a parent vector for the AMPM cassette, the multiple cloning site (MCS) of pBluescript SK (+) (Stratagene) was replaced by a novel MCS in two steps. A pair of chemically synthesized oligonucleotides was first annealed to form MCS1 and inserted into the *Kpn* I-*Eco* RI site, and then MCS2 was similarly produced and inserted into the *Eco* RI-*Xba* I site of pBluescript SK (+). The resulting plasmid was designated as pBS-miR. To generate the AMPM-FL cassette, oligonucleotides corresponding to the antisense strand region (AMPM-FL-A) and the modified sense strand region (AMPM-FL-mS) were inserted into the pBS-miR vector. After digestion with *Pvu* II and *Eco* RV, the AMPM-FL cassette was inserted into the blunted *Bam* HI site and the *Nae* I site in pVITRO1 (Invivogen), generating the exonic and intronic AMPM vectors, pAMPM-FL-exon and pAMPM-FL-intron, respectively. Other AMPM vectors targeting the human p53 mRNA, the human lamin A/C, the HIV-1 integrase and the HIV-1 capsid genes were constructed in a similar manner using appropriate oligonucleotides given in Table 1. Human H1 promoter-driven shRNA vectors targeting the HIV-1 integrase and capsid genes, pH1-sh-IN1 and pH1-sh-CA1, were constructed using

oligonucleotide pairs sh-IN1, and sh-CA1, respectively, as previously described (Brummelkamp et al, 2002) except that the stem regions we used were 26 bp long.

The clusters of AMPM cassettes were constructed in pBS-miR. A pair of oligonucleotides, INT, consisting of partial sequence of an interval sequence in the human miR-17 cluster on chromosome 13 (GenBank accession No. AB176708), and an *Eco* RV recognition sequence, was inserted into the *Eco* RV-*Xba* I site of pBS-AMPM-IN1. The *Eco* RV site was used for further concatenation of the interval sequence unit, which was 24 bp in length. The AMPM-IN1 cassette, obtained by digestion of pBS-AMPM-CA1 with *Pvu* II and *Xba* I, was inserted into the *Eco* RV-*Xba* I site of pBS-AMPM vectors containing the AMPM-IN cassette and various lengths of interval sequences. The AMPM cluster vectors containing the AMPM-IN1 and AMPM-CA1 cassettes were constructed in a similar manner.

Series of reporter vectors, pFL-INs and pFL-CAs, were constructed as follows. Partial sequences encoding integrase (IN) and p24 capsid protein (CA) from several strains of HIV-1 were inserted into the *Xba* I site located in the 3'-UTR 7 bp downstream from the GL3 firefly luciferase open reading frame in the pGL3-Control vector (Promega). The IN1 and CA1 sequences were selected from an HIV-1 strain (GenBank accession No. AF033819). The sequences corresponding to IN1 from different strains of HIV-1, IN2, IN3 and IN4 were from GenBank accession Nos. AF484511, AY713418 and AF055729, respectively. The sequences CA2, CA3 and CA4 corresponding to CA1 were from GenBank accession Nos. AY134925, AY396897 and AY489925, respectively. To generate pFL-IN1-CA1, IN2-CA2, IN3-CA3 and IN4-CA4, oligonucleotides IN1, IN2, IN3 and IN4 were inserted into the *Xba* I site in the pFL-CA1, CA2, CA3 and CA4 vectors, respectively.

Cell culture and transfection

HeLa cells were grown in DMEM (Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (JRH Biosciences) and plated at 2×10^5 cells per well in 12-well plates or 4×10^5 cells per well in 6-well plates 24 hr prior to transfection. The cells were transfected using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions.

To isolate stable transfectants, HeLa cells transfected with pAMPM-p53 or pAMPM-lamin A/C were replated in 100-mm dishes 24 hr post-transfection, and cultured with 200 μ g/ml of hygromycin (Wako) for 2 weeks. Thereafter, colonies were counted, isolated and propagated.

Northern blot analysis

The chemically synthesized DNA oligonucleotide probes to detect the antisense strand region of AMPMs are as follows:

AMPM-FL:
5'-ATCACTTACGCTGAGTACTTCGAAATGT-3'

AMPM-p53:
5'-CTGGAAGACTCCAGTGGTAATCTACT-3'

Table 1. Oligonucleotides for plasmid vector construction

Name	Sense 5' → 3'	Antisense 5' → 3'
Oligonucleotides for pBS-ArmiR		
MCS1	CGGATCCCAGCTGCTCGAGGGATCCACTAGTG	AATTCAGTGGATCCCTCGAGCAGCTGGGATCCGGTAC
MCS2	AATTCAGATCTGTGACAGGATATCAAGCTTT	CTAGAAAGCTTGATATCCTGGTCGACAGATCTG
Oligonucleotides for ArmiR cassettes		
ArmiR-FL-A	TCGAGACATTTTCGAGTACTCAGCGTAAGTGATA	CTAGTATCACTTACGCTGAGTACTTCGAAATGTC
ArmiR-FL-mS	AATTCAGTTCGCTGGTACTTTGAAATGTG	TCGACACATTTCAAAGTACCAGCGGAAGTGG
ArmiR-p53-A	TCGAGAGTAGATTACCAGTGGAGTCTTCCAGTTA	CTAGTAACTGGAAGACTCCAGTGGTAATCTACTC
ArmiR-p53-mS	AATTCCTGGACGACTCAGTGGTAATTTATTG	TCGACAATAAATTACCAGTGGTCCGTCAGG
ArmiR-Lamin AC-A	TCGAGTGTAGATGTTCTTCTGGAAGTCCAGTTA	CTAGTAACTGGACTTCCAGAAGAATCTACAC
ArmiR-Lamin AC-mS	AATTCAGTGGCTTCCGAAGAATTTATAG	TCGACTATAAATATTTCTCGGAAGGCCAGTG
ArmiR-CA1-A	TCGAGATCATTCTTCTAGTGTAGCCGCTGGCTA	CTAGTAGCCAGCGCTACACTAGAAGAAATGATC
ArmiR-CA1-mS	AATTCAGCAGCTACCTAGAAGAAATGATG	TCGACATCATTCTTCTAGGTAGCTGCTGGG
ArmiR-IN1-A	TCGAGGAAAATATGCTGTTTCTGCGCTGTTCTA	CTAGTAGAACAGGGCAGGAAACAGCATATTTTCC
ArmiR-IN1-mS	AATTCACAGTGCAGGAACAGCATATTTTTG	TCGACAAAATATGCTGTTCTGCACTGTTG
Oligonucleotides for shRNA vectors		
Sh-IN1	GATCCCCGAAAATATGCTGTTTCTGCGCTGTTTTCAAG	AGCTTTTCCAAAAGAAAATATGCTGTTTCTGCGCTGT
	AGAAACAGGGCAGGAAACAGCATATTTTCTTTTGGAAA	TTCTCTTGA AAAACAGGGCAGGAAACAGCATATTTTCGGG
Sh-CA1	GATCCCCATCATTCTTCTAGTGTAGCCGCTGGTTCAAG	AGCTTTTCCAAAATCATTCTTCTAGTGTAGCCGCTG
	AGACCAGCGGCTACACTAGAAGAAATGATTTTTTGGAAA	GTCTCTTGAACCAGCGGCTACACTAGAAGAAATGATGGG
Oligonucleotides for interval sequence in ArmiR cluster		
INT	CATCCAATAATTCAAGCCAAAGATATCAAGCTTT	CTAGAAAGCTTGATATCTTTGGCTTGAATTATTGGATG
Oligonucleotides for target sequences of HIV-1		
IN1	CTAGAAACAGGGCAGGAAACAGCATATTTTCC	CTAGGGAAAATATGCTGTTTCTGCGCTGTTTT
IN2	CTAGAAACAGGGCAGGAAACAGCCTATTTTCC	CTAGGGAAAATAGGCTGTTTCTGCGCTGTTTT
IN3	CTAGAAACAGGGCAGGAAACAGCCTATTTTCC	CTAGGGAAAATAGGCTGTTTCTGCGCTGTTTT
IN4	CTAGAAACAGGACAGGAAACTGCCTATTTTCC	CTAGGGAAAATAGGCTGTTTCTGCTGCTGTTTT
CA1	CTAGACCAGCGGCTACACTAGAAGAAATGATC	CTAGGATCATTCTTCTAGTGTAGCCGCTGGT
CA2	CTAGACCAGCGGCTACCTAGAAGAAATGATC	CTAGGATCATTCTTCTAGGCTAGCCGCTGGT
CA3	CTAGACCAGCAGCTACCTAGAAGAAATGATC	CTAGGATCATTCTTCTAGGCTAGCTGCTGGT
CA4	CTAGACCAGGAGCCTCCTAGAAGAAATGATC	CTAGGATCATTCTTCTAAGGAGGCTCCTGGT

AMPM-lamin A/C:

5'-ACTGGACTTCCAGAAGAACATCTACA-3'

AMPM-IN1:

5'-AACAGGGCAGGAAACAGCATATTTTC-3'

AMPM-CA1:

5'-CCAGCGGCTACACTAGAAGAAATGAT-3'

A probe for the modified sense strand region of AMPM-FL was 5'-ACATTTCAAAGTACCAGCGGAAGTG-3'. These probes were labeled with γ -³²P ATP (MP Biomedicals) using T4 polynucleotide kinase (TOYOBO). HeLa cells were grown in 6-well plates and transfected with 8 μ g of each plasmid per well. After 48 hr of transfection, the cells were harvested and used for total

RNA extraction using Isogen (Nippongene). Twenty microgram total RNA was separated by electrophoresis in a 15% (w/v) polyacrylamide/8M urea gel, and electrotransferred onto a Hybond-N+ membrane (Amersham Pharmacia Biotech). After UV crosslinking, the membrane was hybridized with the ³²P-labeled DNA oligonucleotide probe in Church solution (1 mM EDTA, 0.5 M phosphate buffer and 7% (w/v) SDS) at 37°C overnight. Washing was carried out at 37°C twice in 2 x SSC and 0.1% (w/v) SDS for 15 min. The signals were detected using the FLA-8000 (Fujifilm).

Luciferase assay

HeLa cells were transfected in 24-well culture plates with 2 μ g of either AMPM expression vector or mock vector, 0.2 μ g of the pGL3-Control (Promega), pFL-IN1-4, pFL-

CA1-4 or pFL-IN1-4-CA1-4 reporter vectors, and 0.1 μ g of the pRL-TK (Promega) transfection control vector. The cells were harvested at 48 hr post-transfection. The luciferase activities were measured with the Dual Luciferase assay kit (Promega) using a Lumat LB9501 luminometer (Berthold). The luciferase activity was defined as the ratio of the reporter firefly luciferase activity to the internal control *Renilla* luciferase activity.

RT-PCR

Total RNA was extracted as described above. Reverse transcription was carried out using a GeneAmp RNA PCR kit (Applied Biosystems) with random hexamer primer according to the manufacturer's instructions. Polymerase chain reactions were carried out with the following primers: 5'-TCTTTTTTCGCAACGGGTTT-3' and 5'-GACCTCCGGTCACCTATTCA-3' for the hygromycin resistance gene (*Hyg^r*) (cycling parameters: 94°C for 2 min, followed by 28 cycles of 94°C for 20sec, 55°C for 10 sec, 72°C for 30 sec, and a final extension step of 72°C for 5 min) and 5'-GTCAGTGGTGGACCTGACCT-3' and 5'-AGGGGTCTACATGGCAACTG-3' for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (cycling parameters: 94°C for 2 min, followed by 28 cycles of 94°C for 20sec, 60°C for 10 sec, 72°C for 30 sec, and a final extension step of 72°C for 5 min). PCR products were separated in a 1.5% (w/v) agarose gel by electrophoresis, and photographed after staining with ethidium bromide.

Western blot analysis

HeLa cells stably expressing AMPMs against endogenous targets, the p53 or lamin A/C gene, were harvested and lysed in SDS sample buffer. Cell pellets were homogenized by brief sonication and protein content was determined by modified Lowry's procedure (Markwell et al, 1978). Twenty micrograms of total proteins were separated on 12.5% (w/v) SDS-PAGE gels and electrophoretically blotted onto Immobilon-P membranes (Millipore). Proteins were detected using specific primary antibodies, peroxidase-labeled secondary antibodies and the ECL Plus Western Blotting Detection System (Amersham Biosciences), and photographed using LAS-1000 (Fujifilm). The primary antibodies were mouse anti-p53 monoclonal antibody (DO-1) (Santa Cruz), rabbit anti-lamin A/C polyclonal antibody (H-110) (Santa Cruz) and rabbit anti-GAPDH polyclonal antibody (FL-335) (Santa Cruz), which were used at dilutions of 1:400, 1:800 and 1:200, respectively. The secondary antibodies were horseradish peroxidase-linked sheep anti-mouse Ig and donkey anti-rabbit Ig antibodies (Amersham Bioscience), which were used at dilutions of 1:5000 and 1:8000, respectively. We used the immuno-reactivity of GAPDH as an internal control. We quantified the signals in the resulting blots by densitometric analysis using Image Gauge (Fujifilm).

RESULTS

Design of the artificial miRNA precursors and cloning of the AMPM cassettes

AMPMs against the GL3 firefly luciferase (AMPM-FL), human p53 (AMPM-p53) and human lamin A/C (AMPM-lamin A/C) genes were designed and their sequences and a

predicted secondary structures are shown in Figure 1A. The target sites of AMPMs were selected as described in Materials and Methods. AMPMs were designed to mimic the secondary structure of the human miR-21 precursor (Zeng et al., 2005b) but they do not contain any sequence of miR-21 or any other endogenous miRNAs. Since a loop of >10 nt is essential for effective processing by Droscha (Zeng et al., 2005b), we used neighboring restriction sequences, *Spe* I and *Eco* RI, as the loop. The first two nucleotides of the loop structure were substituted to form a loop of 14 nt long in the mfold estimations. The antisense regions of AMPMs were designed to be fully complementary to the target sequences, but cytosine residues in the 3' half of the sense strand regions were substituted by uridine residues to enhance asymmetric incorporation of the antisense strand into RISC, so called 'strand bias' (Khvorova et al, 2003). Additionally, a mismatch and a bulge structure in their stem regions were produced by modification of the sense strand sequences to mimic miR-21. At the terminus of AMPM, a part of the *Pvu* II and *Xho* I sequences at the 5' terminus and the *Sal* I site and additional CAG sequences at the 3' terminus form an extended double-stranded region. We confirmed the secondary structures of AMPM, including a mismatch and a bulge structure in the stem region and a loop of 14 nt by mfold analysis.

Prior to construction of the AMPM expression system, we prepared a cloning vector, pBS-miR, containing multiple cloning sites appropriate for the construction of an AMPM cassette. The oligonucleotides corresponding to the antisense region of the target gene and its modified sense region were introduced into the *Xho* I-*Spe* I and *Eco* RI-*Sal* I sites, respectively, generating pBS-AMPM (Figure 1B).

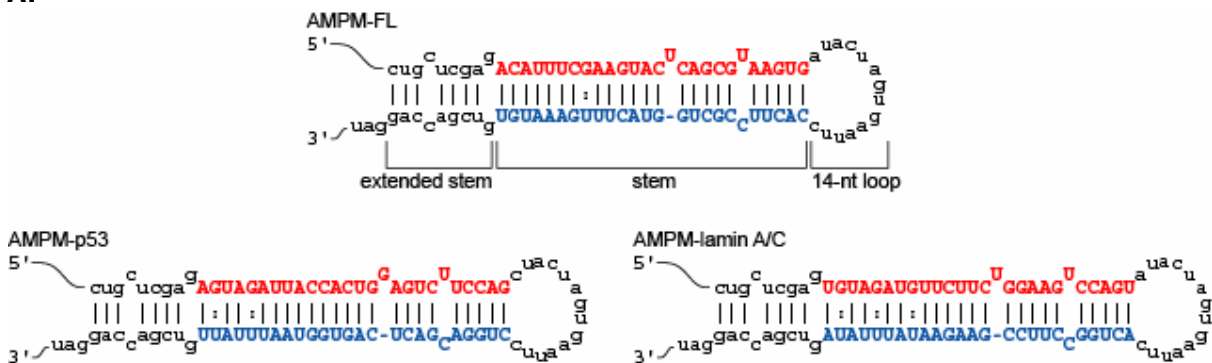
Construction of the artificial miRNA expression system

We generate an AMPM expression vector under the control of the EF1a promoter, we initially constructed an AMPM cassette targeting GL3 firefly luciferase (AMPM-FL). The AMPM-FL cassette (Figure 1) was excised from pBS-AMPM-FL by digestion with *Pvu* II and *Eco* RV, and was then inserted into either the exon (5'-UTR) or the intron of *Hyg^r* in pVITRO1. The resulting plasmids were designated pAMPM-FL-exon and pAMPM-FL-intron, respectively (Figure 2A).

To assess whether AMPM was appropriately processed into the mature miRNA, pAMPM-FL-exon and pAMPM-FL-intron were transfected into HeLa cells and the total RNA was isolated. Northern blot analysis showed a single band around 22 nt in length due to the expression of a shortly processed antisense region of AMPM-FL from both AMPM-FL vectors (Figure 2B). This data suggests that AMPM-FL is recognized as miRNA precursor and processed into mature miRNA, probably by Droscha and Dicer, regardless the location within a transcript. Moreover, a strand bias was observed because RNA molecule corresponding to the modified sense region could not be detected by northern blot analysis (data not shown).

When the AMPM-FL vectors were transfected together with the firefly luciferase reporter vector, luciferase assays showed that both vectors effectively suppressed the

A:



B:

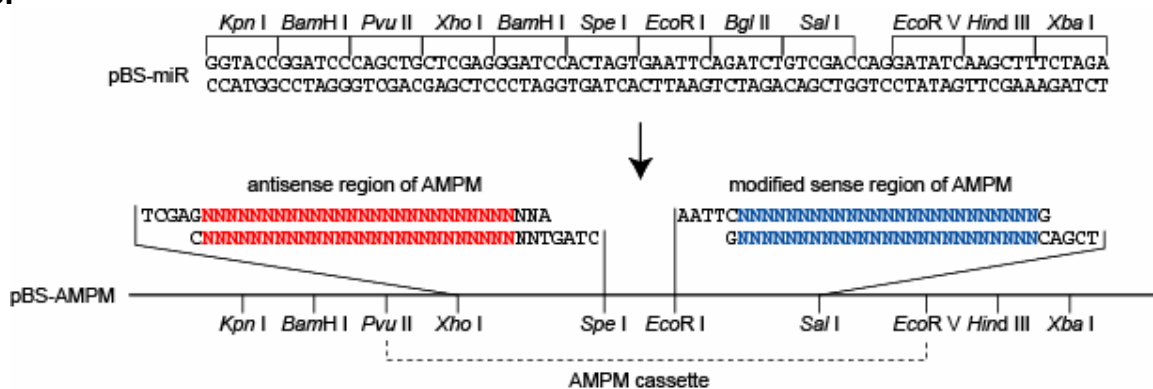


Figure 1. AMPM design and construction of AMPM cassettes. **A.** Nucleotide sequence and predicted secondary structure of AMPMs targeting the GL3 firefly luciferase (AMPM-FL), human p53 (AMPM-p53) and human lamin A/C (AMPM-lamin A/C) genes. The secondary structures of AMPMs were predicted using mfold program (see Materials and Methods). Each AMPM is composed of a 14 nt loop, a stem region containing a mismatch and a bulge structure, and an extended stem region containing two mismatches. The antisense regions indicated as red letters are fully complementary to the target sequences. The modified sense regions are indicated as blue letters. **B.** The schematic representation of the construction of the AMPM cassette. The multiple cloning sites of the parental vector, pBS-miR, are shown at the top. The oligonucleotide fragments containing the antisense region (red letters) and the modified sense region (blue letters) of AMPM were inserted into pBS-miR in two steps to generate the pBS-AMPM vectors.

luciferase activity (Figure 2C), suggesting that both the exonic and the intronic AMPMs can mediate gene silencing.

RT-PCR analysis showed that *Hyg^r* was expressed from the pAMPM-FL-intron as well as the empty control vector, but not from the pAMPM-FL-exon vector (Figure 2D). The expression of *Hyg^r* was further confirmed by colony formation assays. HeLa cells transfected with pAMPM-FL-intron normally formed colonies for 2 weeks under hygromycin selection, while no colonies were observed with pAMPM-FL-exon (data not shown). The ablation of the *Hyg^r* expression suggested the extremely effective processing of AMPM-FL located in the exon by Drosha in HeLa cells. Although northern blot analysis showed similar levels of AMPM-FL expression from the exonic and intronic AMPM-FL vectors, the processing of AMPM-FL in the intron did not affect the expression of

Hyg^r, suggesting that the splicing event probably precedes the processing of AMPM-FL by Drosha. Therefore, we used the intronic AMPM system for further gene silencing experiments to achieve both effective gene silencing and expression of the selection marker gene.

Silencing of endogenous genes by AMPM

We examined whether AMPMs could suppress the expression of endogenous genes. We used as targets the human p53 gene and the human lamin A/C gene, which were effectively suppressed in cultured cells by shRNAs (Brummelkamp et al, 2002; Sui et al, 2002). The intronic AMPM vectors targeting the p53 and the lamin A/C genes, pAMPM-p53 and pAMPM-lamin A/C, respectively, were each transfected separately into HeLa cells. After 2-week selection with hygromycin, independent clones of the cells stably expressing AMPM-p53 (Figure 3A) and AMPM-lamin A/C (Figure 3C) were obtained. Western blot

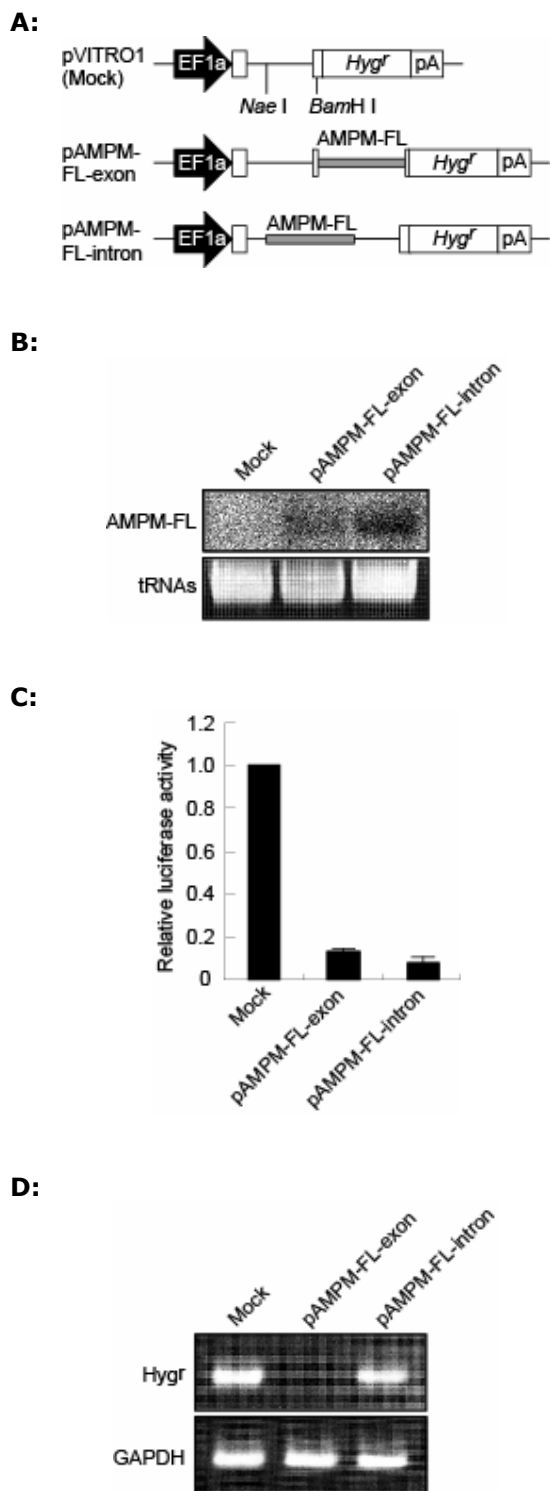


Figure 2. Evaluation of AMPM expression systems by comparison of the level of expression of AMPM and of silencing efficacy. **A.** Schematic representation of the AMPM expression vectors. To generate the pAMPM-FL-exon and pAMPM-FL-intron, the AMPM-FL cassette was inserted into the 5'-UTR and intron of *Hyg^r*, respectively, in pVITRO1. Gray boxes in the pAMPM-FL-exon and pAMPM-FL-intron indicate the AMPM-FL cassettes. EF1a, the promoter of the rat translation elongation factor 1a gene; *Hyg^r*, the hygromycin resistance gene; pA, the human elongation factor 1a polyadenylation signal. **B.** The

expression of a mature artificial miRNA targeting firefly luciferase. The pAMPM-FL-exon and pAMPM-FL-intron vectors were transiently transfected into HeLa cells and the total RNAs were isolated. Northern blot analysis using a radiolabeled oligonucleotide probe detected a band around 22 nt in length corresponding to the antisense strand region of AMPM-FL. Transfer RNAs (tRNAs) served as a loading control. **C.** Silencing efficacy of exonic and intronic AMPM-FL. The pAMPM-FL-exon, pAMPM-FL-intron or pVITRO1 vector was transfected with the pGL3-Control reporter vector and pRL-TK normalization vector into HeLa cells. Normalized luciferase activities were standardized relative to levels in lysates from cells transfected with the empty vector, pVITRO1. The values are means with S.E.M. (n=3). **D.** The influence of the insertion of the AMPM cassette on the expression of *Hyg^r*. The pAMPM-FL-exon, pAMPM-FL-intron or pVITRO1 vector was transfected into HeLa cells and the total RNAs were isolated 48 hours after transfection. RT-PCR was carried out with primers to detect *Hyg^r* and GAPDH mRNA. GAPDH served as a control.

analysis showed significantly reduced expression of each target gene in these cell lines (Figures 3B and 3D), indicating that AMPM can silence the expression of endogenous genes.

Mutations in target sequences reduce RNAi efficacy of AMPMs

As natural miRNAs mediate RNAi despite their partial complementarity to their targets (Lagos-Quintana et al, 2001; Lau et al, 2001; Lee and Ambros, 2001), we assumed that AMPMs could mediate gene silencing even if the target sequences were mutated. HIV-1 is known to mutate easily, resulting in various mutant strains. When HIV-1 was targeted by RNAi in previous studies, HIV-1 could escape from RNAi by mutations in its RNA genome (Boden et al, 2003; Das et al, 2004). We therefore inserted partial sequences of the HIV-1 integrase (IN1) and capsid (CA1) genes and their mutant sequences derived from different HIV-1 strains (see Materials and Methods) into the 3'-UTR of the firefly luciferase gene (Figures 4A and 4B), and used them as reporter vectors to assess the sequence-specificity of AMPMs. The intronic AMPM vectors, pAMPM-IN1 and pAMPM-CA1, were constructed to express AMPMs fully complementary to IN1 and CA1 sequences, respectively (Figures 4C and 4D). Additionally, to compare AMPMs with shRNAs, human H1 promoter-driven shRNA vectors targeting the IN1 and CA1 sequences, pH1-sh-IN1 and pH1-sh-CA1, were constructed (Figures 4E and 4F). Luciferase assays showed that both AMPMs and shRNAs suppressed the reporter gene expression when the target sequences were fully complementary. Introduction of mutations in the target sequences attenuated the suppression of the luciferase activity according to the number of the mutations (Figures 4G and 4H), suggesting that AMPMs cannot silence the mismatched targets, like shRNAs.

Targeting multiple sites by the AMPM cluster overcomes effects of mutations in target sequences

How do natural miRNAs mediate RNAi effectively despite their partial complementarity? We next took note of the fact that the target sites of natural miRNAs were often redundant. We assumed that AMPMs could overcome the

effects of mutations in RNAi by targeting multiple sites within a single mRNA. To examine this possibility, we constructed a series of reporter vectors that contained both HIV-1 IN1 and CA1 or their mismatched sequences in the 3'-UTR of the firefly luciferase gene (pFL-IN1-CA1, IN2-CA2, IN3-CA3 and IN4-CA4) (Figure 5A). The design of these constructs was based on the fact that the HIV-1 integrase and capsid proteins are encoded in a single transcription unit of the HIV-1 genome.

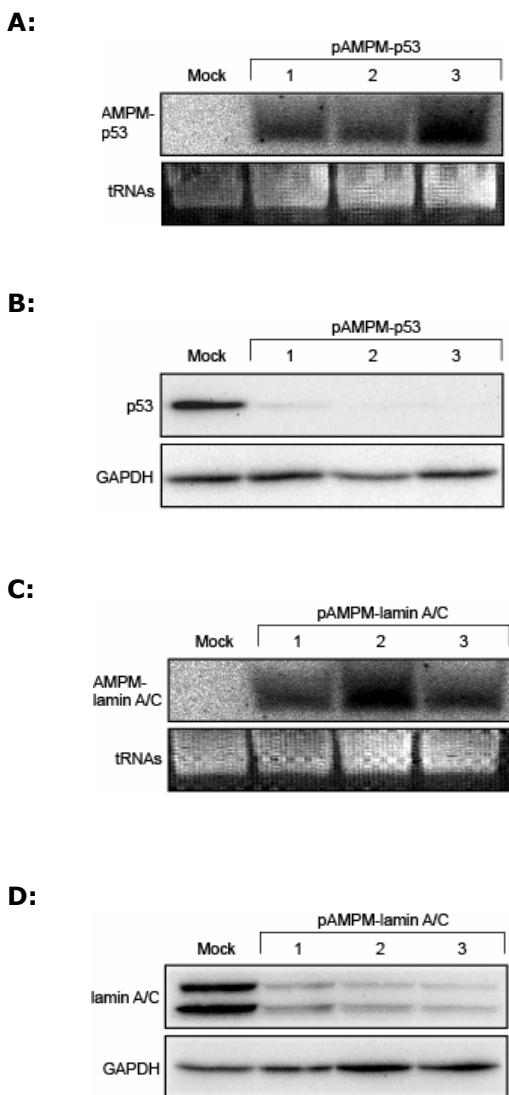


Figure 3. Stable gene silencing of endogenous genes by AMPMs. (A, C) Stable expression of AMPMs targeting endogenous genes. The total RNAs were isolated from stable transfectants of pAMPM-p53 (A) and pAMPM-lamin A/C (C). Northern blot analysis showed the expression of mature artificial miRNAs targeting p53 and lamin A/C as a single band around 22 nt in length. tRNAs served as a loading control. (B, D) Western blot analysis for p53, lamin A/C and GAPDH in cells stably expressing AMPM-p53 (B) and AMPM-lamin A/C (D). Lanes 1 to 3 contained samples from independently isolated cell lines. GAPDH served as a loading control.

The AMPM-IN1 and AMPM-CA1 cassettes were clustered with interval sequences of various lengths (Figure 5B), and the resulting AMPM cluster vectors were transfected into HeLa cells and total RNAs were isolated. Northern blot analysis showed that mature artificial miRNAs for IN1 and CA1 were produced from pAMPM-IN1-48-CA1 and pAMPM-IN1-96-CA1 as well as the monocistronic pAMPM-IN1 and pAMPM-CA1 vectors, while the products from pAMPM-IN1-0-CA1 could not be detected (Figure 5C). The level of IN1 miRNA expression from pAMPM-IN1-24-CA1 was relatively low, although that of CA1 was almost the same as pAMPM-CA1. These results indicate that the length of the interval sequence between two AMPMs can affect the processing of AMPMs.

AMPM cluster vectors were co-transfected with the various pFL-IN-CA reporter vectors into HeLa cells (Figure 5D). When we used pFL-IN1-CA1 as a reporter, the AMPM cluster vectors with interval sequences of 24, 48 and 96 bp showed enhanced gene silencing in comparison with the monocistronic pAMPM-IN1 and pAMPM-CA1 vectors. It is noteworthy that the dicistronic AMPM-IN1-48 and 96-CA1 vectors showed effective suppression even against mismatched target sequences, such as pFL-IN2-CA2 and pFL-IN3-CA3. These results indicate that targeting of multiple sites within a single mRNA by clusters of different AMPMs with an interval sequence of more than 48 nt could overcome the effects of mutations in target sequences. Therefore, this AMPM technology can be a powerful tool for gene therapy against viruses such as HIV-1, which often acquire drug resistance via mutations.

DISCUSSION

We have shown here that our artificially designed miRNA precursor, AMPM, that contains restriction sites in the loop and in the terminus of the stem region within the miRNA precursor motif, is useful as a gene silencing tool. Although AMPMs do not contain any sequence from natural miRNA precursors, they are effectively processed (perhaps by Drosha and Dicer), and the resultant short products can mediate effective gene silencing. Furthermore, the intronic AMPM system enables co-expression of artificial miRNAs and a selection marker gene. Although AMPM cassettes have to be cloned into pBS-miR prior to construction of AMPM expression vectors, this step makes the AMPM system flexible for application to various expression systems. Actually, we could easily insert AMPM cassettes into different vectors and observed coincidence of the expression of a fluorescent marker protein and effective gene silencing in several cell lines (data not shown).

The presence of mutations in the target sequence easily prevents suppression of the target gene expression by both monocistronic AMPMs and shRNAs. This observation indicates that AMPM technology can be applied to gene function analysis and gene therapy in a sequence-specific manner, like the shRNA system. We noticed that mutations in the CA target sequences affected silencing efficacy less than those in the IN target sequences. This may be due to the location of the mutations within the target sequences. In the CA target sequences, the core

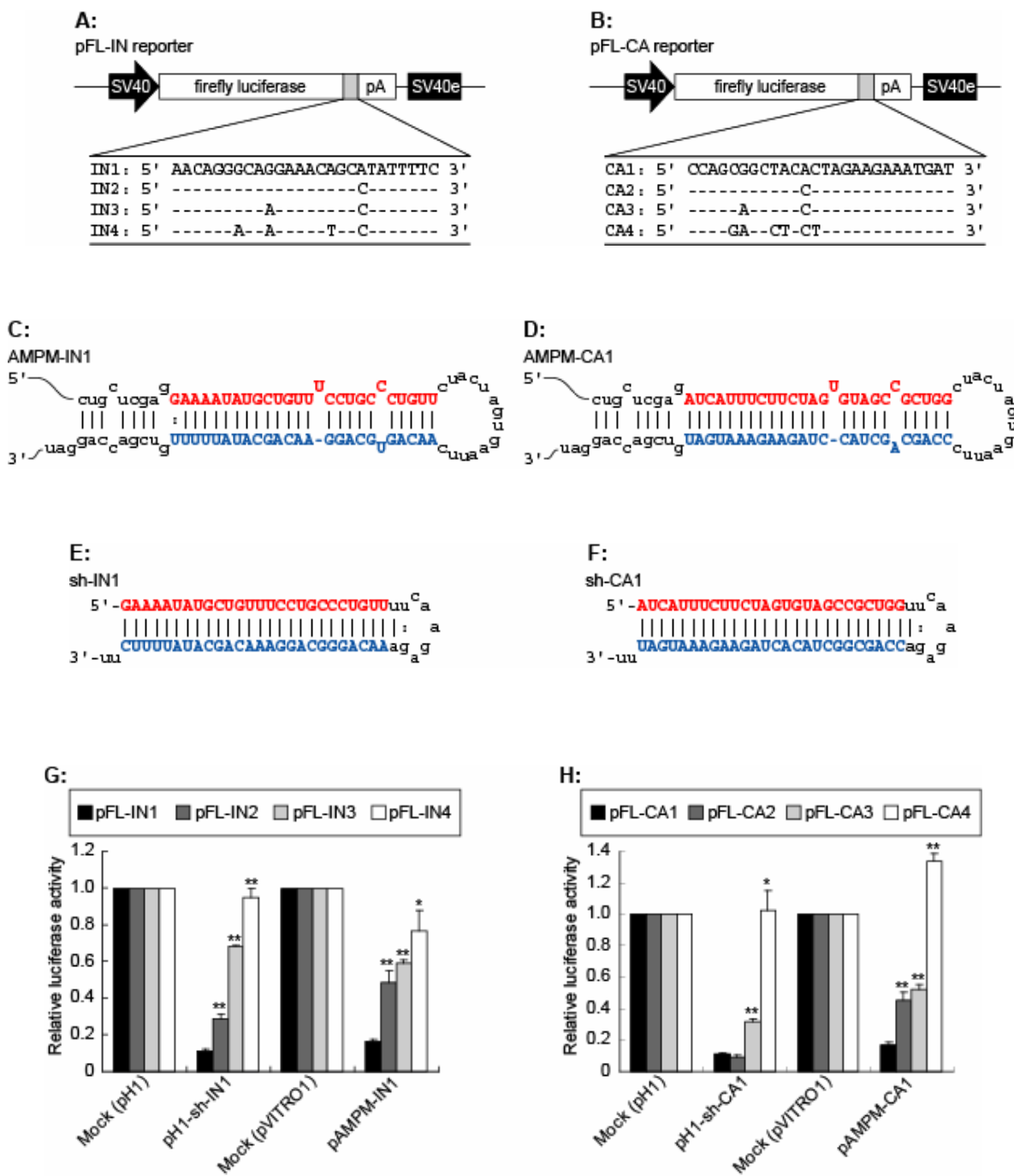


Figure 4. Effects of mutations in the target sequences. **(A, B)** The series of pFL-IN **(A)** and pFL-CA **(B)** reporter vectors containing the target sequences with or without mutations in downstream of the firefly luciferase gene are shown. SV40, the SV40 promoter; IN, a partial sequence of the HIV-1 integrase gene shown as a gray box; CA, a partial sequence of the HIV-1 p24 capsid protein gene shown as a gray box; pA, the SV40 late polyadenylation signal; SV40e, the SV40 enhancer. **(C, D)** The sequences and predicted secondary structures of AMPM-IN1 RNA **(C)** and AMPM-CA1 RNA **(D)**. The antisense regions are indicated as red letters, and the modified sense regions are indicated as blue letters. **(E, F)** The sequences and predicted secondary structures of sh-IN1 RNA **(E)** and sh-CA1 RNA **(F)**. The antisense regions of sh-IN1 and sh-CA1 (red letters) are the same as those of AMPM-IN1 and AMPM-CA1. The sense regions of sh-IN1 and sh-CA1 (blue letters) do not contain any modification, and are fully complementary to the antisense regions. **(G, H)** Silencing efficacy of shRNAs and AMPMs against the target sequences, IN **(G)** and CA **(H)**, with or without mismatches. The pFL-IN and pFL-CA reporter vectors were cotransfected with shRNA and AMPM expression vectors targeting IN and CA sequences, respectively. Normalized luciferase activities were standardized relative to levels in lysates from cells transfected with the empty vector pH1 for shRNAs or pVITRO1 for AMPMs. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, in t-tests comparing luciferase activities of the non-mutated reporter vectors (pFL-IN1 or pFL-CA1) with the mutated reporter vectors (pFL-IN2-4 or pFL-CA2-4). The values are means with S.E.M. (n=3).

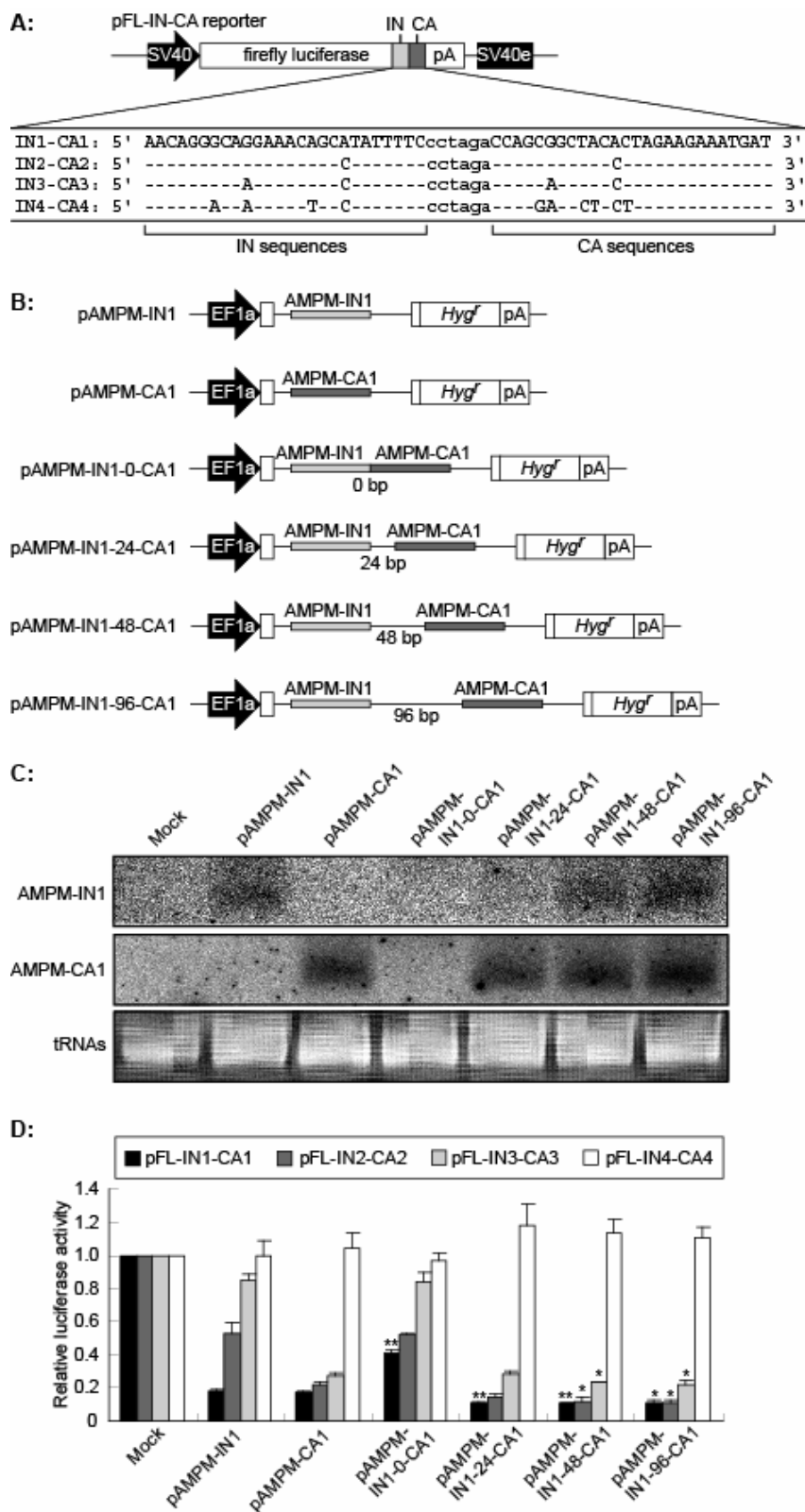


Figure 5. The AMPM cluster vectors targeting different sequences within a single mRNA and their silencing efficacy. **A.** The tandemly arranged target sequences are shown as capital letters below a schematic representation of the reporter vector. The IN and CA target sites were linked via the interval of 6 nt long. SV40, the SV40 promoter; IN, a partial sequence of the HIV-1 integrase gene shown as a light gray box; CA, a partial sequence of the HIV-1 p24 capsid protein gene shown as a dark gray box; pA, the SV40 late polyadenylation signal; SV40e, the SV40 enhancer. **B.** Schematic representation of the AMPM cluster vectors

with various lengths of interval sequences. The AMPM cassettes targeting the IN1 and CA1 sequences are shown as light gray and dark gray thin boxes, respectively. EF1a, the promoter of the rat translation elongation factor 1a gene; *Hyg^r*, the hygromycin resistance gene; pA, the human elongation factor 1a polyadenylation signal. **C.** The expression of mature artificial miRNAs from AMPM cluster vectors. The monocistronic AMPM vectors and AMPM cluster vectors were separately transfected into HeLa cells and the total RNAs were isolated. Northern blot analysis showed the shortly processed RNA derived from antisense strand region of AMPM-IN1 (top) and AMPM-CA1 (middle) around 22 nt in length. tRNAs served as a loading control. **D.** Silencing efficacy of each construct. The AMPM cluster vectors were co-transfected with the pFL-IN-CA reporter vectors. Normalized luciferase activities were standardized relative to levels in the lysate from cells transfected with the empty vector pVITRO1. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, in t-tests comparing the AMPM cluster vectors with the monocistronic AMPM vectors. The values are means with S.E.M. (n=3).

sequence for miRNA-mRNA interaction, located in the 3' half of the target sequences, is conserved, suggesting that the "seed" sequence of AMPM-CA1 is preserved (Tomari and Zamore, 2005).

In the AMPM cluster system, the interval sequence between two AMPMs affect the efficacy of RNAi. An interval sequence of 24 nt between two AMPMs was sufficient to mediate RNAi, although interval sequences of 48 nt and 96 nt were more efficient. Northern blot analysis showed the maximum production of mature artificial miRNAs when the interval sequences were more than 48 nt in length. These observations are consistent with the fact that the interval sequences between the nearest miRNA precursor motifs in the human miR-17 cluster (GenBank accession no. AB176708) are around 30-60 nt long. This may reflect the efficacy of recognition and processing of AMPM by Droscha. Indeed, Zeng and Cullen reported that flanking non-structured sequences are essential for efficient processing of pri-miRNA by Droscha (Zeng and Cullen, 2005).

We also showed that use of the AMPM cluster system overcame the effects of mutations in the multiple target sites within a single mRNA (Figure 5), suggesting the possible therapeutic usefulness of the AMPM technology against highly mutable targets such as HIV-1. Natural miRNAs often bind to clusters of repetitive target sites with partial complementarity to each other (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001), suggesting that miRNAs accomplish the fine-tuning of gene expression by the binding of multiple sites with partial complementarity to target mRNAs.

The AMPM system can also be used to temporally and spatially suppress gene expression based on the fact that it is a Pol II-driven system. A recent study suggested that excessive shRNA in cells causes oversaturation of endogenous miRNA pathways and often results in lethality in mice (Grimm et al., 2006). The AMPM system may prevent such side effects because the transcriptional levels of AMPMs could be controlled by the use of appropriate Pol II promoters.

CONCLUSIONS

- A newly designed artificial miRNA, named AMPM, can be efficiently processed perhaps by Droscha and Dicer, despite the absence of any sequence of natural miRNA precursors.

- AMPM can accomplish stable silencing of endogenous target genes in transfected cells.

- AMPM clusters targeting multiple sites within a single transcript can overcome mutations in the target sequences and be applied for the suppression of highly mutable targets such as HIV-1.

ACKNOWLEDGMENTS

This work was supported in part by the 21st Century COE Program from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

STATEMENT OF COMPETING INTERESTS

The authors declared no competing interests.

LIST OF ABBREVIATIONS

AMPM: Artificial miRNA precursor motif
MCS: Multiple cloning site
Hygr: Hygromycin resistance gene
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
HIV-1: Human immunodeficiency virus type 1

REFERENCES

- Altuvia Y, Landgraf P, Lithwick G et al. 2005. Clustering and conservation patterns of human microRNAs. *Nucleic Acid Res*, 33, 2697-2706.
- Bagga S, Bracht J, Hunter S et al. 2005. Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell*, 122, 553-563.
- Bartel DP. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, 116, 281-297.
- Boden D, Pusch O, Lee F, Tucker L and Ramratnam B. 2003. Human immunodeficiency virus type 1 escape from RNA interference. *J Virol*, 77, 11531-11535.
- Boden D, Pusch O, Silbermann R, Lee F, Tucker L and Ramratnam B. 2004. Enhanced gene silencing of HIV-1 specific siRNA using microRNA designed hairpins. *Nucleic Acid Res*, 32, 1154-1158.
- Bohnsack MT, Czaplinski K and Gorlich D. 2004. Exportin-5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-microRNAs. *RNA*, 10, 185-191.
- Brummelkamp TR, Bernard R and Agami R. 2002. A system for stable expression of short interfering RNAs in mammalian cells. *Science*, 296, 550-553.
- Chung KH, Hart CC, Al-Bassam S et al. 2006. Polycistronic RNA polymerase II expression vectors for RNA interference based on BIC/miR-155. *Nucleic Acid Res*, 34, e53.

- Das AT, Brummelkamp TR, Westerhout EM et al. 2004. Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. *J. Virol*, 78, 2601-2605.
- Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K and Tuschl T. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*, 411, 494-498.
- Filipowicz W. 2005. RNAi: the nuts and bolts of the RISC machine. *Cell*, 122, 17-20.
- Grimm D, Streetz KL, Jopling CL et al. 2006. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature*, 441, 537-541.
- He L, Thomson JM, Hemann MT et al. 2005. A microRNA polycistron as a potential human oncogene. *Nature*, 435, 828-833.
- Khvorovova A, Reynolds A and Jayasena SD. 2005. Functional siRNAs and miRNAs exhibit strand bias. *Cell*, 115, 209-216.
- Lagos-Quintana M, Rauhut R, Lendeckel W and Tuschl T. 2001. Identification of novel genes coding for small expressed RNAs. *Science*, 294, 853-858.
- Lau NC, Lim LP, Weinstein EG and Bartel DP. 2001. An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science*, 294, 858-862.
- Lee RC and Ambros V. 2001. An extensive class of small RNAs in *Caenorhabditis elegans*. *Science*, 294, 862-864.
- Lee Y, Jeon K, Lee JT, Kim S and Kim VN. 2002. MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J*, 21, 4663-4670.
- Li Z, Zhan W, Wang Z et al. 2006. Inhibition of PRL-3 gene expression in gastric cancer cell line SGC7901 via microRNA suppressed reduces peritoneal metastasis. *Biochem Biophys Res Commun*, 348, 229-237.
- Markwell MAK, Haas SM, Bieber LL and Tolbert LE. 1978. A modification of Lowry procedure to simplify protein determination in membranes and lipoprotein samples. *Anal Biochem*, 87, 206-210.
- Murchison EP and Hannon GJ. 2004. miRNAs on the move: miRNA biogenesis and the RNAi machinery. *Curr Opin Cell Biol*, 16, 223-229.
- Petersen CP, Bordeleau ME, Pelletier J and Sharp PA. 2006. Short RNAs repress translation after initiation in mammalian cells. *Mol Cell*, 21, 533-542.
- Rao MK, Pham J, Imam JS et al. 2006. Tissue-specific RNAi reveals that WT1 expression in nurse cells controls germ cell survival and spermatogenesis. *Genes Dev*, 20, 147-152.
- Sandy P, Ventura A and Jacks T. 2005. Mammalian RNAi: a practical guide. *Biotechniques*, 39, 215-224.
- Stegmeier F, Hu G, Rickles RJ, Hannon GJ and Elledge SJ. 2005. A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells. *Proc Natl Acad Sci USA*, 102, 13212-13217.
- Suh MR, Lee Y, Kim JY et al. 2004. Human embryonic stem cells express a unique set of microRNAs. *Dev Biol*, 270, 488-498.
- Sui G, Soohoo C, Affar EB et al. 2002. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc Natl Acad Sci USA*, 99, 5515-5520.
- Tomari Y and Zamore PD. 2005. Perspective: Machines for RNAi. *Genes Dev*, 19, 517-529.
- Weber MJ. 2005. New human and mouse microRNA genes found by homology search. *FEBS J*, 272, 59-73.
- Xia XG, Zhou H, Samper E, Melov S and Xu Z. 2006. Pol II-expressed shRNA knocks down Sod2 gene expression and causes phenotypes of the gene knockout in mice. *PLoS Genet*, 2, e10.
- Yekta S, Shih IH and Bartel DP. 2004. MicroRNA-directed cleavage of HOXB8 mRNA. *Science*, 304, 594-596.
- Yi R, Qin Y, Macara IG and Cullen BR. 2003. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev*, 17, 3011-3016.
- Zeng Y, Cai X and Cullen BR. 2005a. Use of RNA polymerase II to transcribe artificial microRNAs. *Methods Enzymol*, 392, 371-380.
- Zeng Y and Cullen BR. 2005. Efficient processing of primary microRNA hairpins by Drosha requires flanking nonstructured RNA sequences. *J Biol Chem*, 280, 27595-27603.
- Zeng Y, Wagner EJ and Cullen BR. 2002. Both natural and designed microRNAs can inhibit the expression of cognate mRNAs when expressed in human cells. *Mol Cell*, 9, 1327-1333.
- Zeng Y, Yi R and Cullen BR. 2005b. Recognition and cleavage of primary microRNA precursors by the nuclear processing enzyme Drosha. *EMBO J*, 24, 138-148.
- Zhou H, Xia XG and Xu Z. 2005. An RNA polymerase II construct synthesizes short-hairpin RNA with a quantitative indicator and mediates highly efficient RNAi. *Nucleic Acid Res*, 33, e62.
- Zuker M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res*, 31, 3406-3415.

SHORT COPYRIGHT STATEMENT

This is an open access article, published under the terms of the Licence for Users available at <http://www.libpubmedia.co.uk/RNAi/LicenceForUsers.pdf>. This licence permits non-commercial use, distribution and reproduction of the article, provided the original work is appropriately acknowledged with correct citation details.