SiRNAs do not induce RNA-dependent transcriptional silencing of retrovirus in human cells

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Abstract RNA-dependent transcriptional silencing (RdTS) has been reported to operate even in human cell lines. It is tempting to speculate that RdTS plays a role in retroviral gene silencing, considering that retroviral RNA transcripts harbor a U3 promoter sequence that is a potentially good source of double-stranded RNAs. To test this possibility, we constructed several model HeLaS3 cell lines expressing GFP driven by murine leukaemia virus (MLV)-long terminal repeat (LTR) and introduced a series of shRNAs that target the U3 region of the MLV-LTR. However, transcriptional gene silencing was not induced in most instances, in spite of the fact that processed shRNA was found in cellular nuclei, indicating that RdTS does not contribute to MLV gene silencing in host cells.

Keywords: RNA interference; Gene silencing; Retrovirus; MLV; siRNA; shRNA

1. Introduction

Small RNAs, such as small interfering RNAs (siRNAs) and microRNAs (miRNAs), function as guide molecules in many inhibitory pathways that target the transcripts of coding genes [1–5], a phenomenon known as RNA interference. In this process, these tiny RNA species induce either mRNA degradation or translational repression (post-transcriptional gene silencing (PTGS)) of mRNAs through specific base pairing.

In plants, dsRNA molecules that are homologous to a region of a gene promoter can induce de novo DNA methylation which results in transcriptional gene silencing (TGS). This phenomenon is termed RNA-dependent DNA methylation (RdDM) [6,7]. In yeasts, RNAs transcribed from the centromeric regions of the genome in both orientations form dsRNAs that are processed to siRNAs, which are finally embedded in an RNA-induced transcriptional silencing (RITS) complex. The RITS complexes are recruited to the centromere and induce heterochromatin formation in this region [8,9].

In human cell lines, such as HeLa, several studies have now reported that the transfection of synthetic siRNAs that target promoter regions can induce TGS of the corresponding gene such as EF1A and RASSF1A [10,11]. Since in some cases, the gene silencing mechanism does not seem to necessarily require efficient CpG DNA methylation in human cells, it has been designated as RNA-dependent transcriptional silencing (RdTS), rather than RdDM [12]. In light of these developments, it is now important to determine whether these small regulatory RNAs truly function as transcriptional suppressors in human cells in a more biologically relevant setting.

Retroviruses initiate the expression of their genomic RNAs just after integration into the host genome, but this expression is often suppressed. This repression is known as retroviral gene silencing and seems to involve a variety of different molecular mechanisms. It is noteworthy in this regard that retroviruses possess the remarkable characteristic that their RNA transcripts contain a U3 sequence that is homologous to the promoter DNA sequence within the 5′LTR (long terminal repeat) region of the provirus. Murine leukaemia virus (MLV) integrates into its host chromosomes in an almost random manner and it is now well known that there are many endogenous retroviruses in the genome of its natural hosts. It is therefore possible to speculate that there would be significant opportunities for either exogenous or endogenous DNA sequences that are homologous to the U3 region of retroviruses to be transcribed from strong promoters of adjacent host genes in an antisense orientation. The siRNAs produced from the resulting dsRNA molecules harboring U3 sequences would then target retroviral RNA transcripts and induce PTGS. Furthermore, if RdTS is indeed widely operating in mammalian cells, we can further speculate that such siRNAs that target U3 promoter sequences would induce retroviral TGS.

In our present study, we have investigated using MLV-based expression vectors whether siRNAs play any significant role in the TGS of retroviruses. Because, unlike the mouse genome, the human genome does not contain significant levels of endogenous retroviruses that are homologous to MLV, we utilized the human cell line, HeLaS3, in our assay system. We report a series of expression analyses of the MLV promoter targeted by short hairpin RNAs (shRNAs) that were expressed from HIV-1-based lentivirus vector in these human cells.

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Abbreviations: TGS, transcriptional gene silencing; PTGS, post-transcriptional gene silencing; RdTS, RNA-dependent transcriptional silencing; RISC, RNA induced silencing complex; RITS, RNA induced transcriptional silencing; MLV, murine leukaemia virus; LTR, long terminal repeat; FBS, fetal bovine serum
2. Material and methods

2.1. Plasmid construction

The 0.7-kb BamHI–EcoRI fragment of pCS2-Venus [13], that encodes a variant of GFP with improved fluorescence, was inserted into the BamHI–EcoRI site of pBluescriptII-SPK+ to generate pMXs-GFP. For the construction of shRNA expression plasmid, pIRES-Neo was inserted into the EcoRI–XbaI site of pBluescriptII-SK+ to generate pMXs-GFP. The 1.8-kb EcoRI–XbaI fragment of the reporter cassette in its genome for further analysis.

2.2. Cell culture, transfection and cloning

HeLaS3 cells cultured at 37 °C in DMEM containing 10% fetal bovine serum (FBS) were seeded at 8 × 103 cells per well in six-well plates. After 24 h, the cultures were transfected with pMLVLTR-GIN-polyA (1 μg) using 6 μl Lipofectamine 2000 (Invitrogen), and selected with G418 (1 mg/ml) from 24 h after the transfection. To establish cellular clones stably expressing GFP, neomycin resistant cells expressing GFP were sorted, and single cells were then isolated into well-plates, using FACS Aria (BD). We isolated several stable clones and selected one of these lines, HeLaS3-LTRGFPP#1, which harbors a single copy of the reporter cassette in its genome for further analysis.

2.3. Virus transduction and FACS analysis

HeLaS3-LTRGFPP#1 cells were seeded at 1 × 105 cells per well in six-well plates in DMEM containing 10% FBS and, after 24 h, transduced with each shRNA expression virus stock (3 × 105 TU) in the presence of 8 μg/ml of Polybrene. The medium was then changed to DMEM containing 10% FBS and puromycin (1 μg/ml) after a further 24 h. After 6 days of selection, the puromycin was removed from the medium. GFP expression levels were measured using FACS Calibur (BD).

2.4. Purification of nuclear and cytoplasmic small RNAs

Nuclear and cytoplasmic RNA was isolated from untransduced HeLaS3-LTRGFPP#1 cells, or from HeLaS3-LTRGFPP#1 cells that were transduced with a shRNA expression lentivirus vector and grown for more than one month. Twelve dishes (10 cm in diameter) for each cell culture were rinsed twice with ice-cold PBS, harvested in a further 2 ml ice-cold PBS by scraping, and centrifuged for 5 min at 1500 rpm at 4 °C. Cell pellets were suspended with NP40 lysis buffer [10 mM Tris–HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl2, 0.5% Nonidet P-40], incubated on ice for 10 min, and then centrifuged for 5 min at 1500 rpm at 4 °C. The cytoplasmic RNA fraction was prepared from the supernatant by adding ISOGEN (Wako Pure Chemical Industries, Osaka, Japan). Nuclear pellets underwent two additional washes with 4 ml NP40 lysis buffer. RNAs smaller than 200 bp were purified with mirVana mRNA Isolation Kit (Ambion, Austin, TX) from purified cytoplasmic RNA and nuclear pellets, respectively, according to the manufacturer’s protocol.

2.5. Northern blot analysis

Ten percent of the total yield of small RNAs from nuclear and cytoplasmic fractions was analyzed by Northern blotting to allow for a comparison with the same cell numbers. These fractions were separated on denaturing 18% polyacrylamide gels, transferred to Hybond-XL membranes (Amersham BioSciences) and crosslinked with 60 mJ/cm² UV. The synthesized oligonucleotide probe sequences are listed in Supplementary Table S2 and all probes were 5’ end-labeled with [α-32P] ATP using T4 polynucleotide kinase (TAKARA). Hybridizations were performed using UltraHyb-Oligo buffer (Ambion) at 37 °C according to manufacturer’s instructions and membranes were washed using 2× SSC, 0.5% SDS buffer. Radioactive signals were visualized using a FLA-5100 (FUJIFILM).

2.6. Quantitative RT-PCR

Seven days after transduction, total cellular RNAs were prepared with RNeasy Mini kit (QIAGEN) and treated with DNaseI (QIAGEN). Quantitative RT-PCR was performed with Superscript One-Step RT-PCR with Platinum Taq Kit (Invitrogen Corp.). The primer pairs used to detect mRNA expressions are listed in Supplementary Table S3. RNA was reverse-transcribed at 50 °C for 30 min, and after an initial denaturation at 94 °C for 3 min, cDNA amplification procedures were performed as follows: for GFP, 18 cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min; for RASSF1A, 26 cycles of 94 °C for 30 s, 56 °C for 1 min, and 72 °C for 1 min; for GAPDH, 25 cycles of 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min. After polyacrylamide gel electrophoresis, the band density of the PCR products was quantified by densitometry (ATTO Densiograph3). Amounts of each cellular RNA were adjusted so as to give the same band density of PCR products of GAPDH.

3. Results

3.1. Experimental design and preparation of cell assay systems

As shown schematically in Fig. 1A, mRNA transcribed from the U3 promoter of a replication-defective MLV-based retrovirus vector, pMXs-GIN, contains a region that is homologous to the U3 promoter in its 3’UTR. Since shRNAs that are designed to target U3 promoter DNA regions will also target such RNA transcripts and cleave them, it is difficult to subsequently assay for RdTS of the retroviral promoter using natural retroviruses, or retroviral vectors derived from them, as targets. To accurately assay for TGS of the U3 promoter, we therefore constructed an expression plasmid, pMLVLTR-GIN-polyA (Fig. 1A), in which, the 3’LTR region of pMXs-GIN has been substituted with the polyadenylation signal derived from the bovine growth hormone gene. In this setting, shRNAs that target the U3 promoter DNA region would not suppress GFP expression through PTGS. TGS of the U3 promoter would therefore be detectable by determining the GFP expression levels. We transfected HeLaS3 cells with this construct and established six stable clones. Among these, we identified a clone, designated HeLaS3-LTRGFPP#1, which was determined to harbour a single copy of the reporter cassette in its genome by Southern blot analysis.

3.2. ShRNA expressing lentiviral vectors that target several regions of the U3 promoter

We designed 12 shRNA species (shU3-1–12) that would target different parts of the U3 promoter region (Fig. 1B). Eight of these molecules (shU3-1,2,4–9) were designed using guidelines previously developed for the selection of highly effective target sequences [16,17], where antisense RNA (complementary to the target mRNA) was designed to be selectively assembled into the RNA induced silencing complex (RISC). We separately introduced 12 different pairs of synthetic oligonucleotides (Supplementary Table S1) to generate the shRNA expression cassette, driven by the mouse U6 promoter, which is present in a self-inactivating HIV-1-based lentivirus vector. Because the degree of sequence homology between the retrovirus vector and lentivirus vector used here is marginal, we expected that unintended mutual interactions would be minimized.
We analyzed the processing of shRNA and subcellular localization of them and their products in HeLaS3 following introduction of two of these shRNAs, shU3-4 and shU3-7. Days (50–60) after transduction, both nuclear and cytoplasmic RNA fractions were prepared. In the shRNA producing cells, unprocessed molecules (56nt) were undetectable in either fraction. As putative guide RNAs, exclusively antisense RNA was detectable in both shU3-4 and shU3-7 producing cells (Fig. 2), indicating algorithm used to design these shRNAs worked as predicted [16,17]. These results also indicate that the processing of shRNA to siRNA and subsequent loading of the specific strand onto RISCs occurs very rapidly. Most importantly, antisense RNAs originating from either shU3-4 or shU3-7 were present in both the nuclear and cytoplasmic fractions (Fig. 2). This indicates that shRNA processed by dicer in the cytoplasm [18] is efficiently recruited to the cellular nuclei and our shRNA expression system is suitable to demonstrate RdTS.

We next tested whether these shRNAs could efficiently degrade homologous mRNAs. We transduced pMXs-GIN into HeLaS3 cells and subsequently obtained a stable GFP expressing cell population designated as HeLaS3-ViralGFP. After

Fig. 2. Analysis of the processing and subcellular localization of RNAs derived from transduced and untransduced HeLaS3-LTRGFP#1 cells. Y4 small cytoplasmic RNA and ACA1 small nucleolar RNA served as marker RNAs of cytoplasmic and nuclear fractions, respectively.
transduction of each shRNA lentiviral vector into the HeLaS3-ViralGFP cells and selection with puromycin, the GFP expression profiles were examined. ShRNA that targeted coding region of the Cre recombinase gene (shCre) was used as negative control as it has low homology to the U3 sequence. The results of these experiments showed that each shRNA suppressed exogenous GFP expression. The suppression efficiency was found to be high in most cases, although that of shU3-11 and 12 was not as strong (Fig. 3A).

3.3. The effects of the U3 shRNAs on GFP transcription driven from the MLV-LTR promoter

The shRNA expression lentivirus vectors were transduced into HeLaS3-LTRGFP#1 cells, followed by puromycin selection, and the resulting GFP expression profiles were assessed (Fig. 3B). Eleven out of the 12 shRNAs did not cause any significant changes in the GFP expression levels. Only one shRNA, shU3-7, produced 35% reduction in GFP protein expression. Using quantitative RT-PCR, we next assessed GFP mRNA abundance in cells transduced with shU3-7 or shCre as well as in untransduced cells. The results clearly showed 38% reduction in GFP mRNA expression specifically in shU3-7 transduced cells, indicating that the observed suppression by shU3-7 occurred at the transcriptional level (Fig. 3C).

It has been reported in HeLa cells that shRNA molecules induce CpG methylation of homologous DNA at very low frequencies, and it is thus possible that the establishment of TGS takes some time [11]. We therefore cultured shRNA transduced HeLaS3-LTRGFP#1 cells for over 1 month and then determined the GFP expression levels. However, we found no differences with the earlier findings, and the suppressive effects of shU3-7 were also unchanged after more than 7 weeks of culture (Fig. 4A).

In both plants and Drosophila, TGS caused by siRNA is detected in circumstances where PTGS by the same siRNA is also operating [19,20]. We thus speculated that it might be possible that the RNA interference effects that we observed in Fig. 3A would be enhanced in a long-term culture if TGS is also triggered. We therefore also cultured shRNA transduced HeLaS3-ViralGFP cells for over 1 month but could detect no additional suppressive effects upon GFP expression in cells expressing either shU3-7 or other shRNAs targeting the U3 promoter region (Fig. 4B).

3.4. The effects of shRNA targeting the promoter region of the endogenous RASSF1A gene

It has been reported that the RASSF1A promoter was CpG methylated and transcriptionally suppressed in HeLa cells by expressing shRNA containing 21-mer sequences complemen-
RASSF1A promoter CpG island [11,21]. We therefore tested whether our shRNA expression system induced RdTS on the RASSF1A gene in HeLa cells. We designed shRASSF1A which targets the same sequence that caused RdTS in the previous report [11]. The vector carrying shRASSF1A expression unit as well as a control vector expressing shCre was transduced to HeLa cells and abundance of RASSF1A mRNA in the stably transduced cells was determined by quantitative RT-PCR. The level of RASSF1A mRNA in shRASSF1A transduced cells was 97 ± 21% of that in the shCre transduced cells. According to the previous report, the level of methylation triggered by shRNA targeting RASSF1A is very low [11]. So we speculate the frequency of RdTS induced by shRASSF1A would be too low to be detected in the mixed cellular populations transduced with the shRNA.

3.5. The effects of U3 shRNAs on GFP transcription driven by the MLV-LTR promoter in non-tumour cells

We next tested whether the results obtained using HeLaS3 cells (Fig. 3B) could be extended to non-tumour cell lines such as 3Y1 and TIG-3/E/TERT [22]. 3Y1 is a rat fibroblast cell line with marginal oncogenic potential that does not express any significant MLV-related endogenous viruses. TIG-3/E/TERT originates from a human diploid embryonic lung fibroblast cell line, TIG-3, and was immortalized by the introduction of the hTERT gene. However, TIG-3/E/TERT cells are resistant to transformation and remain diploid [22]. We transfected these two cell lines with pMLVLTR-GIN-polyA and prepared two cellular populations stably expressing GFP, 3Y1-LTRGFP and TIG-3/E-TERT-LTRGFP.

When a number of our shRNAs were transduced into 3Y1-LTRGFP cells, very similar effects upon GFP expression were observed when compared with the HeLaS3-LTRGFP#1 cells, i.e. most of the shRNAs had no effect on GFP expression, whereas a slight suppression was observed in cells transduced with shU3-7 (Supplementary Fig. 2A). We next transduced some of the U3 shRNAs into the TIG-3/E/TERT-LTRGFP cells. Among the shRNAs tested, only shU3-7 showed any cytostatic or cytotoxic effects in these cells and no stable transductants were obtained. The introduction of other shRNAs (shU3-3, 4 and 8) showed no effects upon GFP expression in the TIG-3/E/TERT-LTRGFP cells (Supplementary Fig. 2B).

Considering that shU3-7 showed cytostatic and cytotoxic effects in TIG-3/E/TERT-LTRGFP cells, shU3-7 would induce off-target silencing in TIG-3/E/TERT-LTRGFP cells. We speculate that shU3-7 suppresses unintended mRNA targets encoding transcription factors or chromatin remodelling factors which enhance MLV promoter activity, as well as endogenous gene transcripts important for cellular proliferation. We also expect that these effects might also reduce total amounts of ACA1 snoRNA even in HeLaS3 (Fig. 2).

4. Discussion

To test whether TGS is operating as a mechanism for retroviral gene silencing, we here stably expressed shRNA targeting MLV-LTR in cells harboring a GFP reporter gene driven by MLV-LTR. Our results did not show clear involvement of TGS in retroviral gene silencing (Figs. 3B and 4A), whereas the introduced shRNA was successfully processed to the single stranded guide RNA, which located in both cellular nuclei and cytoplasm (Fig. 2). These observations are apparently different from previous studies that TGS contributes to gene silencing of HIV [23,24], which belongs to lentivirus subfamily (Lentivirinae) of retrovirus family (Retroviridae). In previous reports, siRNAs or shRNAs were, in most cases, introduced by direct transfection of siRNA [23] or transient expression of shRNA expression plasmids [24], whereas in our system, shRNA were stably expressed to achieve more physiological conditions. But in some cases, expression of stable shRNA or even antisense RNA was shown to suppress HIV-LTR transcription in the previous work [24]. So the difference in the methods to express siRNA would not fully explain this discrepancy and therefore we could not fully exclude the possibility that our findings on MLV might not be directly extended to lentiviruses.

Recently, there have been several reports testing RdTS in mammals using approaches other than transient siRNA transfection experiments. In dicer-null mouse ES cells, RNA transcripts corresponding to the centromeric major satellite repeats have been shown to be very much enhanced [25,26]. Other studies of pericentric heterochromatin in these same mutant ES cells, however, have reported contradictory results showing a partially disrupted [25], or a completely intact region [26], as judged by DNA methylation and histone H3K9 trimethylation. RdTS pathways have also been reported not to play any role in the silencing of the transgenic repetitive element in baby hamster kidney cells [27]. Our current data and these earlier findings thus suggest that the RdTS pathway...
selectively induces the silencing of a limited region in the genome such as the centromere, but does not function in a genome-wide manner in mammalian cells.

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Appendix A. Supplementary data


References
