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A lentiviral vector encoding two fluorescent proteins enables imaging of adenoviral infection via adenovirus-encoded miRNAs in single living cells

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MicroRNAs (miRNAs) are non-coding small RNAs that have been found in various kinds of eukaryotes and viruses. Recently, adenovirus non-coding RNAs, VA RNAs, have been reported to generate miRNAs. Here, we developed a lentiviral vector for monitoring adenovirus-derived miRNAs in living cells. By using red and green fluorescent proteins under the control of bi-directional two distinct promoters, adenoviral infection and consequent miRNA expression was successfully visualized and quantified by the reduction in green fluorescence when 3′-untranslated regions were connected to the target sequences of the adenovirus-derived miRNAs. Our functional analysis using a lentiviral vector is a useful method to examine the activity of miRNA in living mammalian cells.

Keywords: adenovirus/lentiviral vector/microRNA/transcriptional interference/VAI RNA.

Abbreviations: CMV, cytomegalovirus; EF-1α, elongation factor-1α; GFP, green fluorescent protein; HIV-1, human immunodeficiency virus-1; miRNA, microRNA; MOI, multiplicity of infection; PGK, phosphoglycerate kinase; RFP, red fluorescent protein; UTR, untranslated region; dsRNA, double-stranded RNA; RT-PCR, Reverse Transcription-Polymerase Chain Reaction.

MicroRNAs (miRNAs) are a class of small regulatory RNAs that post-transcriptionally regulate gene expression through sequence-specific interactions with the 3′-untranslated regions (UTRs) of target mRNAs (*1*–*3*). Some miRNAs show localized expression patterns with limited tissue, cell and spatio-temporal specificities (*4*). Hundreds of miRNAs have been identified in various eukaryotes, and many miRNAs are evolutionarily conserved, suggesting their important roles in biological processes.

Not only endogenous miRNAs in cellular genomes but also exogenous miRNAs in viral genomes have recently been reported (*5*–*7*). Potential precursor miRNAs are predicted based on their stem-loop structure by computational analysis and the corresponding small RNAs of ~22 nt in length are detected in mammalian DNA viruses, most often in the herpesvirus family. Several small RNAs derived from viruses function to reduce their target genes expression as miRNAs. For example, miRNAs derived from simian virus 40 (SV40) regulate their own mRNAs encoding large T antigen with perfect complementarity (*8*). Since large T antigen is recognized by T lymphocytes, the miRNA-mediated reduction in large T antigen levels at the late stage helps the virus to escape from the cellular immune system governed by T cells. Human cytomegalovirus (CMV)-encoded miRNAs also show autoregulation of viral genes with their own miRNAs (*9*).

Several groups have reported that adenovirus non-coding RNAs could be the source of miRNAs (*10*–*12*). Adenovirus type 2 encodes two non-coding RNAs, VAI and VAII, both of which are transcribed by RNA polymerase III. VAI RNA is highly accumulated at the late stage after infection at 10^8 copies per cell (*13*, *14*), and is conserved among adenovirus serotypes (*15*). VA RNAs were first recognized as positive regulators for the neutralization of an interferon-related cellular defense mechanism (*16*, *17*). VAI RNA binds to and blocks the dsRNA-induced protein kinase PKR, which is activated at late stages of infection due to symmetric transcription of the viral genome (*18*, *19*). We previously found that an imperfectly base paired terminal stem of VAI RNA was processed into small RNAs that strongly suppressed the expression of their target genes containing perfectly or imperfectly complementary sequences during viral infection (*10*). However, the specific mechanism of adenoviral miRNA-mediated antagonization of the cellular anti-viral system remains unknown. To understand the molecular mechanism involving adenoviral miRNAs, it is crucial to monitor the dynamics of miRNAs during the course of adenoviral infection.

In addition to direct detection of miRNAs such as RT-PCR and northern analysis, indirect detection of miRNAs has been reported by using reporter genes whose UTRs are connected to the target sequences of the miRNAs (*20*–*23*). Since miRNAs repress protein translation, if the target sequence of the miRNAs is located down-stream of the reporter genes, the presence of miRNAs would induce decreases in the reporter signal. For example, the gene for luciferase or β-galactosidase was connected to the target sequence of an miRNA (*22*, *23*). Recently, Brown *et al.* (*20*, *21*) reported that a lentiviral vector encoding green fluorescent protein (GFP) connected to a target sequence

allowed them to visualize the activity of miR-142-3p followed by immunostaining of an internal control gene using fixed tissues. Such methods including homogenization or fixation of cells or tissues prevent from observation of dynamic action of miRNAs. We have previously developed a retroviral vector to monitor the specific miRNA activity in living cells (*24*). Using two fluorescent proteins as reporters, the muscle-specific miRNA, miR-133, in mouse myoblasts was successfully analysed using fluorescence microscopy. However, our retroviral system could to a certain extent infect dividing rodent cells but not primate cells.

In the present study, a novel lentiviral vector was developed to monitor specific miRNAs in living cells. Since the lentiviral vector becomes integrated into the cellular genome, expression of the transgenes was retained during the course of the adenoviral life cycle. Moreover, the two bi-directionally encoded fluorescent proteins could be coordinately expressed and the risk of fluctuating expression of the transgenes due to random integration of the lentiviral vector into the cellular genome can be reduced. Here, we demonstrate that the adenoviral infection could be followed by visualizing the GFPs and red fluorescent proteins (RFPs) in living cells. This quantitative analysis at the single cell level indicates that our lentiviral system should represent a novel and useful tool for real-time and multicolour monitoring of miRNAs of interest in living cells.

Materials and methods

Lentiviral vector construction

To construct lentiviral vectors, two connected genes were amplified by a fusion PCR method (*25*) using Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA). GFP and RFP were interchangeable by using the same primers and restriction sites.

pLV.WPRE. To make unique restriction sites, the basic vector pLV.WPRE was constructed by rearranging the original vector pCMV-GIN-ZEO (Open Biosystems, Odyssey Drive, AL, USA). The WPRE region was amplified by PCR using pCMV-GIN-ZEO as a template with the primers 5′-AAAGGGCCCTTCGAAGCGG CCGCCCTCGAGGAATCAACCTCTGGATTACAAA-3′, 5′-AT GTTTTTCTAGGTCTCGATGATCAGCGGGGAGGCGGCCC A-3′ and 5′-AAAGGTACCTGAGGTGTGAC-3′. The PCR product was digested with ApaI plus KpnI and introduced into pCMV-GIN-ZEO at ApaI plus KpnI sites to generate pLV.WPRE.

pLV.PtR. The mouse phosphoglycerate kinase (PGK) promoter was amplified using genomic DNA extracted from NIH3T3 cells with the primers 5′-ACAGCGGCCGCAATTCTACCGGGTAGG G-3′ and 5′-CTTGCTCACCATGGTGGCTAGCGAAAGGCCCG GAGATG-3′. RFP connected to the PGK promoter was amplified using the resultant PCR product and pRSET-tdTomato (*26*) with the primers 5′-TTCCTCGAGGATCCCGGGAATTCATTTGTAC AGCTCGTCC-3 and 5′-ACAGCGGCCGCAATTCTACCGGGT AGGG-3′. The PCR product corresponding to the monomer was digested with NotI plus XhoI and introduced into pLV.WPRE at NotI plus XhoI sites to generate pLV.PtR.

pLV.SGx.PtR. GFP connected to the SV40 promoter was amplified using pEGFP (Clontech, Palo Alto, CA, USA) and pMX-puro (*27*) as templates with the primers 5′-AATTGCGGCCGCTGTGGAAT GTGTGTCAGTTA-3′, 5′-TTGCTCACCATGGTGGCATCTCCT TCTAGATTTGCAAAAGCCTAGGCCTCCAAA-3′ and 5′-AAA TTCGAAATCGATTTTGTACAGCTCGTCCAT-3′. The PCR product was digested with NotI plus Csp45I and introduced into pLV.PtR at NotI plus Csp45I sites to generate pLV.SGx.PtR.

pLV.SGb.PtR. The polyadenylation signal connected to the blasticidin-resistance gene was amplified using pTracer-EF/Bsd (Invitrogen, Carlsbad, CA, USA) as a template with the primers 5′-GGGCCCTTCGAACCATAGAGCCCACCGCATC-3′, 5′-CT GGCAACTAGAAGGCACAGACGCGTGGATCCCGGGAATT CAGCCCTCCCACACACATAACC-3′ and 5′-AATCTAGACCA TGGGAATCGATGGCCAGGCCAAGC-3′. The PCR product was digested with Csp45I plus ClaI and introduced into pLV.SGx.PtR at a ClaI site to generate pLV.SGb.PtR.

pHV.SGb.PtR. The RSV promoter was amplified using pLenti6 (Invitrogen) as a template with the primers 5′-TGACGTCGACAA TGTAGTCTTATGCAATACTCT-3′ and 5′-ATGTTTTTCTAGG TCTCGATGATCAGCGGGGAGGCGGCCCA-3′. The PCR product was digested with SalI plus BssHII and introduced into pLV.SGb.PtR at SalI plus BssHII sites to generate pHV.SGb.PtR.

pHV.SiGb.PtR. An artificial intron region was amplified using pBIND (Promega, Madison, WI, USA) as a template with the primers 5′-GGAGGCCTAGGCTTTTGCAAACTTCTGACACAAC AGTCTCG-3′ and 5′-TTGCTCACCATGGTGGCATCTCCTTC TAGATTTGCAAAAGCCTAGGCCTCCAAA-3′. The PCR product was digested with AvrII plus XbaI and introduced into pHV.SGb.PtR at AvrII plus XbaI sites to generate pHV.SiGb.PtR.

pHV.FtRb.PG. The elongation factor-1α (EF-1α) promoter was amplified using pTracer-EF/Bsd as a template with the primers 5′-AAAAAGCGGCCGCGGCTCCGGTGCCCGTCA-3′ and 5′-C CCTCTAGATCACGACACCTGAAATGGAAG-3′. The PCR product was digested with NotI plus XbaI and introduced into pHV.StRb.PG at NotI plus XbaI sites to generate pHV.FtRb.PG.

Construction of miRNA sensor vectors

A fragment corresponding to a three tandem repeat of a target sequence that was completely complementary to the double-stranded region of VAI RNA was prepared using the self-extension reaction of a primer by T7 DNA polymerase (New England Biolabs) with the following individual primers: VAI T5, 5′-AAGAATTCACCAGAC CACGGAAGAGTGCCCACCAGACCACGGAAGAGTGCCCA CCAGACCACGGAAGAGTGCCCGGATCCGG-3′; VAI T3, 5′-AAGAATTCAAGGAGCGCTCCCCCGTTGTCTGAAGGAG CGCTCCCCCGTTGTCTGAAGGAGCGCTCCCCCGTTGTCT GGATCCAG-3′; VAI A5, 5′-AAGAATTCAACCCCGGTCGTCC GCCATGATAACCCCGGTCGTCCGCCATGATAACCCCGGT CGTCCGCCATGATAGGATCCTA-3′; VAI A3, 5′-AAGAATTC ATCACGGCGGACGGCCGGATCTATCACGGCGGACGGCC GGATCTATCACGGCGGACGGCCGGATCCGG-3′; miR-1, 5′-AAGAATTCTACATACTTCTTTACATTCCATACATACTTC TTTACATTCCATACATACTTCTTTACATTCCAGGATCCTG-3′; let-7, 5′-AAGAATTCAACTATACAACCTACTACCTCAACT ATACAACCTACTACCTCAACTATACAACCTACTACCTCAG GATCCTG-3′. The resultant fragments were digested with BamHI and EcoRI and introduced into pLV.SGb.PtR at BamHI and EcoRI sites.

Northern blot analyses

Total RNA was extracted and purified with the ISOGEN reagent (Wako, Osaka, Japan). Aliquots of total RNA (50 μg/lane) were electrophoresed in a denaturing 12% polyacrylamide gel of 30 cm in length. A plasmid pAdVAntage (Promega) was used for the analysis. The separated RNA bands were electrotransferred to a Hybond-XL membrane (Amersham Bioscience, Piscataway, NJ, USA). The membrane was stained with 0.02% methylene blue (Sigma, St Louis, MO, USA) in 0.3 M sodium acetate buffer (pH 5.2) to stain tRNAs. The membrane was probed with ^{32}P-labelled RNAs that were complementary to the sequences of the individual dsRNA region.

Cultured cells and lentivirus production

293T and HT1080 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine

serum and a mixture of antibiotics (Invitrogen). Plasmids were transfected with the cationic transfection reagent TransIT-293 (PanVera, Madison, WI, USA) according to the manufacturer's protocol. 293T cells were grown to ~80% confluence in 6-well plates and transfected with 0.8 μg of lentiviral plasmid, 0.4 μg of pMDL-gp-RRE, 0.4 μg of pRSV-Rev and 0.4 μg of pCMV-VSVG (*28*). After 18 h, the culture medium was exchanged for fresh medium. After a further 30 h of culture, the culture medium was collected and passed through a 0.45 μm filter. The viruses in the filtered medium samples were directly added to HT1080 cells with 5 μg/ml polybrene (Sigma). At 2 days after the infection, cells were selected with 100 μg/ml blasticidin (Sigma). Recombinant viruses were handled under the safety guidelines for living modified organisms.

Flow cytometry analysis

Cells were infected with the recombinant adenovirus Ad2/βGal-4, which was kindly provided by Genzyme Corporation (Framingham, MA, USA) (*29*). At 5 days after adenoviral infection, cells were trypsinized and analysed using a FACSAria (Becton Dickinson, Sparks, MD, USA). The fluorescent signals from GFP or RFP excited by a laser at 488 nm were monitored by their emissions using 515–545 nm (FL1) or 562–588 nm (FL2) bandpass filters, respectively. When RFP was highly expressed, leakage of GFP fluorescence to the FL2 filter was negligible without multicolour compensation.

RT-PCR analysis

Cells with or without adenovirus infection were collected and digested with DNase I using a CellAmp Direct RNA Prep Kit (Takara Bio Inc., Shiga, Japan). The resultant cell lysates were subjected to RT-PCR using a PrimeScript One-step RT-PCR Kit ver.2 (Takara Bio Inc.) according to the manufacturer's protocol. GFP mRNA was reverse transcribed and amplified with specific primers (5′-GAGAACAGGGGCATCTTG-3′ and 5′-CAATTCACGAATC CCAACT-3′). RFP mRNA was also reverse transcribed and amplified with specific primers (5′-CGAGGAATCAACCTCTGGA-3′ and 5′-TGCGTCAGCAAACACAGT-3′). The obtained products were analysed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

Results

Design of lentiviral vectors

In general, large amounts of total RNA extracts from cells should be prepared for northern blot analyses of miRNAs, which are expressed at lower levels in cells. However, such mixtures of cellular extracts will compromise analyses of miRNAs in individual cells among large cell populations or large amounts of tissues. To monitor the expression of miRNAs in living human cells, we newly constructed a dual-colour monitoring system using a human immunodeficiency virus-1 (HIV-1)-based lentiviral vector encoding two different fluorescent proteins (Fig. 1). The provirus designed by us provides two independent transcripts: one mRNA encodes RFP [dTomato (*26*)] in the forward direction, and the other mRNA encodes GFP (EGFP) in the reverse direction relative to the vector LTR. The GFP is fused with the antibiotic-resistant gene for blasticidin to select infected cells (*24*).

This divergent transcription system allows us to establish coordinated and robust expression of two genes, in comparison with convergent or tandem expression systems that often exhibit transcriptional interference (*30*, *31*). In particular, when two closely located genes are positioned in convergent or tandem orientations, transcription is severely suppressed (*30*, *31*). Indeed, expression of tandemly located two genes in our retroviral vector was not coordinated due to the transcriptional interference. Since uncorrelated gene expression of GFP and RFP were more or less observed, we had to screen the cell clone for further analysis (*24*). Hence, coordinated expressions of two genes under a divergent transcription unit are necessary for quantitative analysis of protein expression. Moreover, HIV-1-based lentiviral vector has broader infectivity than that of our conventional retroviral vector, which is limited to dividing rodent cells.

To explore the optimal combination of promoters with different directions for transgenes, we first constructed seven kinds of vectors. To avoid variation in the expression levels of the two fluorescent proteins, we used robust promoters including the mouse PGK promoter, SV40 promoter and human EF1α promoter, but not the CMV promoter or Moloney murine leukaemia virus LTR promoter because these promoters contain a binding element for the YY1 transcription factor, which is known to lead to fluctuating expression of genes (*32*, *33*). Based on the results of simple assays of the GFP and RFP expression levels, and estimation of the titres of each lentiviral vector, we adopted the most suitable vector, designated pLV.SGb.PtR [Fig. 1B(a)].

In the following experiments, we utilized a flow cytometry apparatus to quantify the expression levels of GFP and RFP. In flow cytometry analyses for optimized detection of RFP, dTomato was detected at the highest intensity among RFPs, including dsRed, mCherry and mOrange (*26*) (data not shown).

VAI RNA produces small RNAs from both strands of the terminal stem

In previous study, adenovirus VAI RNA have reported to produce functional miRNAs, which can reduce target gene expression. VAI RNA forms tight structure that include two double-stranded regions referred to as the terminal and apical stems, respectively (Fig. 2A). According to our previous observations (*10*), the processed miRNAs can be detected from the 3′-strand (T3), but not the 5′-strand (T5), of the terminal stem (Fig. 2A). To further explore the processing of VAI RNA, we performed precise northern blot analyses, which increased the duration of exposure for autoradiography. As shown in Fig. 2B, we successfully detected small RNAs of 22–26 nt from the 5′-strand of the terminal stem (T5), which were not detectable in previous experiments of northern blotting and cloning procedure. Quantification of the individual bands for the miRNAs in northern blotting (Fig. 2B) showed that the processing efficiencies of VAI RNA into the T5 and T3 miRNAs were 0.07 and 0.5%, respectively. Asymmetric accumulation of miRNAs is well-known as a characteristic of the RNA interference machinery at the unwinding step of dsRNA (*34*). Both miRNAs, which were estimated to be present at $\sim 1 \times 10^5$ molecules/cell, were expressed at sufficiently high levels to function comparably as highly expressed endogenous miRNAs (*35*). Small RNA bands from either the 5′-strand (A5) or 3′-strand (A3) of the apical stem were not detected regardless of double-stranded region as reported earlier.

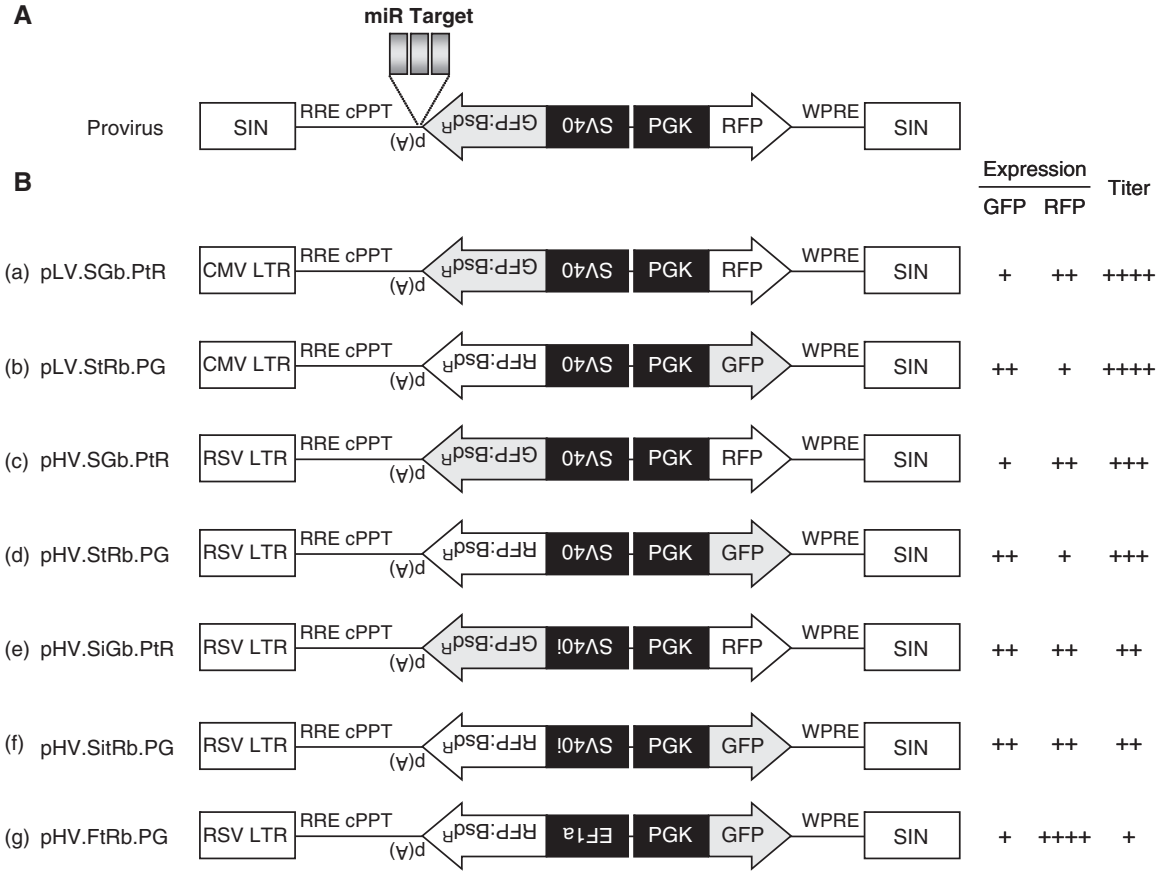

Fig. 1 Scheme of the dual-colour lentiviral vector system. (A) The proviral form of the lentiviral vector is shown. The three tandem repeats with perfect complementarity to miRNAs were inserted between the downstream of GFP and the polyadenylation signal. (B) Structures of the dual-colour lentiviral vectors used in this study. Vectors with different promoters and orientations were tested to analyse the expression levels and titres. SIN, self-inactivating LTR; RRE, rev-responsive element; cPPT, central polypurine tract; p(A), polyadenylation signal; SV40i, SV40 promoter with a synthetic intron; GFP, enhanced green fluorescent protein; RFP, dTomato; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

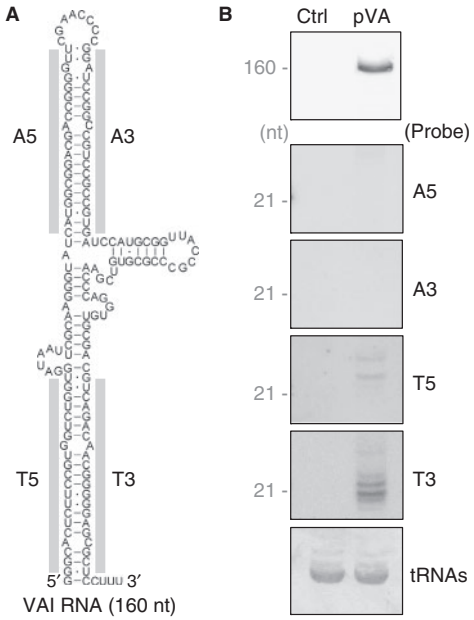

Fig. 2 Small RNAs are processed from both strands of the terminal stem of VAI RNA. (A) Secondary structure of VAI RNA. The regions complementary to probes used in northern blotting analyses are indicated by grey bars. (B) Detection of VAI RNA in 293T cells transfected with VA-expressing vectors (lane pVA) or without transfection (lane Ctrl). tRNAs stained with methylene blue were used as a loading control.

Monitoring of adenoviral miRNAs in single cells

To monitor the expression of adenovirus-derived miRNAs using our lentiviral vector system, complementary sequences against individual miRNAs were inserted into pLV.SGb.PtR between GFP and the poly(A) signal to yield pLV.SGb[A5].PtR, pLV.SGb[A3].PtR, pLV.SGb[T5].PtR and pLV.SGb[T3].PtR, respectively (Fig. 1A). Since miRNAs suppress the expression of target mRNAs with sequence complementarity, the expression of adenovirus-derived miRNAs was expected to induce a reduction in GFP mRNAs containing the target sequences of the miRNAs, without affecting the expression of RFP. Independent expression of RFP from GFP ensures an internal control in the same vector to avoid integrated-site dependency of proviral vector.

After infection of recombinant lentivirus at a multiplicity of infection (MOI) of 10 into HT1080 fibrosarcoma cell line and selection with blasticidin antibiotics, resultant pools of HT1080 cells were used in the following experiments. Fluorescent signals from both GFP and RFP were detectable in HT1080 cells containing SGb.PtR or SGb[T3].PtR (Fig. 3, −AdV). After further infection with a recombinant adenovirus (Ad2/βGal-4) at a MOI of 10, we observed a

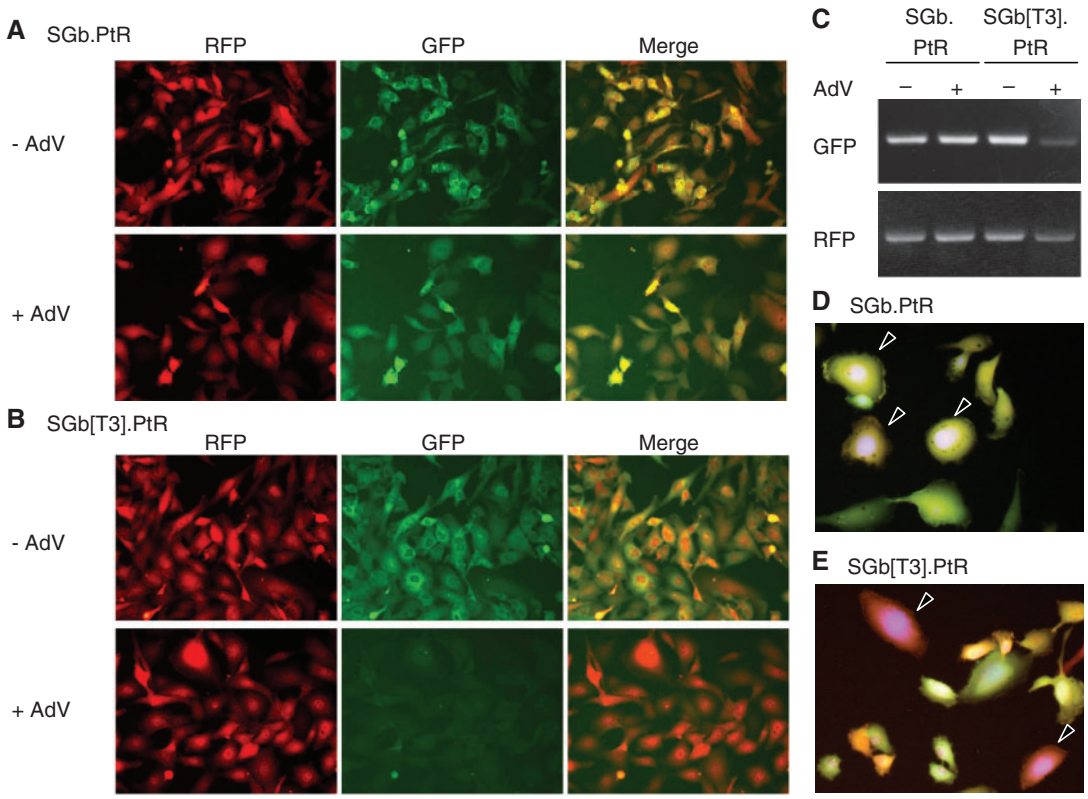

Fig. 3 Dual-colour imaging of adenovirus-derived miRNAs in living cells. HT1080 cells were transduced with SGb.PtR (A) or SGb[T3].PtR (B). Strong expression of both GFP and RFP indicates that the lentiviral vector was efficiently transduced. At 5 days after adenovirus infection (+AdV), the fluorescence of GFP in SGb[T3].PtR cells was reduced, while the fluorescence of RFP was retained. Both colours of fluorescence do not change in SGb.PtR cells, regardless of adenoviral infection. (C) The expression levels of GFP and RFP mRNAs were analysed by RT-PCR. (D and E) Cells were stained with a fluorogenic substrate of β-galactosidase that produces a blue product (arrowheads).

significant reduction in GFP expression in HT1080 cells with SGb[T3].PtR (Fig. 3B, +AdV). The suppression effect was specific for GFP connected to the target sequence because the control RFP fluorescent signals were not reduced by the adenoviral infection. In contrast, the activity of GFP from SGb.PtR without a target sequence was not significantly altered by the adenoviral infection (Fig. 3A). Since the GFP fluorescent signals were only reduced in HT1080 cells containing SGb[T3].PtR, the reduction was due to the expression of miRNAs derived from the adenovirus, but not due to the difference of promoters. RT-PCR analysis showed that these reductions in GFP expression occurred by RNA degradation (Fig. 3C), as expected from previous observations that miRNAs direct the cleavage of their target mRNAs when the target sequence is perfectly complementary to the miRNA in mammals (*23*, *36*). These results are consistent with our previous data from transient transfection experiments using luciferase genes as reporters (*10*). In addition to the cellular analyses using GFP and RFP, recombinant adenovirus infection was monitored by the activity of β-galactosidase, which is encoded in the recombinant adenovirus. A Coumarin-derived synthetic substrate for β-galactosidase was loaded into the cells after infection with the recombinant adenovirus at a low MOI (0.5). A reduction in GFP, but not RFP, was only observed in cells infected with the recombinant adenovirus, which caused blue fluorescence as a result of the β-galactosidase activity (Fig. 3D and E). These observations are consistent with our previous result, and suggest that the native adenovirus can be detected in single living cells by our lentiviral system, even without the β-galactosidase reporter or immunostaining using fixed cells.

Two-dimensional flow cytometry analysis

Next, we quantified the miRNA activity in a large population of living cells by flow cytometry. As a negative control, HT1080 cells alone did not exhibit fluorescence of either GFP or RFP, and were present at the left-bottom region in a two-dimensional (2D) flow cytometry plot (Fig. 4A). When we used HT1080 cells containing SGb[1].PtR, in which GFP was connected to the target sequence of miR-1, the population was detected at the right-upper region in the 2D plot (Fig. 4B). In contrast, HT1080 cells containing SGb[let-7].PtR, in which the target sequence of let-7 was inserted, were detected at the left-upper, indicating that let-7, but not miR-1, is endogenously expressed in HT1080 fibrosarcoma (*37*) (Supplementary Fig. S1). After 5 days of adenoviral infection in cells containing SGb[1].PtR, the population of cells was not significantly shifted (Fig. 4B). When HT1080 cells harbouring SGb[1].PtR were transfected with a synthetic dsRNA of miR-1, GFP was significantly reduced as expected (Supplementary Fig. S1). On the other

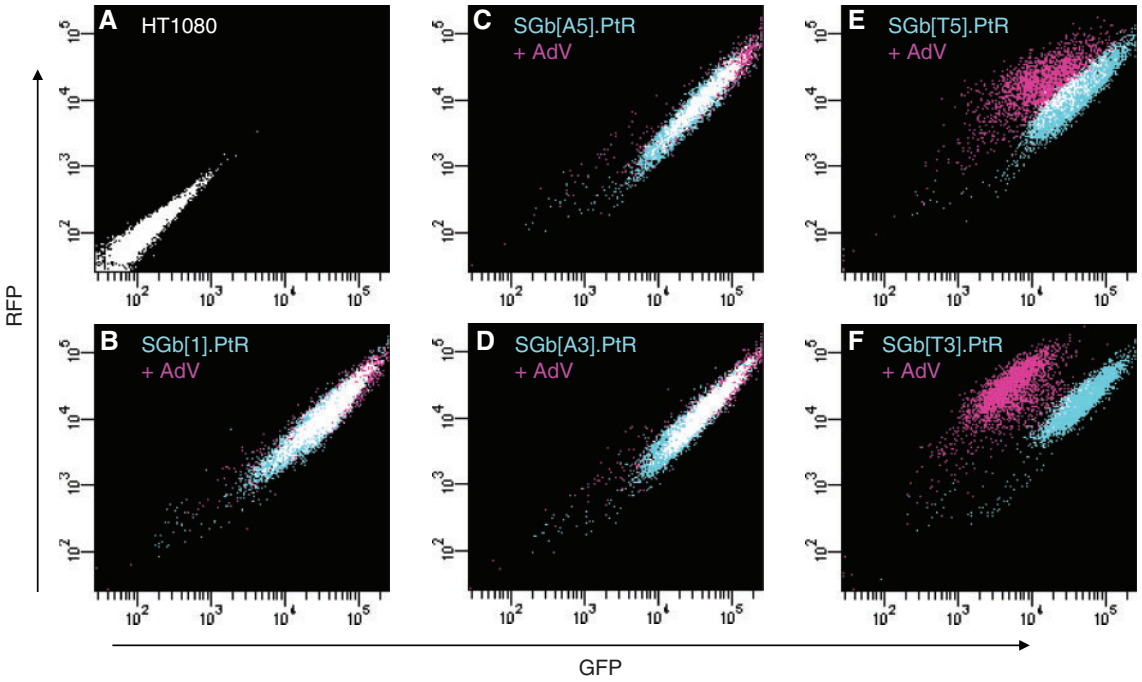

Fig. 4 2D flow cytometry analyses. The fluorescence intensities from two kinds of fluorescent proteins were quantified before and after adenoviral infection, indicated by cyan and magenta dots, respectively. Overlapping populations appear as white dots. The fluorescent signals of GFP and RFP were detected using FL1 and FL2 filters, respectively.

hand, quantitative calculation of the fluorescence from each 1D analysis clarified that both GFP and RFP were increased by ~1.6-fold (Table 1), in accordance with earlier reports that adenoviral infection enhances the expression of transgenes non-specifically (*38*). In addition, we did not observe any reduction in GFP when we analysed cells containing SGb[A5].PtR or SGb[A3].PtR, in which GFP is connected to the complementary sequence of the A5 or A3 region in the apical stem of VAI RNA, respectively (Fig. 4C and D), even though the conditions of adenoviral infection at a MOI of 10 represented intense physiological conditions.

In contrast, we observed a significant reduction in GFP expression after adenoviral infection when we analysed cells containing SGb[T5].PtR or SGb[T3].PtR, in which GFP is connected to the complementary sequence of the T5 or T3 region in the terminal stem of VAI RNA, respectively. After adenoviral infection, the populations of HT1080 cells containing SGb[T5].PtR and SGb[T3].PtR were shifted from the right-upper region to the centre-upper region in the 2D plots (Fig. 4E and F). The levels of GFP expression in cells containing SGb[T3].PtR and SGb[T5].PtR were reduced to 9.7 and 37.5% after adenoviral infection, respectively. These reductions were normalized by the corresponding intensity of RFP (Table 1). These results apparently indicate that functional miRNAs can be produced from the 5′-strand as well as the 3′-strand of the terminal stem of VAI RNA during adenoviral infection. The populations of these cells were observed as sharp diagonal lines in the 2D plots. Quantification of the fluorescence intensity from individual cells confirmed that our lentiviral vector system exhibited less variation in the expression levels of two genes.

Discussion

Highly abundant adenoviral miRNAs may have distinct roles from other viral miRNAs owing to the lack of genes with perfect homology to the VA RNAs in the adenoviral genome for auto-regulation of the virus itself. Very recently, Xu *et al.* (*39*) attempted to clone miRNAs from adenovirus-infected cells using small RNAs that had been immunoprecipitated with an Ago-2-containing RISC. In their sequence analyses, 80% of the cloned miRNAs were derived from VA RNAs, suggesting that adenoviral miRNAs expel endogenous miRNAs via huge expression of VA RNAs. Moreover, VAI RNA directly binds to Dicer and exportin-5, both of which are responsible for processing of pre-miRNAs, implies suppression of endogenous miRNA activity after adenoviral infection (*5*). Previous reports have also indicated essential roles for adenovirus-derived miRNAs in the adenovirus life cycle. For example, mutations in the T3 region (3′-side of the terminal stem) caused 10-fold decreases in viral replication regardless of the global RNA structure (*40*). Moreover, antagonizing adenoviral miRNAs with 2′-*O*-methylated antisense oligonucleotides resulted in 10-fold reductions in the viral titres (*11*). Nevertheless, the exact roles of the small RNAs derived from VAI RNA remain unclear.

GFP has been used to monitor the activity of miRNA in various organisms including plant (*41*), fly (*42*) and zebrafish (*43*). More than one fluorescent protein needs to be used to discriminate the

Table 1. Quantitative analyses of the fluorescence intensities from flow cytometry analyses.

Vector [Target]	Adenovirus	GFP[a]	RFP[a]	GFP/RFP (%)[b]
SGb[1].PtR	−	35,325	17,802	100
	+	58,148	30,242	96.9
SGb[A5].PtR	−	38,121	15,851	100
	+	72,965	32,714	92.7
SGb[A3].PtR	−	31,737	14,282	100
	+	63,781	30,882	92.9
SGb[T5].PtR	−	46,723	21,529	100
	+	22,594	27,747	37.5
SGb[T3].PtR	−	52,625	29,027	100
	+	6,896	39,310	9.7

[a]Mean of fluorescence intensity from 1D analyses. [b]Relative fluorescence of the value without adenovirus infection was set as 100%.

suppression of fluorescent reporter gene due to miRNA activity from global gene suppression by some stimuli or from the failure of the introduction of reporter genes. Moreover, genes for fluorescent proteins can be encoded in a single cassette to avoid differentially controlled expression depending on the introduced loci. To validate the biological functions of miRNAs, we designed a lentiviral vector system that enabled the quantitative monitoring of miRNAs using differently coloured fluorescent proteins under the control of a bi-directional promoter.

In a divergent expression system for two fluorescent proteins, a promoter in the reverse orientation to the lentiviral LTRs can possess, by design, an intron under the control of the promoter. However, the other promoter in the forward orientation should not possess an intron because the transcript from the 5′-LTR promoter may be processed non-uniformly in packaging cells, which may result in heterogeneous expression of the two proteins. Interestingly, when we tested the intron-containing expression cassette in the reverse orientation to the LTR, the virus titres of the constructs with EF1α [Fig. 1B(g)], which has an endogenous intron, and SV40 with a synthetic intron element [SV40i; Fig. 1B(e) and (f)] were lower than those of the constructs without the intron, although the intron-containing constructs exhibited stronger expression. We are not sure whether these observations are due to the effects of the intron on the titres at the present time. Given the structural flexibility in constructing the lentiviral vector, such an element possessing translational enhancer activity is the system of choice in the reverse orientation to the LTR. In addition, the virus titres from the constructs containing the CMV-LTR chimeric promoter [Fig. 1B(a) and (b)] were greater than those of the constructs containing the RSV-LTR chimeric promoter [Fig. 1B(c)–(g)]. These findings are contradictory to observations in a previous study (*44*). Since our vectors have the SV40 promoter that is convergent to the chimeric LTR promoter, transcription from the chimeric promoters for viral packaging may be affected depending on the directions of the promoters in the context of the lentiviral vector.

Since lentiviral vector integration occurs at random, the expression of transgenes of interest may be unexpectedly affected by control regions of the cellular genome near the integration sites. In fact, as shown in Fig. 4, the expression levels of GFP and RFP from individual cells with randomly integrated lentiviral vectors varied by >10-fold. Our bi-directional expression of two distinct transgenes revealed correlated expression of the two genes, regardless of the integration sites, when we used mixed populations of cells separate from the cloned cells. Indeed, we detected 1.6-fold activation of the expression levels of the two fluorescent proteins after adenoviral infection by flow cytometry analyses, but such differences in the expression levels were difficult to distinguish by microscopic observations. Moreover, variance of the data from cell populations could be visualized as dots in the 2D plots as a result of fluorescence data from individual cells. Our system should be a useful tool for real-time monitoring of not only adenoviral miRNAs during viral infection but also other miRNAs, including endogenous miRNAs, in various biological events. Moreover, the broader infectivity of the lentiviral vector to various vertebrates not only for cultured cells but also for tissues (*28*) shows more promise for extensive studies of miRNAs.

Supplementary Data

Supplementary Data are available at *JB* online.

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Conflict of interest

None declared.

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