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Stable suppression of gene expression by short interfering RNAs targeted to promoter in a mouse embryonal carcinoma stem cell line

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Abstract RNA interference (RNAi) can induce gene silencing via two pathways: post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS). The mediators of gene inactivation in both pathways are 21-bp small interfering RNAs (siRNAs) generated from longer double-stranded RNA (dsRNA). PTGS involves siRNA-mediated targeting and degradation of mRNA. However, siRNAs induce TGS via DNA methylation at the targeted promoter. Synthetic siRNAs can induce loss of gene activity comparable to long dsRNA. The limitation of this method is that the transfected synthetic siRNA works for only a few days. In this study, we tested the RNAi response to siRNA (PTGS pathway) by using a plasmid containing an enhanced green fluorescent protein (eGFP) gene as a target as well as a plasmid creates siRNA transcript, in a form of a hairpin, against eGFP gene. To investigate TGS pathway via RNAi, we also used a plasmid creates hairpin siRNA transcript against *pgk-1* promoter. The data presented here indicated long-lasting inhibition in expression of eGFP and *puro*mycin genes, both under the control of the murine *Pgk-1* promoter. However, Southern blot analysis showed no methylation in *pgk-1* promoter.

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Introduction

Double-stranded RNA (dsRNA) induces sequence-specific gene silencing in many organisms by a process known as RNA interference (RNAi; Fire et al. 1998). RNAi has been linked to many previously described silencing phenomena such as post-transcriptional gene silencing (PTGS) in plants (Jorgensen 1990) and quelling in fungi (Romano and Macino 1992; Bernstein et al. 2002). It is also evident in unicellular organism, metazoans, such as *Drosophila*, and mammals (Flavell 1994; Pal-Bhadra et al. 1997; Elbashir et al. 2001a; Esmaeili 2009). Biochemical analysis of the mechanism of RNAi has indicated that the mediators of gene silencing are 21-bp small interfering RNAs (siRNAs) generated from longer dsRNA by the RNase III-like enzyme, Dicer (Hammond et al. 2000). RNAi can induce gene silencing via two pathways: PTGS and transcriptional gene silencing (TGS; Sijen et al. 2001; Pal-Bhadra et al. 2002). Each pathway involves the action of siRNAs. PTGS involves siRNA-mediated targeting and degradation of mRNA, which occurs predominantly in the cytoplasm (Langlois et al. 2005; Robb et al. 2005). However, siRNAs were demonstrated to induce TGS via DNA methylation at the targeted promoter (Pickford and Cogoni 2003; Castanotto et al. 2005; Han et al. 2007) and through heterochromatin assembly in mammalian cells (Ting et al. 2005; Weinberg et al. 2006; Kim and Rossi 2007). Recently, siRNAs targeted to gene promoters were demonstrated to induce TGS via DNA methylation in human cells (Han et al. 2007).

In this study, we used undifferentiated mouse embryonal carcinoma (EC) cell line, P19, to investigate the RNAi

response to siRNA by using a transgene containing an enhanced green fluorescent protein (eGFP) gene as a target, driven by a *pgk-1* promoter. In previous studies, it has been shown that the synthetic siRNAs were functional in vivo and could induce loss of gene reporter activity comparable to long dsRNA (Romano and Macino 1992; Caplen et al. 2000; Hammond et al. 2000; Elbashir et al. 2001a). However, the limitation of this method is that the transfected synthetic siRNA works for only a few days in mammalian cells. To overcome the limitation of using siRNA, we used a vector, based on suppression of endogenous RNA (pSUPER) system that directs the synthesis of siRNA-like transcripts (Brummelkamp et al. 2002).

In 1994, McBurney and colleagues have shown that the murine *Pgk-1* promoter is very active when used to drive expression of reporter genes in pluripotent embryonic cells. *Pgk-1* is an X-linked gene encoding 3-phosphoglycerate kinase, an enzyme necessary in every cell for glycolysis (McBurney et al. 1994). Previous works on the *Pgk-1* gene established that its promoter was very active in driving the expression of exogenous genes following transient and stable transformation of various cell lines (McBurney et al. 1991). To investigate TGS pathway via RNAi, we also used a siRNA transcript against *pgk-1* promoter.

Materials and Methods

Plasmids The plasmids used in this project were pML8 (a vector encoding eGFP and *puromycin* (*puro*) resistance gene under the control of the murine *Pgk-1* promoter), pSUPER (contains a polymerase-III H1-RNA promoter to make the siRNA transcript), pJC2 (expressing specific siRNA transcript for eGFP), pKJ274 (expressing specific siRNA transcript for *pgk-1* sequence), and pKJ303 (contains *hygromycin* (*hygro*) resistance gene and ego-1 under the control of the cmv promoter).

Cell culture and transfection of cells The studies reported here were carried out with the P19 line of murine EC stem cells (McBurney 1993). The cells were plated in a 60-mm culture dish at concentration of 10^6 cells and transfected with 5 μg of circular plasmid DNAs by a modified calcium phosphate ($CaPO_4$) coprecipitation method (Chen and Okayama 1987). Briefly, cells were split 24 h before the transfection. Then $CaPO_4$–DNA solution was added dropwise onto the cells. The cells then incubated for 7 to 9 h at 37°C in 5% CO_2. After incubation period, the growth medium was replaced by fresh α-MEM. In stable transfection, the cells were assayed after 8 d.

siRNA-mediated inhibition of eGFP reporter gene in P19 EC cells Initially, we tested the effect of siRNA on expression of eGFP reporter gene. The cells of the first group were cotransfected with equal ratio of a plasmid expressing eGFP (pML8) together with either pSUPER or a plasmid expressing siRNA specific for eGFP (pJC2; Fig. 1) as follows:

$$\mathrm{pML8} + \mathrm{pSUPER(control)}$$
$$\mathrm{pML8} + \mathrm{pJC2}$$

The following sequence of eGFP was selected to use in the oligonucleotide:

5′-GCTGACCCTGAAGTTCATCT-3′

The cells were selected with medium containing 2 μg/ml *puro* and were assayed after 8 d.

siRNA-mediated inhibition of gene expression via pgk-1 promoter in P19 EC cells To determine whether siRNA could affect the *pgk-1* promoter, we next used the vector pKJ274 (Fig. 2). The cells of the second group were cotransfected with equal ratio of a plasmid expressing eGFP (pML8) together with either pSUPER or a plasmid expressing siRNA specific for *Pgk-1* promoter (pKJ274) as follows:

$$\mathrm{pML8} + \mathrm{pSUPER(control)}$$
$$\mathrm{pML8} + \mathrm{pKJ274}$$

The following sequence of *pgk-1* was selected to use in the oligonucleotide:

5′-TAGCAGCTTTGCTCCTTCGC-3′

The cells were selected with medium containing 2 μg/ml *puro* and were assayed after 8 d.

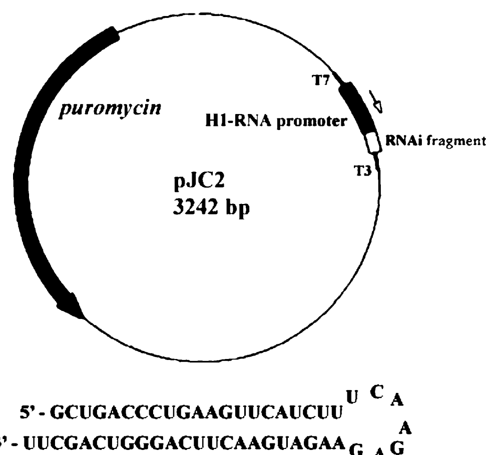

Figure 1 Schematic drawing of pJC2 vector and its predicted transcript: The H1-RNA promoter is used to derive the small interfering RNA which contains the same 20-nt sequence in sense and antisense format from eGFP. The predicted transcript forms a hairpin with the complementary pairing of the two 20-nt sequences separated by a short loop region.

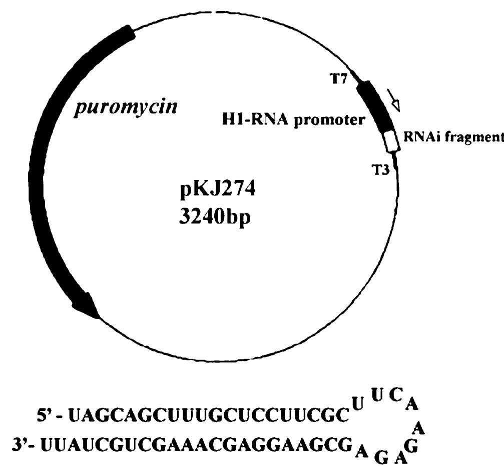

Figure 2 Schematic drawing of pKJ274 vector and its predicted transcript: The H1-RNA promoter is used to derive the small interfering RNA which contains the same 20-nt sequence in sense and antisense format from *pgk-1* promoter. The predicted transcript forms a hairpin with the complementary pairing of the two 20-nt sequences separated by a short loop region.

Finally, to evaluate *puro* gene suppression, the brightest individual colonies (transfected by pML8 plasmid, with the highest eGFP expression) were picked and expanded. The cells cultured continuously in the absence of *puro*. We called these cells as P19(eGFP$^+$) cells. P19(eGFP$^+$) cells were cotransfected with equal ratio of the plasmids pKJ303 and one of the three plasmids, pSUPER, pJC2, and pKJ274, as follows:

pKJ303 + pSUPER(ascontrol)
pKJ303 + pJC2
pKJ303 + pKJ274

For this group, the cells were selected with medium containing 2 μg/ml *hygro* after 8 d. All of the assays were repeated at least three times.

Fluorescence microscopy Fluorescent intensity of eGFP-positive cells was visualized by fluorescence microscopy at 8 d post-transfection in living cells. P19 cells (1.5×10^6/60 mm tissue culture dish) were plated and transfected with 5 μg of each plasmid DNA (pML8 and either pSUPER or pJC2) by the $CaPO_4$ method. eGFP expression was clearly visualized under viable conditions. Fluorescence micrographs were taken 8 d after transfection.

Fluorescent-activated cell sorting For flow cytometry, P19 EC cells (transfected and untransfected) were washed with PBS. Single cell suspensions were prepared and the cells were directly analyzed for eGFP expression. Detector settings were adjusted with untransfected cells. Determinations of the percentage of eGFP-positive cells and fluorescent intensity were carried out on fluorescent-activated cell sorting (FACS; LSR, Becton Dickenson, Mississauga, Ontario, Canada) with 10,000 events capture per sample. The cells were gated for positive eGFP signals. The fluorescent intensity in graphic output was plotted in log scale. The acquisition and analysis of the FACS data were performed with CELLQUEST software (Becton Dickenson). FACS analysis was done at least in triplicates.

Isolation of DNA and Southern blot analysis The cells were stably transfected with plasmid pML8 and one of the three plasmids, pSUPER (control), pJC2, or pKJ274. DNA was isolated from transfected cells (Abcam, ab65358, Cambridge) and dissolved in TE buffer. Purified DNA was digested with restriction enzymes at 10 U/μg (Sph1, Sph1 plus Xma1, or Sph1 plus Sma1). Digested genomic DNA was separated by electrophoresis through 1.2% agarose gels in TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The DNA was then transferred to Hybond-N membranes and treated with ultraviolet light at 110 mJ using a GS Gene Linker UV chamber. These blots were probed for eGFP or *puro* sequences.

Results

siRNA-mediated inhibition of eGFP reporter gene in P19 EC cells We used EC cell line, P19, to study the response of the cells to siRNA. Using pJC2 (a plasmid expressing specific siRNA transcript for eGFP), we tested the effect of the plasmid on stable expression of eGFP from pML8 (a vector encoding *puro* resistance gene and eGFP under the control of the murine *Pgk-1* promoter). The number of cells expressing eGFP was analyzed by FACS, 8 d after transfection. About 84.04% of the cells in the control samples (pML8 + pSUPER) were eGFP positive (Fig. 3*B*), while only 59.20% remained positive in the pJC2 samples (Fig. 3*C*). Figure 3*A* shows FACS analysis of untransfected P19 cells.

The specificity of the siRNA on eGFP expression was confirmed by fluorescence microscopy performed with living cells (Fig. 4). Imaging of transfected EC cells indicated a decrease in eGFP fluorescence in cells transfected with pJC2 (Fig. 4*B*) but not pSUPER (Fig. 4*A*).

siRNA-mediated inhibition of gene expression via pgk-1 promoter in P19 EC cells To determine whether siRNA could affect on gene expression via *pgk-1* promoter, we next used the vector pKJ274 (expressing specific siRNA transcript for *pgk-1* sequence) to transfect P19 cells. FACS analysis indicated that with pKJ274, the percentage of the eGFP-positive cells decreased to 57.26% (Fig. 3*D*).

Another interesting observation from the samples was the difference in the number of colonies (data not shown).

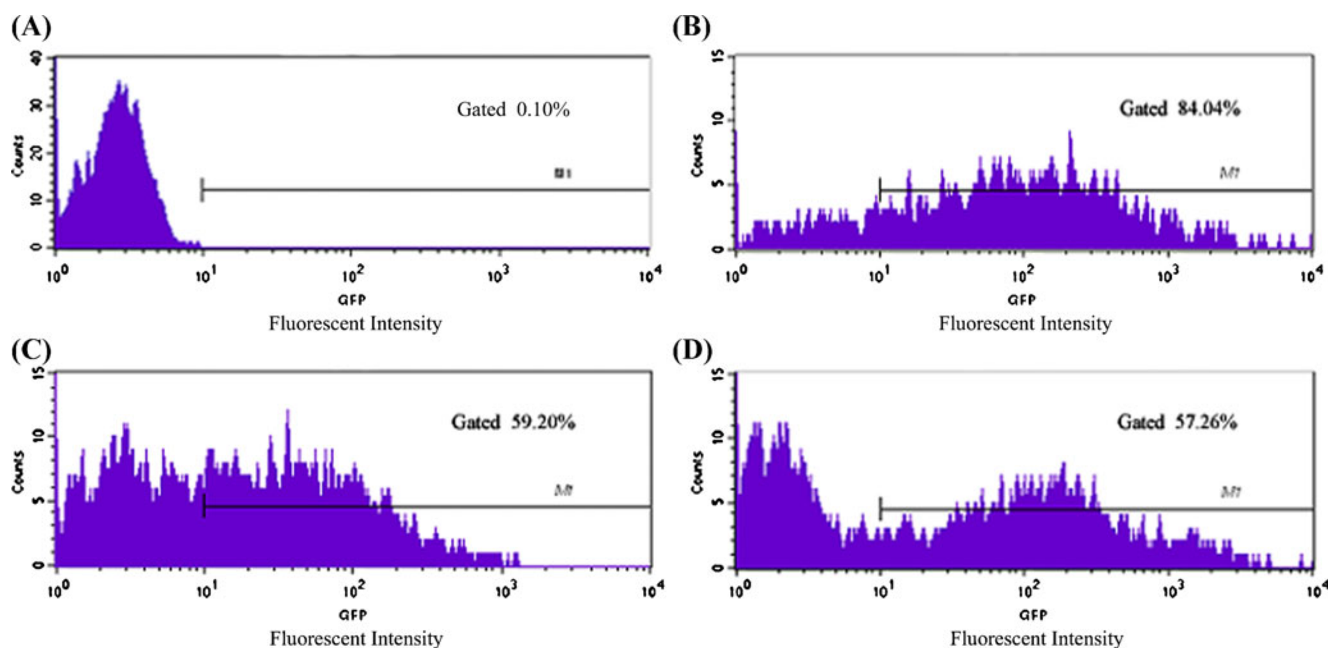

Figure 3 Stable RNAi effect in EC cells. FACS analysis of EC cells 8 d after initial *puro* selection at 48 h after transfection. Untransfected P19 cells (*A*). The cells were transfected with pML8 and pSUPER (control; *B*), pJC2 (*C*), or pKJ274 (*D*). (*C*) and (*D*) show the effect of siRNA transcripts in stable on the number of eGFP-positive cells. The peak patterns represent the fluorescent profile of the cells. The *bar* indicates the gated region for positive eGFP cells. The *number* reflects the percentage of the positive cells.

Results of colony counting, after *puro* selection, showed no difference between controls and the pJC2 samples. However, the number of the colonies was less in pKJ274-transfected cells. To test whether there is a correlation between colony numbers and colony selection system based on *puro* resistance gene, pKJ303 construct (contains *hygro* resistance gene and ego-1 under the control of the cmv promoter) was used to transfect P19(GFP$^+$) cells. Colony counting, after *hygro* selection, showed no differences between controls, pJC2 and pKJ274 samples (data not shown). FACS analysis showed that about 70.91% of the cells in the control were eGFP positive (Fig. 5*A*), while only 42.67% remained positive in the pJC2 samples (Fig. 5*B*). However, there was no significant difference between eGFP fluorescent intensity of controls and pKJ274 (79.42%) samples (Fig. 5*C*).

Southern blot analysis To evaluate the methylation of the enzyme sites in transgene DNA, we used Southern blot analysis with methylation-sensitive restriction endonuclease Sma1 and methylation-insensitive isoschizomer Xma1 (Fig. 6). DNA isolated from the transfected cells with pML8 and pSUPER, pJC2 or pKJ274 and digested with restriction enzymes Sph1, Sph1 plus Xma1, or Sph1 plus Sma1. The Southern blot probed for eGFP (Fig. 6*A*) and *puro* (Fig. 6*B*) revealed a single band at 1.275 and 1.17 kbp, respectively.

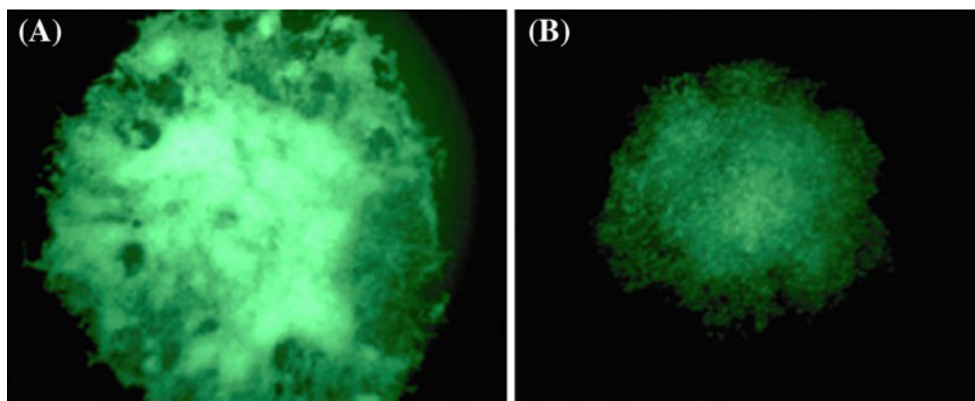

Figure 4 Fluorescent intensity of eGFP-positive cells was visualized by fluorescence microscopy at 8 d post-transfection, in living P19 cells, transfected with pML8 and either pSUPER (*A*) or pJC2 (*B*).

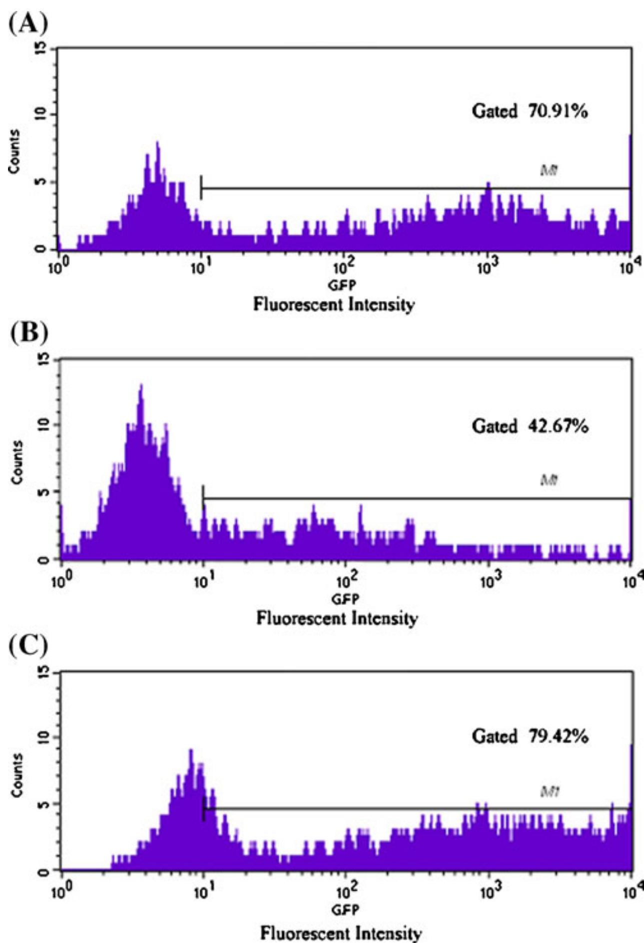

Figure 5 Stable RNAi effect in P19(eGFP+) cells (transfected with pML8). FACS analysis of P19(eGFP+) cells 8 d after initial *hygromycin* selection at 48 h after transfection. The cells were transfected with pKJ303 and pSUPER (control; *A*), pJC2 (*B*), or pKJ274. (*B*) shows the effect of pJC2 in stable on the number of positive eGFP cells. There is no significant difference between (*C*) and control. The peak patterns represent the fluorescent profile of the cells. The *bar* indicates the gated region for positive eGFP cells. The *number* reflects the percentage of the positive cells.

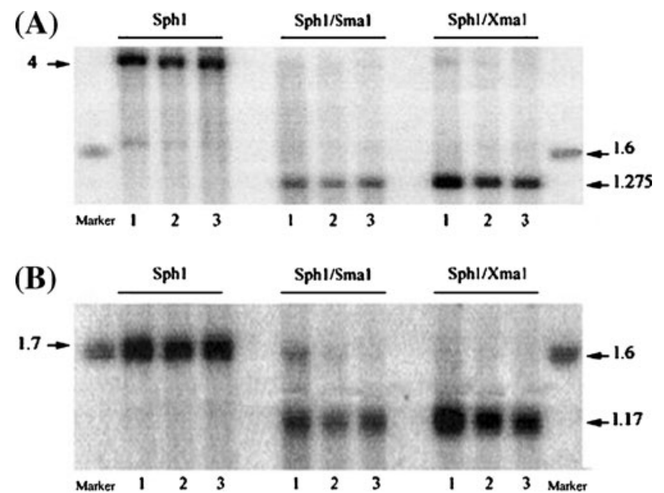

Figure 6 Southern blot analysis. The cells were transfected with pML8 and pSUPER (as control: *lane 1*), pJC2 (*lane 2*), or pKJ274 (*lane 3*). DNA was isolated from the transfected cells and digested with restriction enzymes, Sph1, Sph1 plus Xma1, or Sph1 plus Sma1. The digestion products were electrophoresed on gels, blotted, and probed for eGFP (*A*) or for *puro* (*B*) sequences. The *arrows* in (*A*) and (*B*) indicate the fragment sizes (kilobase pair).

Discussion

To investigate the feasibility of the RNAi strategy for gene silencing in undifferentiated EC mouse cells, we used siRNA transcripts against eGFP gene and *pgk-1* promoter. The eGFP gene was selected as the target because successful RNAi experiments had been done on this reporter gene (Caplen et al. 2001) and its activity is easily detectable. Based on pSUPER system (Brummelkamp et al. 2002), pJC2 and pKJ274 vectors were used to produce a siRNA bearing a 20-nt sequence from eGFP gene and *pgk-1* promoter, respectively. We demonstrated that RNAi model was feasible in the P19 EC cells. Our results also showed that hairpin siRNA caused long-lasting inhibition of targeted genes in the cells. dsRNA longer than 30 bp cannot be used to induce specific gene knockdown effect in mammalian cells (Elbashir et al. 2001a; Paddison et al. 2002). It is most likely because of the known, nonspecific effects of long dsRNA on gene expression and cell growth, mediated by the interferon response (Billy et al. 2001; Elbashir et al. 2001a). This effect can be circumvented by use of synthetic siRNAs. Elbashir et al. (2001b) reported that in vitro synthesized, 21–23-nt siRNA could induce a potent RNAi effect as effective as long dsRNA without showing the unspecific effect. However, transfection of siRNA or dsRNA into mammalian cells allows for only transient inhibition of gene expression (Billy et al. 2001; Elbashir et al. 2001b). Yang et al. (2001) showed that undifferentiated ES cells have a sequence-specific RNAi activity that disappears as ES cells differentiate, presumably due to dilution of dsRNA per cell. Based on some other reports, when mouse eggs were injected with dsRNA corresponding to GFP, RNA-dependent gene silencing was induced. However, the silencing effect continued for only a few days (Bahramian and Zarbl 1999; Wianny and Zernicka-Goetz 2000). The injection of synthesized siRNA of eGFP into "green" one cell eggs was also effective in silencing the expression of the transgene, but again only for a limited time period and fluorescence began to re-appear after the blastocyst stage (Hasuwa et al. 2002). This was probably due to a dilution of injected siRNA by a rapid increase of embryonic body mass (Hasuwa et al. 2002). To overcome this limitation, we used two mammalian expression pSUPER-based vectors (Brummelkamp et al. 2002), which direct the synthesis of siRNA transcripts. For these vectors, there was a

gene-specific insertion such that it specifies a 20-nt sequence derived from the target transcript, separated by a short spacer from the reverse complement of the same 20-nt sequence. The resulting transcript was predicted to fold back on itself to form a 20-bp hairpin structure. Previous observations have demonstrated that hairpin siRNAs could inhibit sequence-specific gene expression in mammalian cells (Yu et al. 2002; Yu and McMahon 2006). Based on this reports, gene silencing by a hairpin siRNA was more effective than inhibition by two siRNA strands (Yu et al. 2002; Yu and McMahon 2006). It is thought that siRNAs must form a duplex for inhibition of a target gene by RNAi (Boutla et al. 2001; Elbashir et al. 2001a, b; Nykänen et al. 2001).

Our observations demonstrated that siRNA hairpin directed toward *pgk-1* promoter effectively results in stable suppression of the targeted genes, eGFP and *puro*, both deriving by *pgk-1* promoter. Given that TGS is known to be due to the methylation of promoter sequences (Pickford and Cogoni 2003; Morris et al. 2004; Castanotto et al. 2005; Weinberg et al. 2006; Han et al. 2007; Lim et al. 2008; Suzuki et al. 2008), we examined the methylation status of the *pgk-1* promoter in transfected P19 EC cells by digesting DNA with methylation-sensitive restriction enzymes. The Southern blot analysis showed that all transfections resulted in cells with the same average number of inserted eGFP and *puro* genes. Since pML8 contains a copy of eGFP and *puro* genes driving by *pgk-1* promoter, maybe there was a correlation between colony numbers and *puro* resistance gene. Therefore, any differences in eGFP fluorescence or number of the colonies must be due to the effects of the RNAi. We were looking for the possibility that this RNAi would induce DNA methylation and that the promoter would be less active. It should be emphasized that in the current study, a DNA methylation analysis of the *pgk-1* promoter region failed to identify any promoter methylation using methylation-sensitive restriction enzymes on Southern blot analysis. If there were partial methylation, we would expect to see DNA fragments of intermediate size. Southern blot analysis indicated that the enzyme sites are unmethylated in the samples. It would seem that our RNAi constructs do not affect promoter methylation at the enzyme sites. These findings are consistent with the earlier observations (Ting et al. 2005). RNA-directed DNA methylation has been described so far in some other organism including plants and *Drosophila* (Mette et al. 2000; Aufsatz et al. 2002; Pal-Bhadra et al. 2002; Matzke and Birchler 2005). Whether a similar mechanism exists in mammalian systems is controversial, and the mechanisms underlying this process are still being defined (Suzuki et al. 2008). Some studies show that these process correlates with TGS (Morris et al. 2004; Castanotto et al. 2005; Han et al. 2007) while others fail to show this relationship (Ting et al. 2005). Even for organisms that do not methylate their DNA, there is growing evidence that chromatin modifications are targeted by components of the RNAi machinery (Aufsatz et al. 2002). In 2005, Ting et al. (2005) reported that short double-stranded RNA induces TGS in human cancer cells in the absence of DNA methylation. Clearly, further work is required to clarify the biochemical mechanisms of promoter silencing by siRNA in mammals.

Conclusions Overall, this work confirms and extends the previous results, demonstrating that promoter-targeted siRNAs are capable of inducing TGS. These data support a role for siRNAs to inhibit gene expression in mouse EC P19 cell line. We have demonstrated that stable, long-lasting sequence-specific silencing can be induced by siRNA hairpins. The ability should facilitate studies of gene function in transfectable mammalian cell lines. In addition, this capability enables the production of large numbers of silenced cells for biochemical analysis and permits the evaluation of phenotypes over long time spans.

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