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ORIGINAL ARTICLE

Utility of Epstein–Barr virus-encoded small RNA promoters for driving the expression of fusion transcripts harboring short hairpin RNAs

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To induce RNA interference (RNAi), either small interfering RNAs (siRNAs) are directly introduced into the cell or short hairpin RNAs (shRNAs) are expressed from a DNA vector. At present, shRNAs are commonly synthesized by RNA polymerase III (Pol III) promoters of the H1 and U6 RNAs. In this study, we designed and characterized a new set of plasmid vectors driven by promoters of the Epstein–Barr virus (EBV)-encoded small RNAs (EBERs). The EBERs are the most abundant transcript in infected cells and they are transcribed by Pol III. We showed that the EBER promoters were able to drive the expression of shRNA fusion transcripts. siRNAs processed from these fusion transcripts specifically and effectively inhibited the expression of homologous reporter or endogenous genes in various types of cells. The partial EBER sequences in the fusion transcripts did not activate double-stranded RNA-dependent protein kinase or suppress RNAi. In nasopharyngeal carcinoma cells, the EBER2 promoter was stronger than the H1 and U6 promoters in shRNA synthesis, leading to more effective knockdown of the target genes. Taken together, our findings suggest that the EBER promoters fundamentally different from those of H1 and U6 can be used to drive the intracellular expression of shRNAs for effective silencing of target genes in mammalian cells and particularly, in EBV-infected cells.
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Introduction

RNA interference (RNAi) is an evolutionary conserved gene-silencing mechanism, in which sequence-specific degradation of the homologous mRNA is triggered by double-stranded RNA (dsRNA).[1–3] This phenomenon was first discovered in *Caenorhabditis elegans* by injecting long dsRNA.[1] However, introduction of dsRNA longer than 30 bp into mammalian cells induces the interferon (IFN) response, in which the activation of dsRNA-dependent protein kinase (PKR) and 2′,5′-oligoadenylate synthetase (2′,5′-AS) results in nonspecific RNA degradation.[4–6] To circumvent this pathway, specific gene silencing can be achieved by direct introduction of either chemically synthesized or *in vitro* transcribed small interfering RNAs (siRNAs) of 21 nucleotides in length.[7–9] Alternatively, short hairpin RNAs (shRNAs) can be expressed from a DNA vector and subsequently processed into functional siRNAs in the cell by Dicer ribonuclease.[9–16] It is noteworthy that 27-nucleotide long synthetic dsRNAs, which are substrates of Dicer, can elicit RNAi 100-fold more potently than the corresponding 21-nucleotide siRNAs.[17]

Although some RNA polymerase II (Pol II) promoters have been used to express shRNAs in mammalian cells,[18,19] at present shRNAs are more commonly transcribed by mammalian U6 or H1 promoters.[9–16] The U6 and H1 promoters belong to type III RNA polymerase III (Pol III) promoters and have promoter elements located extragenically.[20] In addition, type II Pol III promoters, such as the tRNA promoters having promoter elements located intragenically, can also be used to drive shRNA expression.[21,22] Since the intragenic promoter elements of these promoters are co-transcribed as the 5′ end of the shRNA, the secondary structure formed may confer extra stability to the overall shRNA and increase its accessibility to Dicer in the initiation step of RNAi.

Animal viruses such as Epstein–Barr virus (EBV) encode various forms of small RNAs including microRNAs.[23–25] While the biological functions of most viral small RNAs remain elusive, some of these RNAs, such as adenovirus VAI and EBV-encoded small RNAs (EBERs), are exceedingly abundant in infected cells.[26–28] In light of this, we sought to investigate whether the promoters of viral small RNAs such as EBERs can be used to drive the expression of shRNAs in cultured human cells.

The EBERs with a copy number of approximately 10^7 per cell are by far the most abundant RNAs in EBV-infected cells.[27] EBER1 and EBER2 have 165 and 169

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nucleotides, respectively. The EBER promoters are transcribed by Pol III but they are also regulated by transcription factors Sp1 and activating transcription factor (ATF) that bind normally to Pol II promoters.[27] They contain both extragenic and intragenic promoter elements. The extragenic elements include Sp1 element, ATF motif and EBER TATA box (ETAB), whereas boxes A and B are intragenic.[27–29] The activity of the EBER promoters is dictated by these unique features and their exceedingly high activity in EBV-infected cells is attributed to phosphorylation of ATF2 and induction of Pol III-specific basal transcription factors TFIIIC and BDP1.[30]

In this study, we designed, constructed and characterized a new set of plasmid vectors driven by the EBER promoters. We showed that these promoters were able to drive the synthesis of shRNA fusion transcripts efficiently. In addition, the siRNAs processed from the shRNA fusion transcripts inhibited the expression of reporter and endogenous genes in various types of transiently or stably transfected cells. Importantly, the gene-silencing effect was specific and PKR activation was not induced. Notably, the EBER2-shRNAs induced the most pronounced gene knockdown effect in nasopharyngeal carcinoma cells. Thus, we provided the proof-of-principle that the EBER promoters can be used to drive intracellular expression of shRNAs for the induction of RNAi. These vectors based on the EBER promoters are a useful tool for the delivery of gene-silencing agents into mammalian cells and for developing nucleic acid therapeutics.

Results

Construction of shRNA expression vectors driven by EBER promoters

The EBER promoters are strong viral promoters transcribed by Pol III.[27] Similar to tRNA promoters, these viral promoters contain intragenic elements that are transcribed into RNA. The highly structured transcripts produced could be more resistant to nonspecific degradation.[22] In addition, the Sp1- and ATF-binding elements in the EBER promoters may further enhance transcription.[28,30] In light of all these unique features of the EBER promoters, we asked whether they could be utilized to drive the expression of shRNAs in mammalian cells. As a first step, we set out to construct shRNA expression vectors based on the EBER promoters.

Using PCR cloning, we were able to assemble two shRNA expression vectors pEBER1-shRNA and pEBER2-shRNA controlled by EBER1 and EBER2 promoters, respectively (Figure 1a). These vectors have incorporated

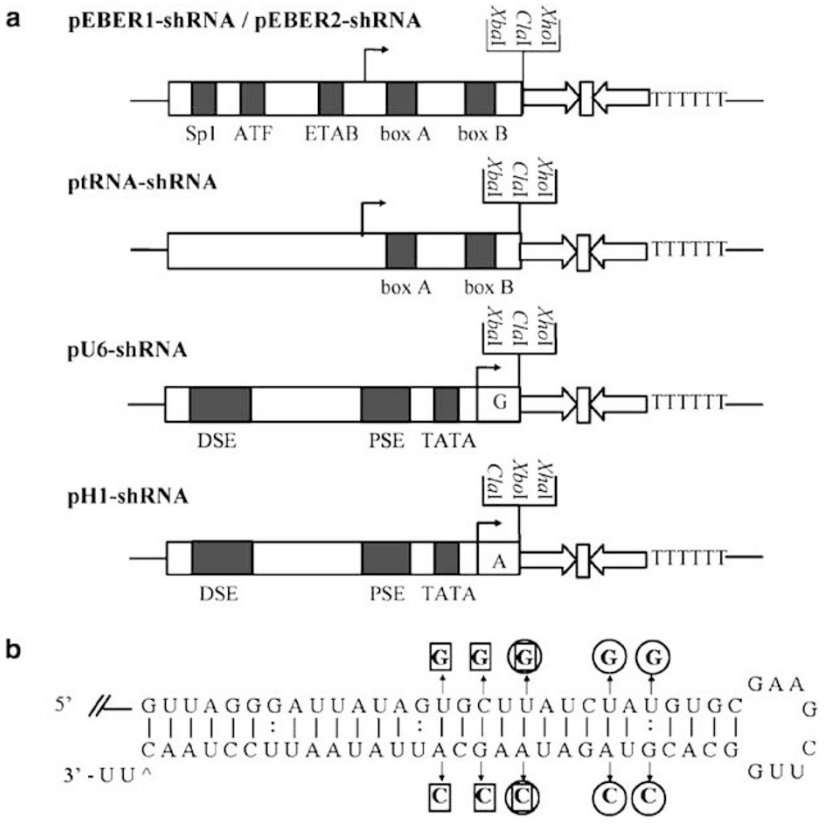

Figure 1 Short hairpin RNA (shRNA) expression vectors based on Epstein–Barr virus (EBV)-encoded small RNA (EBER) promoters. (**a**) Schematic diagram of shRNA expression vectors. All vectors can drive the expression of shRNA (sense-loop-antisense: ⇨⇦; 68 nucleotides), which is terminated by a stretch of six thymidines. Three restriction sites (*Xba*I, *Cla*I and *Xho*I) were inserted upstream of the shRNA sequence to facilitate subcloning. pEBER1-shRNA and pEBER2-shRNA have incorporated all the extragenic (Sp1, activating transcription factor (ATF) and EBER TATA box (ETAB)) and intragenic (boxes A and B) promoter elements of the EBER promoters (~120 nucleotides). DSE: distal sequence element. PSE: proximal sequence element. (**b**) Schematic diagram of an RNA transcript (shRluc) expressed from pEBER2-shRNA. G:U wobble base pairing was introduced into the stem of shRluc to facilitate PCR cloning. Altered nucleotides in EBER2-shRluc m1 are circled, and altered residues in EBER2-shRluc m2 are squared.

all the EBER extragenic and intragenic promoter elements including Sp1 element, ATF motif, ETAB, boxes A and B. shRNAs were placed downstream of the promoter through multiple cloning sites. The transcription of 29-mer shRNA is terminated with a stretch of six thymidines. The shRNA sequence has an eight-nucleotide loop sequence with a *Hin*dIII restriction site situated between the sense and antisense strands of the siRNA sequence. As such, the fusion transcripts produced from these vectors are EBER1-shRNA of 194 nucleotides and EBER2-shRNA of 209 nucleotides, which contain a 68-nucleotide shRNA sequence (Figure 1b) at the 3′ end of the ~120-nucleotide EBER1/2 RNAs. For comparison, we also constructed a series of shRNA expression vectors under the control of human $tRNA^{Val}$, U6 and H1 promoters (Figure 1a).

Production of siRNAs derived from EBER-shRNA fusion transcripts

Next, we investigated whether the expression vectors driven by the EBER promoters might be used to produce shRNAs in cultured human cells. For this purpose, we chose an shRNA directed against firefly luciferase (shFluc), whose effectiveness in knocking down the expression of its target gene has been documented.[9,31,32] The shFluc sequence was subcloned into pEBER1-shRNA and pEBER2-shRNA vectors. To directly analyze the EBER-shRNA fusion transcripts expressed from these vectors, northern blotting was performed (Figure 2a). As expected, ample amounts of EBER-shRluc RNAs of the correct size (194 nucleotides for EBER1-shRluc and 209 nucleotides for EBER2-shRluc) were detected in transfected cells (Figure 2a, lanes 2 and 3 as compared to lane 1).

shRNAs expressed in cells are thought to be processed by Dicer ribonuclease to generate siRNAs, which induce RNAi effect. Above we demonstrated the expression of EBER-shRNAs (Figure 2a). However, it remained to be determined whether siRNAs could be produced from these EBER-shRNA transcripts. To address this issue, we performed RNase protection assay using enriched small RNAs prepared from HEK293 cells transfected with shRluc expression vectors driven by the EBER and U6 promoters (Figure 2b). We noted that the steady-state amounts of shRNA in cells transfected individually with pEBER1-shRluc, pEBER2-shRluc or pU6-shRluc were comparable (Figure 2b, upper panel, lanes 2 and 3 as compared to lane 4). Importantly, siRNAs specifically hybridized to the *Renilla* luciferase (Rluc) sense probe were found in cells transfected with pEBER1-shRluc or pEBER2-shRluc (Figure 2b, upper panel, lanes 2 and 3). siRNAs were also detected in cells carrying pU6-shRluc (Figure 2b, upper panel, lane 4), but not in cells receiving pLuc reporter plasmids alone (lane 1). Thus, effector siRNAs were generated from the EBER-shRluc fusion transcripts.

Induction of RNAi by EBER-shRNA transcripts

Detection of siRNAs in pEBER-shRNA-transfected cells (Figure 2b) predicts that specific RNAi effect could be induced. To provide evidence for this hypothesis, we co-transfected pEBER-shRNA vectors into HeLa cells together with firefly luciferase (Fluc) and *Renilla* luciferase (Rluc) reporter plasmids. The transfected cells were assayed for Fluc and Rluc activities. In this dual luciferase assay, we used pSHAG-Ff1 plasmid, which contains U6 promoter and has been shown to express shFluc efficiently in cultured cells,[9,31,32] as a positive control.

As shown in Figure 3a, Fluc activity was significantly reduced in cells transfected with pEBER1-shFluc or pEBER2-shFluc. A 70% inhibition of Fluc activity was achieved in pEBER2-shFluc-transfected cells. A similar reduction was also seen in cells carrying pSHAG-Ffl. In contrast, cells having empty vector pEBER1-T6 or pEBER2-T6 were able to express Fluc to the same level as cells transfected with reporter plasmids alone (pFluc). Notably, the readouts of Rluc activity in cells carrying

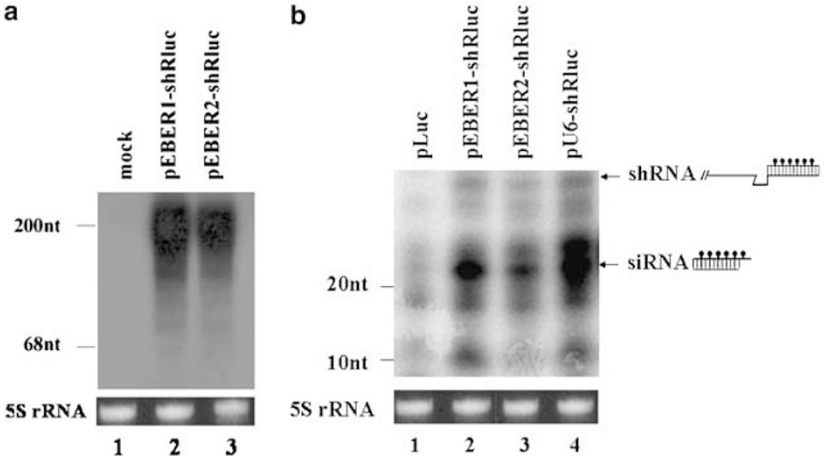

Figure 2 Analysis of Epstein–Barr virus (EBV)-encoded small RNA (EBER)-short hairpin RNAs (shRNAs) and small interfering RNAs (siRNAs) in cells transfected with pEBER1-shRNA targeting Rluc (shRluc) and pEBER2-shRluc vectors. (**a**) Northern blotting. Total cellular RNA was extracted from HeLa cells transfected with the indicated expression plasmids. (**b**) RNase protection assay. RNA was extracted and enriched from HEK293 cells transfected with the indicated expression plasmids. Solution hybridization and RNase treatment were carried out, followed by analysis of protected fragments on a 15% urea gel. Because a ^{32}P-labeled probe of 29 nucleotides was used, protected fragments of siRNA contained 21 or 22 nucleotides, while all protected fragments of shRNA contained 29 nucleotides (see diagrams on the right for reference). To ensure equal loading, 5S rRNA was also stained. nt: nucleotides.

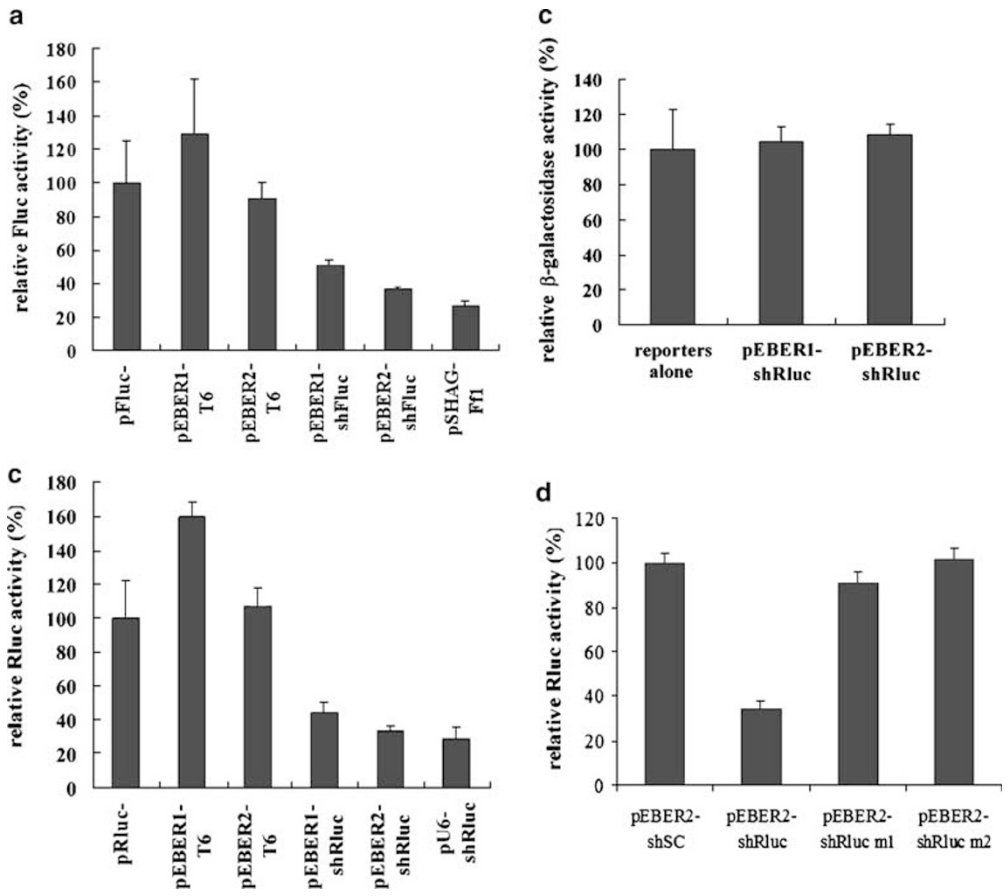

Figure 3 Gene-silencing activity of shRNAs expressed from pEBER1-shRNA and pEBER2-shRNA vectors. (**a**) Silencing of firefly luciferase (Fluc) expression. HeLa cells were transfected with luciferase reporter plasmids pFluc alone or pFluc plus the indicated expression vectors. The relative Fluc activity was obtained by normalizing Fluc readouts with those of *Renilla* luciferase (Rluc). The relative Fluc activity recovered from cells receiving pFluc alone was set as 100%. Results represent the average of triplicate experiments and the error bars indicate s.d. (**b**) Inhibition of Rluc expression. pEBER1-shRluc and pEBER2-shRluc were expression vectors for shRNA targeting Rluc (shRluc) driven by Epstein–Barr virus (EBV)-encoded small RNA (EBER) promoters. The relative Rluc activity was obtained by normalizing Rluc readouts with those of Fluc. The relative Rluc activity recovered from cells transfected with pRluc alone was set as 100%. Results represent the average of triplicate experiments and the error bars indicate s.d. (**c**) Gene-specific RNAi activity of EBER-shRluc. Cells were transfected with the Fluc and *lac*Z reporter plasmids alone (reporters alone) or the reporter plasmids plus the indicated expression vectors. The relative β-galactosidase activity was obtained by normalizing β-galactosidase activity readouts with those of Fluc. The relative β-galactosidase activity of cells having reporters alone was set as 100%. Results represent the average of triplicate experiments and the error bars indicate s.d. (**d**) Sequence-specific RNAi activity of EBER2-shRluc. An expression vector for scrambled shRNA, pEBER2-shSC, was used as a negative control and set as 100%. The shRluc stem region in EBER2-shRluc m1 and m2 mutants contains point mutations as indicated in Figure 1b.

pEBER1-shFluc, pEBER2-shFluc or pSHAG-Ff1 did not drop significantly (data not shown), indicating that the reduction in Fluc activity was rather specific. These results suggest that the EBER promoters can be used to express shRNAs.

To further verify this, we employed the EBER promoters to express another shRNA targeting Rluc (shRluc) but not Fluc. In this case, significant knockdown of Rluc activity was observed in HeLa cells transfected with pEBER1-shRluc or pEBER2-shRluc, when compared to cells carrying plasmid pU6-shRluc, a positive control for expression of shRluc.[31,32] The inhibition of Rluc activity attributed to plasmid pEBER2-shRluc was $>60\%$ (Figure 3b). This inhibition was not seen in cells carrying reporter plasmids alone (pRluc) or an empty vector (pEBER1-T6 or pEBER2-T6). In addition, the original readouts of Fluc activity in cells receiving pEBER1-shRluc, pEBER2-shRluc or pU6-shRluc were comparable to those in cells carrying pFluc/pRluc reporter plasmids alone (data not shown). Consistent with this, the β-galactosidase activity in cells transfected with pEBER1-shRluc and pEBER2-shRluc did not decrease when compared with cells bearing Fluc and *lacZ* reporters alone (Figure 3c), further demonstrating the specificity of RNAi effect. Finally, when point mutations were introduced into the stem of the shRluc (Figure 1b), the gene-silencing effect was abolished (Figure 3d). Thus, EBER-shRluc RNAs were able to mediate gene- and sequence-specific silencing in mammalian cells.

The fusion transcripts produced from pEBER1-shRluc and pEBER2-shRluc contain partial EBER1 and EBER2 sequences at the 5′ end (Figure 1a). While the function of EBER RNAs remains obscure, they have been shown to interact with cellular partners including PKR, L22 and La.[33–35] EBER RNAs can also inhibit apoptosis induced by various signals including IFN-α, but it remains controversial as to whether this antiapoptotic activity is mediated through the inhibition of PKR.[36–38] To address

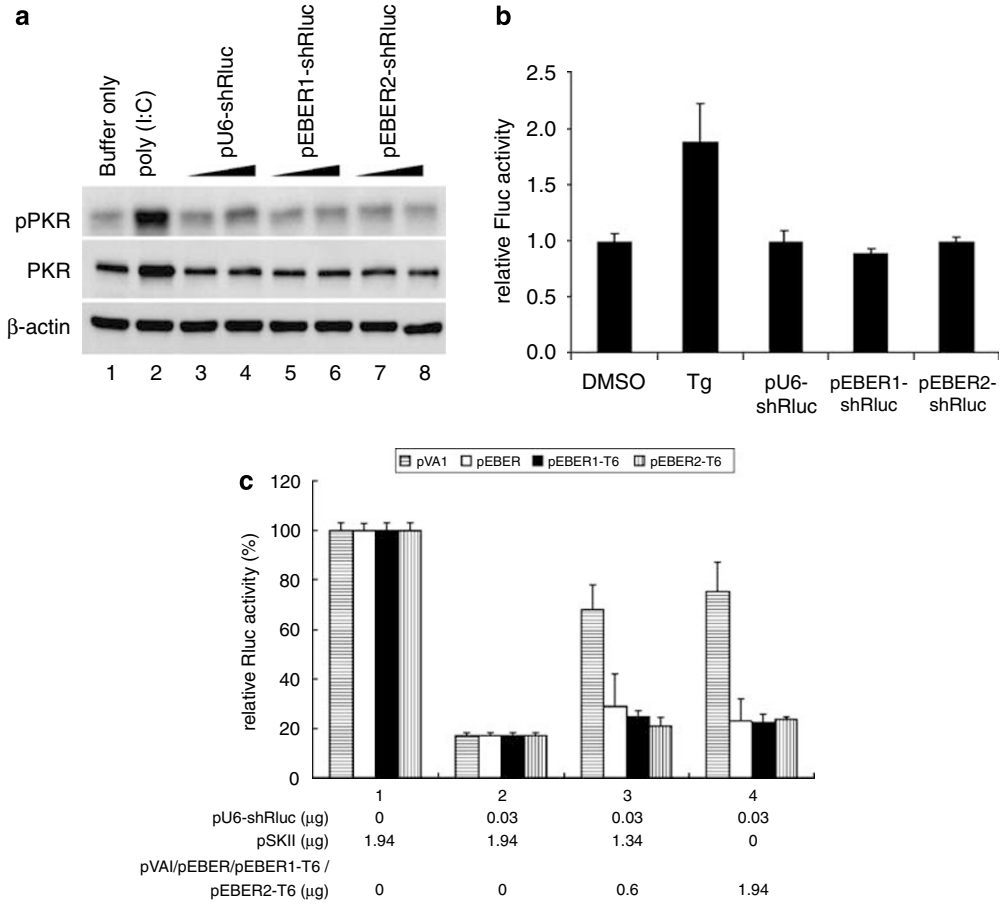

Figure 4 Influence of the partial Epstein–Barr virus (EBV)-encoded small RNA (EBER) sequences on dsRNA-dependent protein kinase (PKR) activation and RNAi. (**a**) Phosphorylation of PKR. Increasing amounts of the indicated plasmids were transfected into HeLa cells. For the positive control group, 100 μg ml^{-1} of poly (I:C) was added directly to the medium. The negative control group received only the buffer that was used to dissolve poly (I:C). (**b**) Activation of eIF2α. pATF4-UTR-Luc is a firefly luciferase (Fluc) reporter with the 5′ UTR of human ATF4 fused to the coding region of Fluc. When eIF2α is activated, stimulation of ATF4 translation through upstream open reading frames in the 5′ UTR can be observed, as shown in the positive control group, in which 600 nM thapsigargin (Tg) was added to the medium. The negative control group received dimethyl sulfoxide (DMSO) only. The relative Fluc activity was obtained by normalizing with the readings of the *Renilla* luciferase (Rluc) activity. Results represent the average of triplicate experiments and the error bars indicate s.d. (**c**) Influence of EBER RNAs on RNAi. Indicated amounts of the different expression vectors were transfected into 293 T cells. Results represent the average of triplicate experiments and the error bars indicate s.d.

whether the partial EBER sequences could activate PKR, we carried out two experiments. First, we checked for the phosphorylated and active form of PKR in pEBER1/2-shRNA-transfected cells (Figure 4a). While treatment with poly(I:C) potently induced phosphorylation of PKR (Figure 4a, lane 2 as compared to lane 1), the steady-state levels of phosphorylated PKR were unchanged in pEBER-shRluc-expressing cells (Figure 4a, lanes 5–8 as compared to lane 1). In this setting, the shRluc expressed from U6 promoter had a weak stimulatory effect on PKR (Figure 4a, lane 4 as compared to lanes 3 and 1). In the second experiment, we assessed the activation of eukaryotic initiation factor 2α (eIF2α) using the pATF4-UTR-Luc reporter construct. eIF2α is a substrate of PKR and phosphorylation of eIF2α stimulates the translation of ATF4 through two upstream open reading frames in the 5′ untranslated region (UTR).[5,39–41] Based on these previous findings, the pATF4-UTR-Luc construct was used as an indicator of eIF2α phosphorylation and activation in our experiment.[41] We noted that treatment with thapsigargin, a known stimulator of eIF2α kinase,[5] induced a two-fold activation of luciferase reporter activity controlled by the 5′ UTR of ATF4 (Figure 4b). In contrast, no activation of this reporter and thus the activity of eIF2α were observed in cells expressing EBER1-shRluc or EBER2-shRluc (Figure 4b). Hence, our two experiments consistently demonstrated that the partial EBER sequences did not activate PKR in mammalian cells.

On the other hand, EBER RNAs are highly structured[42] and resemble adenovirus VAI RNA and human immunodeficiency virus TAR RNA, which have recently been found to be inhibitors of RNAi by exhausting Dicer and sequestering dsRNA-binding protein TRBP, respectively.[43,44] In light of this, we carried out additional experiments to address whether EBER RNAs might influence RNAi. Results from our control experiments shown in Figures 3a and b indicated that the partial EBER sequences produced from pEBER1-T6 and pEBER2-T6 had minimal influence on the expression of luciferase reporter. To determine the effect of EBER RNAs on the activity of shRluc, we co-expressed

different combinations of RNAs in 293 T cells. In our experimental setting, adenovirus VAI was fully competent in suppressing the activity of shRluc (Figure 4c; groups 3 and 4 as compared to groups 1 and 2). In contrast, neither full-length (expressed from pEBER) nor partial (expressed from pEBER1/2-T6) EBER1/2 sequences inhibited the suppressive activity of shRluc (Figure 4c; groups 3 and 4 as compared to groups 1 and 2). These results suggested that EBER RNAs unlikely acted as viral suppressors of RNAi. Thus, the suppressive activities of fusion transcripts EBER1-shRluc and EBER2-shRluc on reporter expression were attributed to the shRluc sequences at the 3′ end, but not to the partial EBER sequences.

Comparison of EBER2 promoter with other Pol III promoters for shRNA expression

Above, we showed that EBER promoters were able to drive the expression of shRNAs in HeLa cells (Figure 3). To assess the utility of this type of viral promoters in different contexts, we extended our analysis to other cell lines and made a comparison between EBER2 promoter and other human Pol III promoters commonly used in shRNA expression. All these promoters (that is, EBER2, $tRNA^{Val}$, U6 and H1 promoters) were inserted into the same vector backbone (Figure 1a). These shRNA expression vectors were then compared for their shRNA-mediated gene-silencing activities in four different cell lines. The pEBER2-shSC vector expressing scrambled shRNAs was used as a negative control.

The four cell lines used were HeLa (human epithelial cervical carcinoma), HEK293 (human embryonic kidney epithelial cells), C666-1 (human nasopharyngeal carcinoma) and HK1 (human nasopharyngeal carcinoma). The C666-1 cell line was derived from undifferentiated nasopharyngeal carcinoma and it consistently harbors EBV, resembling the EBV latency II status.[45] The HK1 cell line was derived from well-differentiated nasopharyngeal carcinoma and it does not carry EBV.[46] The expression of EBER2 genes in the EBV^+ C666-1 cells as well as the EBV^- HK1 cells was examined by RT–PCR (Figure 5, inset). Judging from the gene-silencing activities in all four cell lines, the expression of shRluc from EBER2 promoter was more effective than from the other commonly used Pol III promoters including $tRNA^{Val}$, U6 and H1 promoters (Figure 5). As such, pEBER2-shRluc was able to sustain a 60–80% knockdown of Rluc activity and among all vectors tested it delivered the greatest gene-silencing effect in nasopharyngeal carcinoma cell lines C666-1 and HK1.

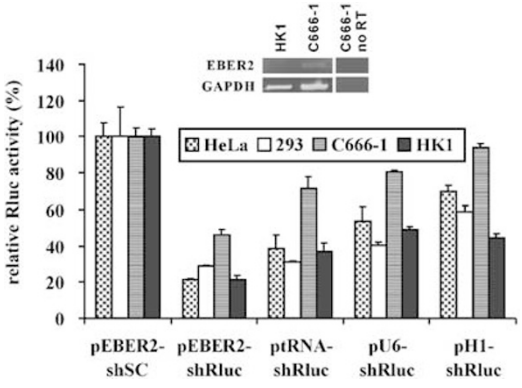

Figure 5 Comparison of shRNA expression vectors. Four different cell lines (HeLa, HEK293, C666-1 and HK1) were transfected with reporter plasmids pRluc and the indicated shRNA expression vectors (see Figure 1 for reference). Results were normalized to Fluc activity and the average of triplicate experiments was shown. The error bars indicate s.d. The relative *Renilla* luciferase (Rluc) activity recovered from cells with pEBER2-shSC, a vector having scrambled shRNA sequence expressed, was set as 100%. Shown in the inset are results from RT–PCR analysis of EBER2 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) genes performed with EBV^- HK1 cells, EBV^+ C666-1 cells, as well as C666-1 cells without the addition of reverse transcriptase (RT) (C666-1 no RT).

Analysis of mRNA target in cells carrying EBER vectors

Results from dual luciferase assays support the notion that the EBER promoters can be used to drive the expression of shRNAs at least as effectively as the commonly used H1, U6 and $tRNA^{Val}$ promoters and in many cases worked even better (Figures 3 and 5). While reporter assays are sensitive, they only indirectly reflect the gene-silencing activities of siRNAs derived from the fusion EBER-shRNA transcripts (Figure 2). The detection of strong RNAi effect induced by ERER-shRNAs and the detection of siRNAs in cells expressing EBER-shRNAs prompted us to ask whether the gene-silencing effect observed was due to siRNA-induced degradation of the target mRNA. To address this question, we performed quantitative RT–PCR using total RNA extracted from pEBER1-shRluc- and pEBER2-shRluc-transfected HEK293 cells (Figure 6). Another siRNA expression plasmid pU6-shRluc was chosen for comparison. Additionally, an empty siRNA expression vector pGEM-U6 was used as a negative control. We noted that the relative Rluc mRNA level did decrease in pEBER1-shRluc-transfected cells. The Rluc mRNA level dropped to a more significant degree in cells having pEBER2-shRluc. Around 85% of the targeted Rluc mRNA was cleaved and this ratio was comparable to that observed in pU6-shRluc-transfected cells. Hence, shRNAs transcribed from vectors with the EBER promoters were effectively processed into siRNAs that induce sequence-specific mRNA degradation.

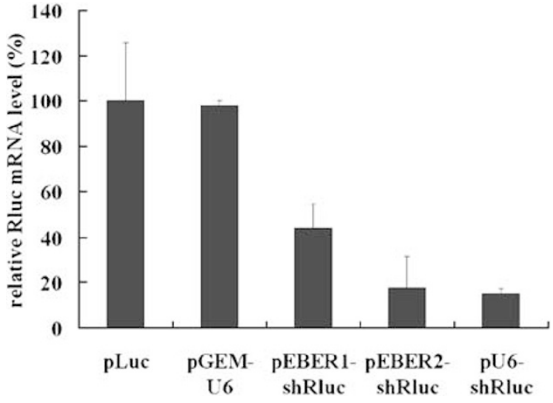

Figure 6 Quantitative PCR analysis of messenger ribonucleic acid (mRNA) degradation induced by shRNAs expressed from pEBER1-shRNA targeting Rluc (shRluc) and pEBER2-shRluc vectors. Each bar represents the average of triplicate experiments and s.d. was also plotted. Normalization with the Fluc mRNA level was carried out to obtain the relative Rluc mRNA level. The relative Rluc mRNA level of pLuc-transfected cells was set as 100%.

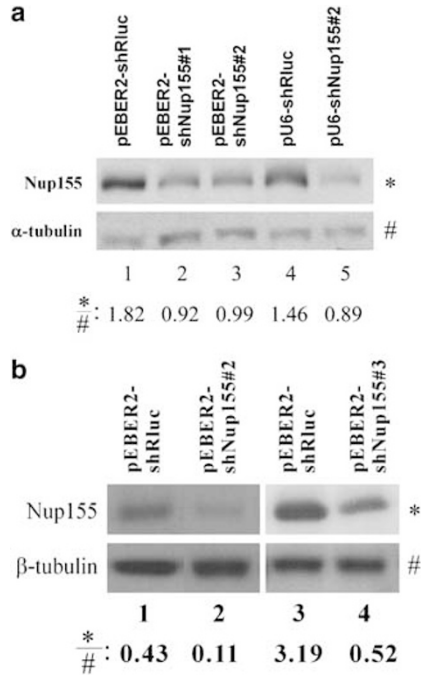

Figure 7 Knockdown of cell-endogenous Nup155 transcript using shRNA-expressing vector driven by the Epstein–Barr virus-encoded small RNA 2 (EBER2) promoter. (**a**) Western blotting using 293 T cells transiently transfected with the shRNA-expressing vector. The relative Nup155 protein amount for each sample was analyzed using Scion Image software. (**b**) Western blotting using HeLa cells stably harboring the shRNA-expressing vector.

Silencing of an endogenous transcript with EBER2 vector

After checking for the silencing of exogenously introduced reporter genes with a series of assays, we were also interested in assessing the effectiveness of the EBER vectors in delivering siRNAs that target cell-endogenous transcripts. We designed two independent shRNAs against human nucleoporin Nup155, a component of the nuclear pore complex required for the formation of nuclear membrane.[47] In 293 T cells transfected with pEBER2-shNup155 vector, the expression of Nup155 protein was diminished by approximately 50% (Figure 7a, lanes 2 and 3 as compared to lane 1). Remarkably, when the two shNup155s expressed from the EBER2 promoter were compared to each other, their efficiencies in gene silencing were found to be similar (Figure 7a, lanes 2 as compared to lane 3). In addition, when shNup155 no. 2 was separately expressed from the EBER2 and the U6 promoters, the silencing efficiencies were also comparable (Figure 7a, lane 3 as compared to lane 5). Thus, the EBER2 promoter is highly efficient in driving the expression of shNup155s.

Because the above assays on the EBER2 promoter were carried out via transient transfection of cells, it will be of interest to see whether the expression of shRNAs from the EBER2 promoter could be sustained in established cell lines stably carrying the shRNA expression vector. Thus, we transfected the EBER2 promoter-based expression vector into HeLa cells and selected stable clones with the addition of G418. Western blot analyses indicated that the expression of Nup155 was constitutively suppressed in several clones. It is noteworthy that this inhibition of Nup155 expression is not restricted to a single clone. For example, the amount of Nup155 recovered from two representative stable clones carrying pEBER2-shNup155 (clones nos. 2 and 3) was significantly less than from cells containing pEBER2-shRluc (Figure 7b, lane 2 as compared to lane 1, and lane 4 as compared to lane 3). Hence, the EBER2 vector was able to deliver siRNAs targeting exogenous or endogenous transcripts into cultured mammalian cells both efficiently and sustainably.

Discussion

In this feasibility study, we established the utility of the EBER promoters from EBV for the expression of gene-silencing RNAs in cultured mammalian cells. EBER-shRNAs of the correct size were abundantly transcribed from the EBER promoters (Figure 2a) and were subsequently processed presumably by endogenous Dicer nuclease into siRNAs (Figure 2b). The effector siRNAs were able to induce sequence-specific degradation of target mRNA (Figure 6) leading ultimately to the knockdown of protein expression as indicated in the reduction of protein amount and activity (Figures 3 and 7). The gene-silencing effect observed was gene- and sequence specific, with no evidence of PKR activation (Figures 3 and 4). The expression of shRNAs by the EBER promoters was as effective as or even more effective than that from other commonly used Pol III promoters. In addition, the EBER2 promoter was the most efficient among all promoters tested in nasopharyngeal carcinoma cells (Figure 5).

Ever since the discovery that 21-nucelotide siRNAs introduced into mammalian cells effectively induce RNAi without provoking the IFN response,[7] various strategies have been used to achieve the greatest and the most stable gene-silencing effect within the cells. Although some Pol II promoters including CMV and U1 promoters[18,19] have been shown to be able to deliver shRNAs into the cells, up till now one common design of RNAi experiments is still to express shRNAs from a DNA vector driven by Pol III promoters, such as those of U6, H1 and tRNA.[9,11–16,21,22] Our work has added one important group of viral Pol III promoters to the list of promoters that can drive shRNA expression. Our findings establish the concept that shRNA fusion transcripts produced by Pol III-dependent viral promoters are effectively processed by Dicer into functional siRNAs.

While the existing pEBER-shRNA vectors are already powerful, they can be further modified and improved in several aspects. First, because efficient transcription from the EBER promoters depends on the secondary structure of the RNA,[48] further modification of the EBER-shRNA sequences might enhance their transcription from the EBER promoters. Second, other unique features of the EBER promoters can also be utilized to enhance RNA expression from the pEBER-shRNA vectors. Particularly, the EBER promoters have both extragenic (Sp1, ATF and ETAB) and intragenic promoter elements (boxes A and B).[27–29] All these elements are important for the transcriptional activity[27–29] and were incorporated into the existing pEBER-shRNA vectors (Figure 1a). The

extragenic promoter elements of EBER may provide an opportunity for developing inducible shRNA expression vector. Third, since the activation of ATF2 and the elevation of pol III-specific transcription factors in EBV-infected cells are responsible for the exceedingly strong activity of the EBER promoters,[30] RNA expression could be optimized through manipulation of the host cell. For instance, constitutively active versions of ATF2 and other key factors could be stably introduced to the cell in order to maximize RNA production. Finally, it will be of interest to see whether the EBER promoters might still be feasible for the expression of small RNAs when incorporated into viral vectors, such as lentiviral, adenoviral and even EBV-based vectors.

The EBERs localize to the nucleus.[35,49] It will thus be of interest to determine the subcellular localization of EBER-shRNAs that are transcribed from the EBER promoters. In this regard, some shRNAs expressed from the U6 promoter have been found mainly in the nucleus.[22,50] The subcellular localization of RNAs expressed from Pol III promoters depends mainly on the expression cassette.[50] Interestingly, while RNAi mediated by mRNA degradation occurs in the cytoplasm, siRNAs can also localize to the nucleus and induce specific RNA degradation there.[51,52] In addition, nuclear siRNAs are capable of inducing specific gene silencing.[52–55] Thus, shRNAs and siRNAs targeted to the cytoplasm and the nucleus can be used to knockdown different RNAs specifically through different mechanisms.

EBV is etiologically associated with several lymphoid and epithelial malignancies including Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma and gastric cancer.[56,57] While EBV can produce lytic and persistent infection, all EBV-associated cancers involve the establishment of three types (I–III) of latency in target cells. The EBERs are most abundantly found in all types of EBV-infected cells, having 10^7 copies per cell.[27] Previous attempts to design anti-EBV agents based on the specific and high expression of EBERs have already been successful. For example, an engineered recombinant adenovirus which depends on EBERs for replication showed specific oncolytic activity in EBV^+ tumor cells.[58] In our experiments, EBER-shRNAs transcribed from the EBER promoters were highly abundant in cells (Figure 2a). The degradation of target mRNA induced by these shRNAs was also remarkable (Figure 6). In addition, the EBER2 promoter was indeed most potent in nasopharyngeal carcinoma cells C666-1 and HK1 (Figure 5). Although HK1 cells are EBV^-, they were likely derived from EBV^+ cells, in which certain cellular genes were irreversibly altered through genetic and epigenetic mechanisms.[46] As a result, these alterations in HK1 cells might support an elevated activity of the EBER2 promoter. Nevertheless, the high activity of the EBER2 promoter in nasopharyngeal carcinoma cells can be utilized to deliver siRNA-based agents to study and combat this tumor. For example, the EBER2-shRNA expression vector might be particularly powerful in silencing EBV-specific genes, such as EBV nuclear antigen EBNA1, in EBV-infected tumor cells. EBNA1 is required for the maintenance of EBV episomes and the silencing of EBNA1 expression inhibits tumor cell growth.[59–61] Thus, the delivery of EBNA1-silencing siRNAs via pEBER2-shRNA vector might prove useful in the development of new anti-EBV therapeutics.

While the biological functions of EBERs remain obscure and controversial, one of their potential functions is to promote cellular growth.[62] We showed that the EBER sequences remaining in the shRNA fusion transcripts could neither activate PKR nor suppress RNAi (Figure 4), suggesting that these partial EBER sequences might not share the same PKR-modulatory properties as their full-length counterparts. However, it will still be of importance to clarify the influence of these EBER sequences on cell growth and tumorigenesis before the EBER promoter-based vectors could be used to deliver any therapeutic agents into human cells.

Materials and methods

Construction of RNA expression vectors

PCR and TA cloning were employed for the construction of shRNA expression vectors. The shRNA expression cassettes containing the promoters (that is, promoter-sense-loop-antisense-termination signal T6) were amplified by PCR. The EBER promoters were derived from EBV genomic DNA purified from B95-8 cells (GenBank accession number of EBV sequence: V01555; EBER sequences amplified: EBER1: 6510∼6736, EBER2: 6856∼7078). Human H1 promoter and human U6 promoter were PCR-subcloned from plasmids pSUPER (a gift from Reuven Agami, the Netherlands Cancer Institute)[11] and pGEM-U6 (a gift from Greg Hannon, Cold Spring Harbor Laboratory).[9] Human $tRNA^{Val}$ promoter was PCR-amplified from genomic DNA of HeLa cells. Expression vector for adenovirus VAI has been described.[31] The EBER expression vector expresses both EBER1 and EBER2 under the control of their own promoters.

The shRNA against firefly luciferase (shFluc) was derived from plasmid pSHAG-Ff1 (a gift from Greg Hannon).[9] The shRNA targeting Rluc (shRluc) was targeted to 1776 to 1803 nucleotides of Rluc mRNA. The primers used were as follows: EBER1-shFluc, 5′-GGA AAT GAG GGT TAG CAT AGG C-3′ (forward) and 5′-AAA AAA ATC AGG TGG CTC CCG CTG AAT TGG AAT CCC AAG CTT CGG ATT CCA ACT CAG CGA GAG CCA CCC GAT CTC GAG ATC GAT TCT AGA AGA CAA CCA CAG ACA CCG TC-3′ (reverse); EBER2-shFluc, 5′-GCT TAA CGT TGC ATC CCA GAA G-3′ (forward) and 5′- AAA AAA ATC AGG TGG CTC CCG CTG AAT TGG AAT CCC AAG CTT CGG ATT CCA ACT CAG CGA GAG CCA CCC GAT CTC GAG ATC GAT TCT AGA CTG ACT TGC AAA TGC TCT AGG C-3′ (reverse); EBER1-shRluc, 5′-GGA AAT GAG GGT TAG CAT AGG C-3′ (forward) and 5′- AAA AAA GTT AGG AAT TAT AAT GCT TAT CTA CGT GCC AAG CTT CGC ACA TAG ATA AGC ACT ATA ATC CCT AAC CTC GAG ATC GAT TCT AGA AGA CAA CCA CAG ACA CCG TC-3′ (reverse); EBER2-shRluc, 5′-GCT TAA CGT TGC ATC CCA GAA G-3′ (forward) and 5′-AAA AAA GTT AGG AAT TAT AAT GCT TAT CTA CGT GCC AAG CTT CGC ACA TAG ATA AGC ACT ATA ATC CCT AAC CTC GAG ATC GAT TCT AGA CTG ACT TGC AAA TGC TCT AGG C-3′ (reverse); $tRNA^{Val}$-shRluc, 5′-TTG ATC CCG AAA GAT GTC CAG CG-3′ (forward) and 5′-AAA AAA GTT AGG AAT TAT AAT GCT TAT CTA CGT GCC AAG CTT CGC ACA TAG ATA AGC ACT ATA ATC CCT AAC CTC GAG ATC GAT TCT

AGA TTT CCG CCC GGT TTC GAA C-3′ (reverse); U6-shRluc, 5′-ATT TAG GTG ACA CTA TAG-3′ (forward) and 5′-AAA AAA GTT AGG AAT TAT AAT GCT TAT CTA CGT GCC AAG CTT CGC ACA TAG ATA AGC ACT ATA ATC CCT AAC CTC GAG ATC GAT TCT AGA CGG TGT TTC GTC CTT TCC AC-3′ (reverse); H1-shRluc, 5′-TAA TAC GAC TCA CTA TAG GG-3′ (forward) and 5′-AAA AAA GTT AGG AAT TAT AAT GCT TAT CTA CGT GCC AAG CTT CGC ACA TAG ATA AGC ACT ATA ATC CCT AAC TCT AGA CTC GAG ATC GAT GGG AAA GAG TGG TCT CAT ACA GAA CTT ATA AGA TTC CC-3′ (reverse).

EBER2-shRluc mutants were constructed using QuikChange site-directed mutagenesis reagents (Stratagene, La Jolla, CA, USA). The shRNA sequences against Nup155 (shNup155) were designed with the help of the software provided by the Whitehead Institute (URL: http://jura.wi.mit.edu/bioc/siRNAext/). The forward primer used to amplify EBER2-shNup155 was the same as that used for EBER2-shFluc. The reverse primers used were as follows: EBER2-shNup155 no. 1, 5′-GAA GAT CTA AAA AAC ATG CAG GTG TTA GGT TAT ACA AGC TTC TAT AAC CTA ACA CCT GCA TGT GGA TCC CTG ACT TGC AAA TGC TCT AGG C-3′; EBER2-shNup155 no. 2, GAA GAT CTA AAA AAG CCG GTT ATT CAG ACC CTA TAC AAG CTT CTA TAG GGT CTG AAT AAC CGG CGG ATC CCT GAC TTG CAA ATG CTC TAG GC-3′. The forward primers used to PCR the shRNAs with scrambled sequence was those used for EBER2-shRluc and U6-shRluc. The reverse primers are as follows: EBER2-shSC, 5′-AAA AAA GAA GAG AAC AGA ATA AGA GAT TCT CTT GAA ATC TCT TAT TCT GTT CTC TTC CTG ACT TGC AAA TGC TCT AGG C-3′; U6-shSC, 5′-AAA AAA GAA GAG AAC AGA ATA AGA GAT TCT CTT GAA ATC TCT TAT TCT GTT CTC TTC GGT GTT TCG TCC TTT CCA C-3′. The resulting products were then separately cloned either into pGEM-T easy vector (Promega, Madison, WI, USA) or into pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) with the CMV promoter removed.

Cell transfection and establishment of stable Nup155-knockdown cell lines

HeLa, HEK293 and 293 T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). C666-1 and HK1 cells were grown in RPMI1640 medium supplemented with 10% FBS. Cells were transfected at 50% confluence in 12-well plate, 6-well plate or 10 cm dish for 48 h using GeneJuice transfection reagent (Novagen, San Diego, CA, USA), FuGene 6 (Roche, Indianapolis, IN, USA) or Lipofectamine 2000 (Invitrogen). Plasmids expressing Fluc under the control of SV40 promoter (pGL3-Control) or Rluc driven by the CMV enhancer and early promoter (pRL-CMV) were from Promega.

To knockdown the expression of Fluc, pGL3-control, pRL-CMV and shRNA expression vector were co-transfected into cells in a ratio of 1:1:3. As such, cells in each well of the 12-well plate received 0.3 μg of pGL3-Control, 0.3 μg of pRL-CMV and 0.9 μg of shRNA vector. To target Rluc, the above vectors were used with either a ratio of 1:1:2 or 1:1:3. For assessing the specificity of the knockdown effect mediated by shRluc, pRL-CMV was replaced by pcDNA3.1/V5-His/*lac*Z (Invitrogen) in the transfection. To evaluate vector influence on PKR activity, 1 or 2 μg of shRNA expression vector was transfected. Plasmid pATF4-UTR-Luc used in the determination of eIF2α activation has been described.[41]

For the generation of stable Nup155-knockdown cell lines, HeLa cells were transfected with shRNA expression vectors carrying a G418 resistance gene cassette. After 48 h, the cells were selected with G418 (Calbiochem, San Diego, CA, USA) for 2 weeks before the isolation of single colonies.

Dual luciferase reporter assay

Luciferase assays were performed as described.[32,63,64] Briefly, after rinsing with 1 × PBS once, the harvested cells were lysed with 150 μl of passive lysis buffer (Promega) at room temperature for 15 min. Subsequently, 5 or 10 μl of the cell lysate was added to an opaque 96-well plate for measurement of luciferase activity using the Dual-Luciferase reporter assay system (Promega). The readouts of luciferase activity were taken in an LB 96 V microplate luminometer (EG&G, Victoria, Australia).

Luminescent β-galactosidase reporter assay

Cells were harvested and lysed with passive lysis buffer. Measurement of β-galactosidase activity was performed using the luminescent β-galactosidase detection kit II (Clontech, Mountain View, CA, USA). The readouts of β-galactosidase activity were taken in an LB 96 V microplate luminometer (EG&G).

Northern blotting

Total RNA was extracted from cells, 48 h after transfection, using Trizol reagent (Invitrogen). A total of 20 μg of total RNA was then separated by electrophoresis on a 12% polyacrylamide per 8 M urea gel and electroblotted onto a Zeta-Probe GT membrane (Bio-Rad, Hercules, CA, USA) for 1.5 h at 30 V. RNA was immobilized by UV crosslinking. Hybridization was carried out at 42 °C using Ultrahyb-Oligo hybridization buffer (Ambion, Austin, TX, USA) and a ^{32}P-labeled Rluc sense DNA oligonucleotide. Membrane was washed twice for 20 min each at 42 °C with 2 × SSC and 0.2% sodium dodecyl sulfate (SDS). Film was exposed overnight with an intensifying screen.

RNase protection assay

Small RNAs (around 200 nucleotides or smaller) were extracted and enriched from transfected HEK293 in 10 cm dish using *mir*Vana miRNA isolation kit (Ambion). A ^{32}P-labeled Rluc sense RNA probe of 29 nucleotides in length was made using the *mir*Vana miRNA probe construction kit (Ambion). Solution hybridization was carried out overnight at 42 °C with 10 μg of the enriched RNA and the gel-purified probe. After 24 h of hybridization, RNase treatment was performed for 1 h with 40 μg ml^{-1} RNase A (USB) and 2 U of RNase T1 (Ambion). RNase was then inactivated with 5 μl of proteinase K (10 mg ml^{-1}) in 10 μl of 20% SDS. The protected fragments were analyzed by polyacrylamide gel electrophoresis with 15% urea. Results were visualized using a phosphor imager (Molecular Dynamics, Sunnyvale, CA, USA).

Quantitative RT–PCR

Total cellular RNA was extracted using Trizol and proteinase K. Briefly, the cells were incubated with Trizol for 3 min at room temperature. The lysed samples were then treated with 10 μl of preheated proteinase K (10 mg ml^{-1}) at 55 °C for 30 min. Total cellular RNA was extracted using the protocol provided by Invitrogen. After RNA extraction, 2 μg of the total cellular RNA was treated with 2 U of DNase (Ambion) at 37 °C for 15 min. DNase was heat-inactivated at 65 °C for 20 min. The synthesis of cDNA with oligo(dT)$_{20}$ primer was then performed using the ThermoScript RT–PCR system (Invitrogen). Subsequently, 2 μl of the synthesized cDNA was added to the quantitative PCR reaction mix containing 12.5 μl of 2 × Brilliant SYBR Green QPCR master mix (Stratagene), 2.5 μl of forward primer (1 μM), 2.5 μl of reverse primer (1 μM) and 5.5 μl of water. Primers used to quantitate the Rluc mRNA were 5′-ACG CTG AAA GTG TAG TAG A-3′ (forward) and 5′-AGA ATC CTG GGT CCG A-3′ (reverse). For RNA normalization, quantitation of the Fluc mRNA was performed using 5′-TCT ATC CGC TGG AAG ATG-3′ (forward) and 5′-ACT GTT GAG CAA TTC ACG-3′ (reverse). Quantitative PCR was carried out in M × 3000P real-time PCR system (Stratagene) using the following thermal cycling profile: 1 cycle of 95 °C for 10 min, 40 cycles of amplification (95 °C for 30 s, 55 °C for 1 min and 72 °C for 30 s), followed by 41 cycles of incubation where the temperature increased by 1 °C per cycle beginning at 55 °C and ending at 95 °C. For each experimental setup, triplicate measurements were carried out and analyzed using the software (version 1.20c) provided with the M × 3000P system.

Antibodies and western blotting

RIPA buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) was used to lyse transfected HeLa or 293 T cells. To detect PKR and phosphorylated form of PKR, PhosphoStop solution (Roche) was also added to RIPA. The lysates were analyzed by SDS–PAGE. The polyvinylidene fluoride-membrane transferred with proteins was blocked with 5% skim milk in PBS and probed with primary antibodies. Primary antibodies used include rabbit polyclonal antibodies against PKR or phosphorylated form of PKR (Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal antibodies against Nup155 (kindly provided by Iain Mattaj, European Molecular Biology Laboratory),[47] mouse monoclonal antibodies against β-actin (Sigma, St Louis, MO, USA) and mouse monoclonal antibodies against α- or β-tubulin (Sigma). Membranes were washed three times with Tris-buffered saline containing Tween 20 before donkey anti-rabbit or sheep anti-mouse HRP-conjugated secondary antibodies (GE Healthcare Life Sciences, Piscataway, NJ, USA) were added. After washing the membranes three times with TBST, bands were detected using the ECL system (GE Healthcare Life Sciences).

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