

Research Article

A Significant Increase of RNAi Efficiency in Human Cells by the CMV Enhancer with a tRNA^{lys} Promoter

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Received 19 November 2008; Revised 21 June 2009; Accepted 31 July 2009

Recommended by John McGregor

RNA interference (RNAi) is the process of mRNA degradation induced by double-stranded RNA in a sequence-specific manner. Different types of promoters, such as U6, H1, tRNA, and CMV, have been used to control the inhibitory effect of RNAi expression vectors. In the present study, we constructed two shRNA expression vectors, respectively, controlled by tRNA^{lys} and CMV enhancer-tRNA^{lys} promoters. Compared to the vectors with tRNA^{lys} or U6 promoter, the vector with a CMV enhancer-tRNA^{lys} promoter silenced pokémon more efficiently on both the mRNA and the protein levels. Meanwhile, the silencing of pokémon inhibited the proliferation of MCF7 cells, but the induction of apoptosis of MCF7 cells was not observed. We conclude that the CMV enhancer-tRNA^{lys} promoter may be a powerful tool in driving intracellular expression of shRNA which can efficiently silence targeted gene.

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1. Introduction

RNAi was first discovered in the nematode *Caenorhabditis elegans* [1], after which the power and utility of RNAi for specifically gene silencing has driven its incredibly rapid adoption as a tool for reverse genetics at the organismal level. In mammalian cultured cells, RNAi is typically induced by siRNA introduced directly or expressed as a hairpin structure from a DNA construct, such as a plasmid, a viral vector, or a PCR product. It is known that the choice of promoters has been shown to be very important for efficient RNAi [2–4] and thus received more and more concern in vector-based expression systems after the introduction of vectors [4]. The promoters U6 and H1 [5–15], which belong to Pol III, are widely used for inducible knock-down of gene expression in vitro or in vivo [16]. Type II Pol III promoters such as tRNA lead to longer primary dsRNAs than U6 or H1, which might induce interferon response that could be avoided by a careful design of vector system [6, 17, 18]. It has been reported that tRNA promoters are more effective in target gene knock down [6, 17]. Recently, a polymerase I promoter with high

species specific has also been reported, which provides an interesting potential regarding the biosafety of experiments [19].

The CMV enhancer has been used in many vectors since it could increase the downstream DNA element's transcription level [20–22]. Recent studies have compared the inhibition of exogenous SOD1G93A^{GFP} expression by CMV enhancer with U6 promoter and U6 promoter alone, and the results have shown a more efficient inhibition by the use of CMV enhancer [5]. However, silencing by enhancer-promoter constructs has not been compared with promoters alone for endogenous genes.

Pokémon is a POK protein family member, a specific ARF suppressor, whose expression level is a critical determinant of cellular response to oncogenic transformation, Pokémon acts as a genuine proto-oncogene when overexpressed [23]. It is thought that downregulated Pokémon could upregulate ARE, thus blocking MDM2, followed by the increase in p53 expression, and p53's activation may lead to apoptosis [24].

In this study, we constructed two kinds of shRNA expression vectors controlled, respectively, by tRNA^{lys} and

TABLE 1: Sequences of oilgonucleotides used in the research.

shRNA	Sequences of oilgonucleotides	Target site on pokemon mRNA (GenBank accession number NM_01 5898)
1	F1: 5'-ccgtgtacgagatcgacttctcaagagagaagtcgatctctgtacacgtttttt-3'	383–401 nt
	R1: 3'-ccggggcacatgctctagctgaagaagttctctctcagtagagcatgtgcaaaaaatcga-5'	
2	F2: 5'-ccggcttagacttctatgggttcaagaccatagaagtctaagccgtttttt-3'	809–827 nt
	R2: 3'-ccggggccgaatctgaagataccaagttctctgggtatcttcagattcggcaaaaaatcga-5'	
3	F3: 5'-cgggctcatggactactactcaagagagtagtagtccatgacccctttttt-3'	1202–1200 nt
	R3: 3'-ccggggccgcagtagctgatgatgaagttctcatcatcaggtactcgggaaaaaatcga-5'	

shRNA encoding cassettes 1–3 were formed by annealing two corresponding oligonucleotides, respectively. F stands for forward and R for reverse.

CMV enhancer-tRNA^{lys}, in order to examine their efficiency. We then use the best shRNA to knock-down Pokemon expression and to observe the subsequent effect.

2. Materials and Methods

2.1. Plasmid Construction. The human tRNA^{lys} promoter was PCR-amplified from genomic DNA isolated from HeLa cells. Primers used for the generation of tRNA^{lys} were P(Lys1): 5'-GAT GAT ATC TGG CCA CTA GGG ACT GGG-3', and P(Lys2): 5'-GTC GGA TCC AAG CTT GAA TTC GGG CCC AGT CTG ATG CTC TAC CGA CTG-3. The PCR product was digested with BamHI and EcoRV and then named tRNA^{lys} promoter.

The plasmid backbone with CMV enhancer was PCR-amplified from plasmid pCMV5. Primers used were P(MRV1): 5'-GTG GAT ATC GGG GCG GGG TTA TTA CGA C-3', and P(MRV2): 5'-CGA CTC TAG AGG ATC CCG GGT G-3'. The PCR product was digested with BamHI and EcoRV and then named pCBE. Through ligation of tRNA^{lys} promoter and pCBE, a novel vector was created and thus designated as pExsiler, which derived from pCMV5 through replacing CMV minimal promoter with tRNA^{lys} promoter.

In order to obtain the plasmid containing one tRNA^{lys} promoter without CMV enhancer, the pCMV5 plasmid was first digested with BamHI, EcoRI, followed by HindII to completely remove the CMV enhancer sequence and CMV minimal promoter. The processed plasmid fragment was named pBE. Through ligation of tRNA^{lys} promoter and pBE, a new vector was created and thus designated as pSiler, which derived from pCMV5 through replacing CMV promoter (CMV enhancer and CMV minimal promoter) with tRNA^{lys} promoter.

The fragment of human Pokemon cDNA was PCR-amplified from cDNA isolated from MCF7 cells. Primers used in this PCR were P(POK1): 5'-CTTAAGCTTGCCACC-ATGGCCGCGCGCGTGG-3', and P(POK2): 5'-CATGAT-ATCGGCGAGTCCGGCTGTGAAGTTAC-3'. The PCR product was digested with HindIII and EcoRV and then cloned into the HindIII/SmaI sites of pEGFP(N2). The recombinant plasmid was named pEGFP(N2)-Pokemon. SiRNA target sequences of human Pokemon gene were designed using the Takara siRNA Design Support System

(<http://www.takara.com.cn/enter.htm>). Among the 10 target sequences identified by this free online software, three target sequences were selected as follows: (1) CGTGTA-CGAGATCGACTTC; (2) CGGCTTAGACTTCTATGGG; (3) GGGCGTCATGGACTACTAC. According to these three target sequences, the sense strands and antisense strands of duplex shRNA oligonucleotides were synthesized as shown in Table 1.

These single strand oligonucleotides were phosphorylated, and then F1 and R1, F2 and R2, and F3 and R3 oligonucleotides were annealed to generate the duplex oligonucleotides named as FR1, FR2, and FR3. FR1, FR2, and FR3 duplex oligonucleotides were cloned into the ApaI/HindIII sites of pExsiler and pSiler, and the resulting plasmids were named EP1, EP2, EP3, SP1, SP2, and SP3, respectively. FR3 double-stranded oligonucleotides were cloned into the ApaI/HindIII sites of pBS/U6 (kindly provided by Yang Shi of Harvard University) to create the plasmid designated as UP3.

In order to obtain a control plasmid with only TTTTTT sequence at the shRNA site, a PCR was preformed, using P(WW1): 5'-GTC GAT ATC AGA TCT TGA CAT TGA TTA TTG ACT AGT TAT TAA TAG 3', and P(WW2): 5'-GTC GGA TCC AAA AA AGG GCC CAG TCT GAT GCT CTA CCG ACT G-3' as primers and pExsiler as the template. The PCR product was digested with BamHI and EcoRV and then cloned into pBE. The resulting plasmid was named ET as a control plasmid.

2.2. Cell Culture and Transfection of Cells. Human galactophore cancer cell line MCF-7 was cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 2 mM glutamine (Invitrogen), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in a humidified 5%CO₂ incubator.

For western blot and the detection of apoptosis, twenty-four hours before transfection, 5 × 10⁵ MCF-7 cells were trypsinized and transferred to 6-well plates and cultured in DMEM supplemented with 10% FBS without antibiotics. They were grown to a confluency of 90%–95% and then transfected with a total of 4 μg of each of the hairpin vectors using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol, except that the transfection

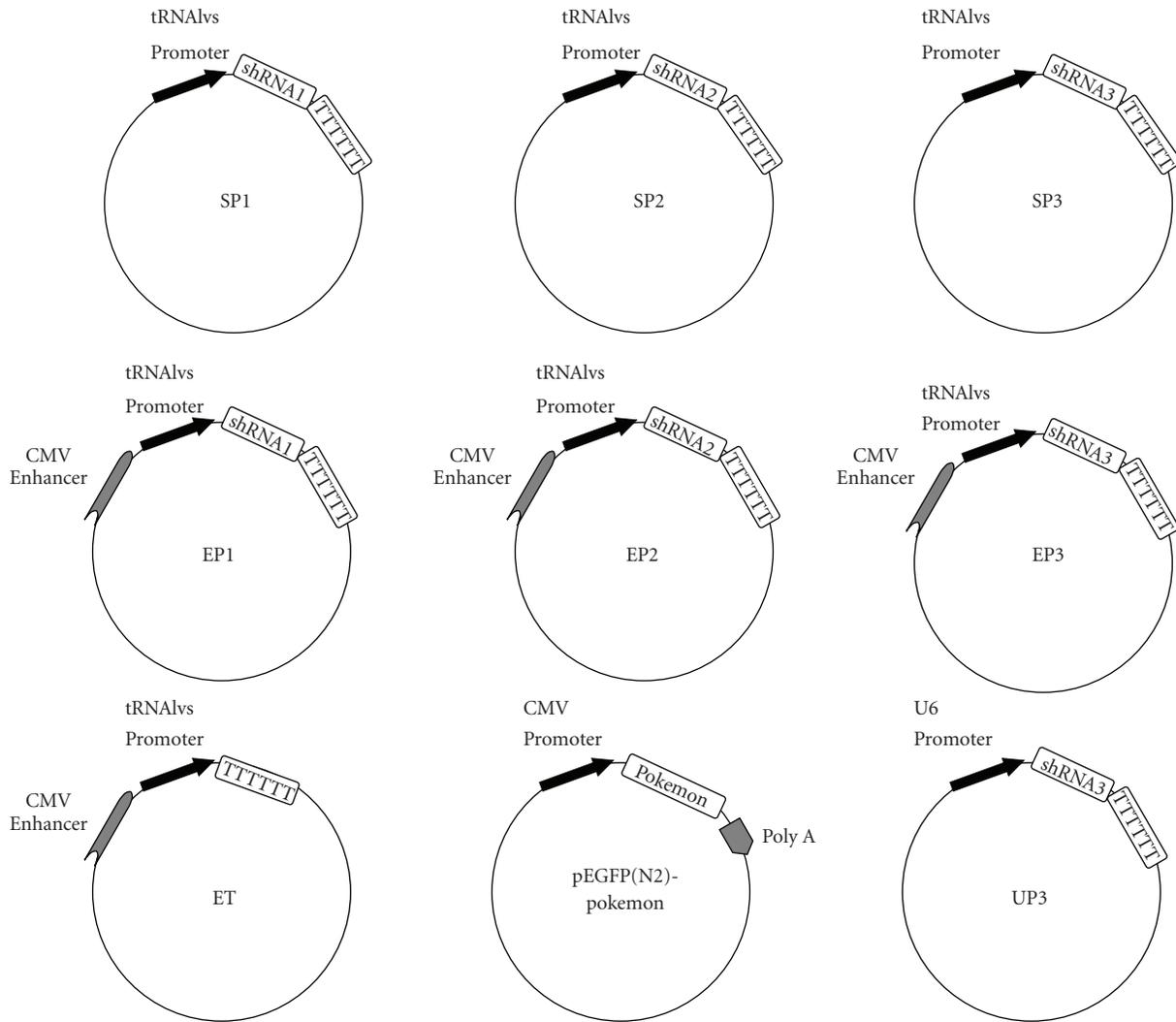


FIGURE 1: Map of constructs used in this work. SP1, SP2 and SP3 plasmids derived from the pSiler plasmid can express shRNA1, shRNA2, and shRNA3, respectively. EP1, EP2 and EP3 plasmids derived from the pExsiler plasmid can express shRNA1, shRNA2, and shRNA3, respectively. UP3 plasmid derived from pBS/U6 plasmid can express shRNA3. As control, ET plasmid derived from the pExsiler plasmid can express no shRNA. pEGFP(N2)-Pokemon derived from pEGFP-N2 vector can express Pokemon-GFP fusion protein.

volume was reduced to 1.5 mL in order to obtain higher transfection efficiency. For the screening of the RNAi plasmids, separate pEGFP(N2)-Pokemon plasmid encoding Pokemon-GFP fusion protein and the RNAi plasmids were generally used at a ratio of 1:1 [16]. Exogenous GFP-Pokemon protein expression levels of EGFP were assessed by green fluorescent signals expression under a microscope. For the detection of cell proliferation, 1×10^4 MCF-7 cells were trypsinized and transferred to 96 well plates, and cultured in DMEM supplemented with 10% FBS without antibiotics. They were immediately transfected with 0.2 μ g of each of the hairpin vectors using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

2.3. Real Time RT-PCR. 36 hours after transfection, MCF-7 cells were collected and washed three times with cold phosphate buffered saline (PBS). Total cellular RNA was

prepared using Trizol reagent (Invitrogen). 1 μ g of total RNA was reverse transcribed (RT) using Superscript II (Invitrogen) and random hexamers according to the manufacturer's protocol. 1 μ L cDNA was added to 19 μ L RealMastermix (SYBR Green) (TIANGEN) containing 1x RealMasterMix, 1x SYBR Green (TIANGEN), 200 nM of sense primer FW (5'-GAA GCC CTA CGA GTG CAA CAT C-3'), and 200 nM of antisense primer ReV (5'-GTG GTT CTT CAG GTC GTA GTT GTG -3') [23]. Real time PCR was carried out in an ABI Applied Biosystem 7500 (Applied Biosystems) using the following thermal cycling profile: 94°C 1 minute, followed by 40 cycles of amplification (94°C 20 seconds, 60°C 20 seconds, 68°C 40 seconds). For RNA normalization, a GAPDH PCR was performed with the same PCR reagents except that the primers were G1 (5'-GGT GGT CTC CTC TGA CTT CAA CA -3') and G2(5'-GTT GCT GTA GCC AAA TTC GTT GT-3') [25].

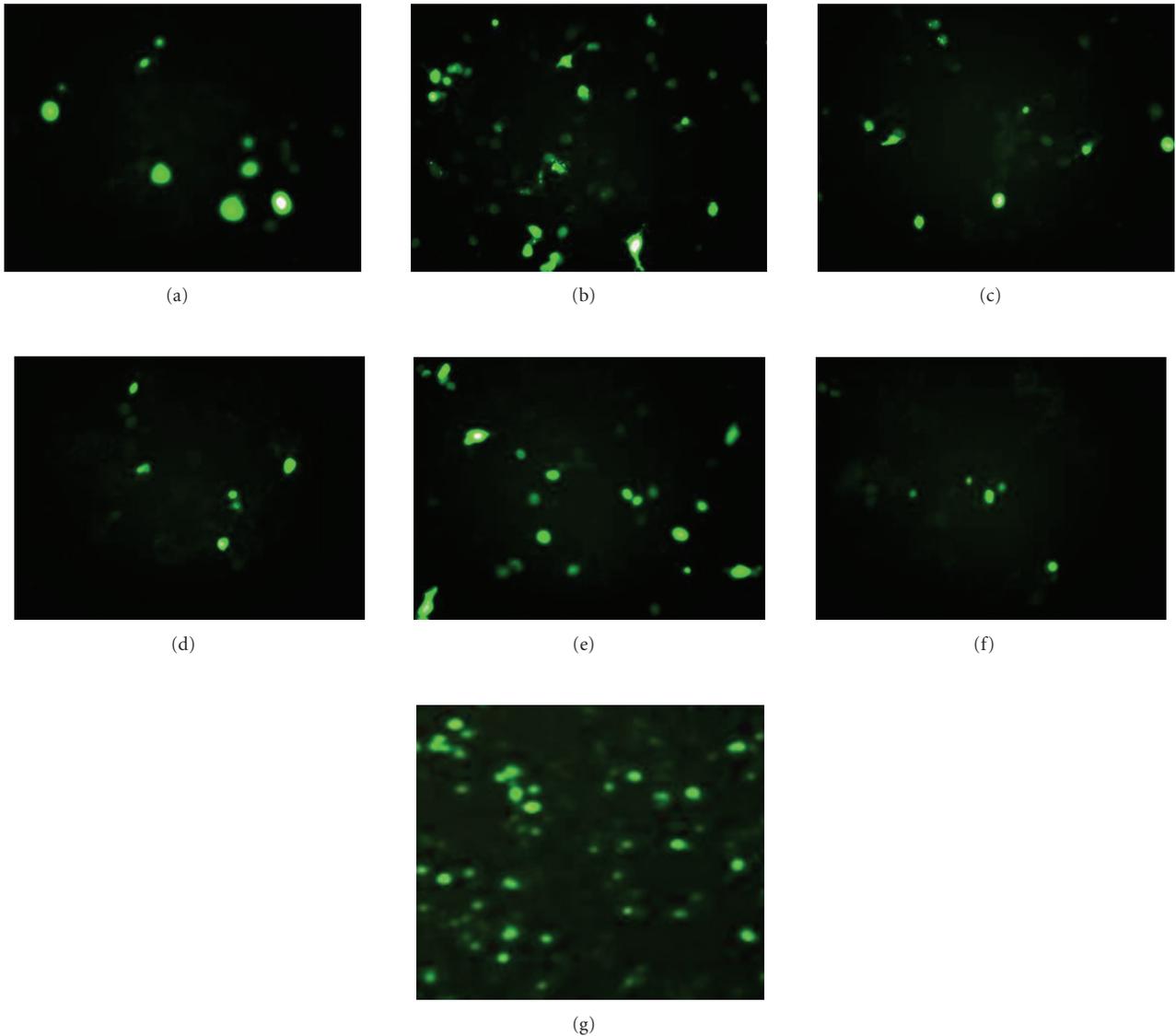


FIGURE 2: Expression of Pokemon-GFP in MCF7 cells cotransfected with the pEGFP(N2)-Pokemon and different shRNA expression plasmids. (a) MCF7 cells cotransfected with the pEGFP(N2)-Pokemon and SP1, (b) with the pEGFP(N2)-Pokemon and SP2, (c) with the pEGFP(N2)-Pokemon and SP3, (d) with the pEGFP(N2)-Pokemon and EP1, (e) with the pEGFP(N2)-Pokemon and EP2, (f) with the pEGFP(N2)-Pokemon and EP3, and with the (g) pEGFP(N2)-Pokemon and ET.

2.4. Western Blot. MCF-7 cells were collected 48 hours after transfection. Cells were washed with cold PBS three times and collected in 500 μ L PBS using a cell scraper and transferred into an Eppendorf tube. Following centrifugation, cells were resuspended in PBS. Cells were lysed in TNE lysis buffer (10 mM Tris, pH 7.8, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40) containing protease inhibitor PMSF. After 30-minutes incubation on ice, the lysates were spun at 12 000 g for 15 minutes to remove insoluble material. Protein concentrations were determined through the Bradford procedure. 20 μ g of total protein lysates were resolved on a 12% SDS-PAGE gel and transferred to a Nitrocellulose membrane (Life sciences). Membranes were blocked with 5% nonfat dried milk powder in TBS/T (0.05% Tween-20) for

2 hours at room temperature. Membranes were sequentially incubated with LRF (E-15) (Santa Cruz sc-34510) and donkey antigoat IgG-HRP (Santa Cruz sc-2020) according to the manufacture's protocol. The protein bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (PIERCE) according to the manufacture's protocol.

For sequential probing, membranes were stripped in Erasure buffer (2% SDS, 62.5 mM Tris-HCl PH 6.8, 100 mM beta-mercaptoethanol) at 70°C for 40 minutes and then incubated in TS buffer (10 mM Tris-HCl PH 7.4, 150 mM NaCl). Actin bands were then detected using polyclone antibody rabbit anti β -actin PR-0255 (ZHONGSHAN GOLDBRIDGE, Beijing, China) and HRP labeled antibody goat antirabbit ZDR-5306 (ZHONGSHAN GOLDBRIDGE).

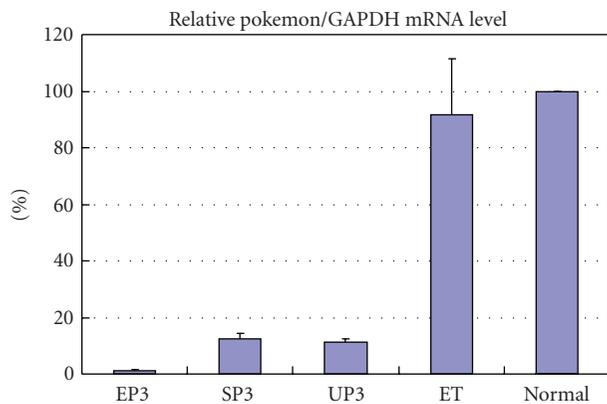


FIGURE 3: Efficiency of different shRNA constructs in the inducing of silencing on Pokemon/GAPDH mRNA level as quantified by real-time RT-PCR. Total RNA was extracted from MCF-7 cells 36 hours after transfection with the indicated constructs. Normal MCF-7 cells were used as a negative control. An external standard curve was created to make sure that Pokemon and GAPDH were amplified at similar efficiency. Data shown represents, three replicated experiments with error bar indicated.

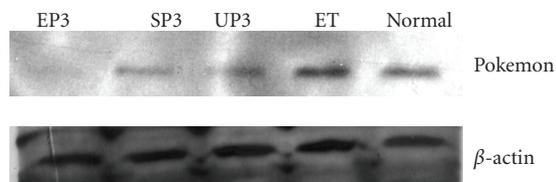


FIGURE 4: Efficiency of different shRNA constructs in the inducing of silencing of endogenous gene expression. The western blot shows the Pokemon bands and the β -actin bands which serve as an endogenous control.

2.5. Detection of Cell Proliferation. The proliferation rate of cell line MCF-7 was measured with a Cell proliferation Kit II (Roche Ltd, Switzerland) according to the manufacturer's protocol.

2.6. Flow Cytometric Determination of Apoptosis. 60 hours after transfection, MCF-7 cells were trypsinized and resuspended in PBS. Cells from 2 wells of a 6-well plate were collected and centrifuged, and the pellets were used for the flow cytometric determination of apoptosis using ANNEXIN V-FITC APOPTOSIS DETECTION KIT I (BD biosciences) according to the manufacturer's protocol. Cells treated with 10 μ g/mL cisplatin served as a positive control.

3. Results

3.1. Construction of Plasmids. To compare the efficiencies of CMV enhancer-tRNA^{lys} promoter and tRNA^{lys} promoter, we constructed two kinds of expression vectors (pExsiler and pSiler) and attached dsRNA to the 3' end of each promoter. All plasmids (pExsiler, pSiler, pEGFP(N2)-Pokemon, EP1, EP2, EP3, SP1, SP2, SP3, UP3, and ET) were sequenced (Invitrogen, China). Results of sequencing verified that the

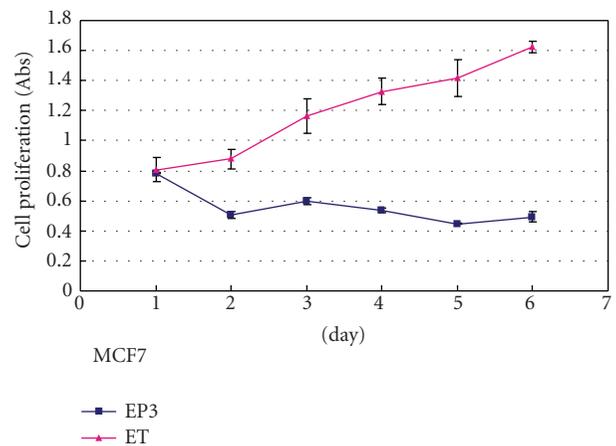


FIGURE 5: Effect of proliferation of cells transfected with different shRNA constructs. Rates of proliferation were determined as described in the text. Data are mean values from three replicates with error bar indicated. MCF-7 cells transfected with EP3 proliferate significantly more slowly than cells transfected with control plasmid ET. The wild type MCF-7 cells were not shown.

sequences of the all aforementioned plasmids were consistent with what we had designed (Figure 1).

3.2. Efficiency of RNAi between Different shRNA Expression Plasmids. For selection of most efficient shRNA from the three shRNA sequences of human Pokemon gene, the EP1, EP2, EP3, SP1, SP2, SP3, and ET plasmids were respectively, cotransfected together with the pEGFP(N2)-Pokemon into MCF7 cells. Pokemon-GFP expression within the transfected cells was assayed by green fluorescent signals (Figure 2). Whereas EP2 and SP2 had less effect on Pokemon-GFP expression, EP3 and SP3 greatly diminished its expression. Therefore, the shRNA derived from target sequences (GGGCGTCATGGACTACTAC) has most efficiency in knockdown of Pokemon expression.

3.3. Efficiency of RNAi between Different Expression Plasmids. To examine the efficiency of RNAi in MCF-7 cells, we introduced expression plasmids EP3/SP3/UP3/ET into MCF-7 cells which express Pokemon. We examined Pokemon mRNA levels through real time RT-PCR. As shown in Figure 3, there is no obvious decrease of Pokemon/GAPDH mRNA level in cells transfected with ET. However, cells transfected with EP3, SP3, and UP3 show decreased relative Pokemon/GAPDH mRNA levels to $1.2\% \pm 0.2\%$, $12.3\% \pm 2.2\%$, and $11.1\% \pm 1.3\%$, respectively, compared to normal MCF-7 cells.

Western blot was used to examine the Pokemon expression level. Figure 4 shows that the Pokemon expression level in MCF7 cells transfected with EP3 was significantly lower than those with SP3, UP3 and ET, whereas the β -actin expression levels remained unchanged. These results suggest that the enhancer-promoter construct can specifically and efficiently silence Pokemon on both mRNA and protein levels in MCF-7 cells.

3.4. Effects of Pokemon Inhibition. To examine the phenotype of cells that were transfected with different constructs,

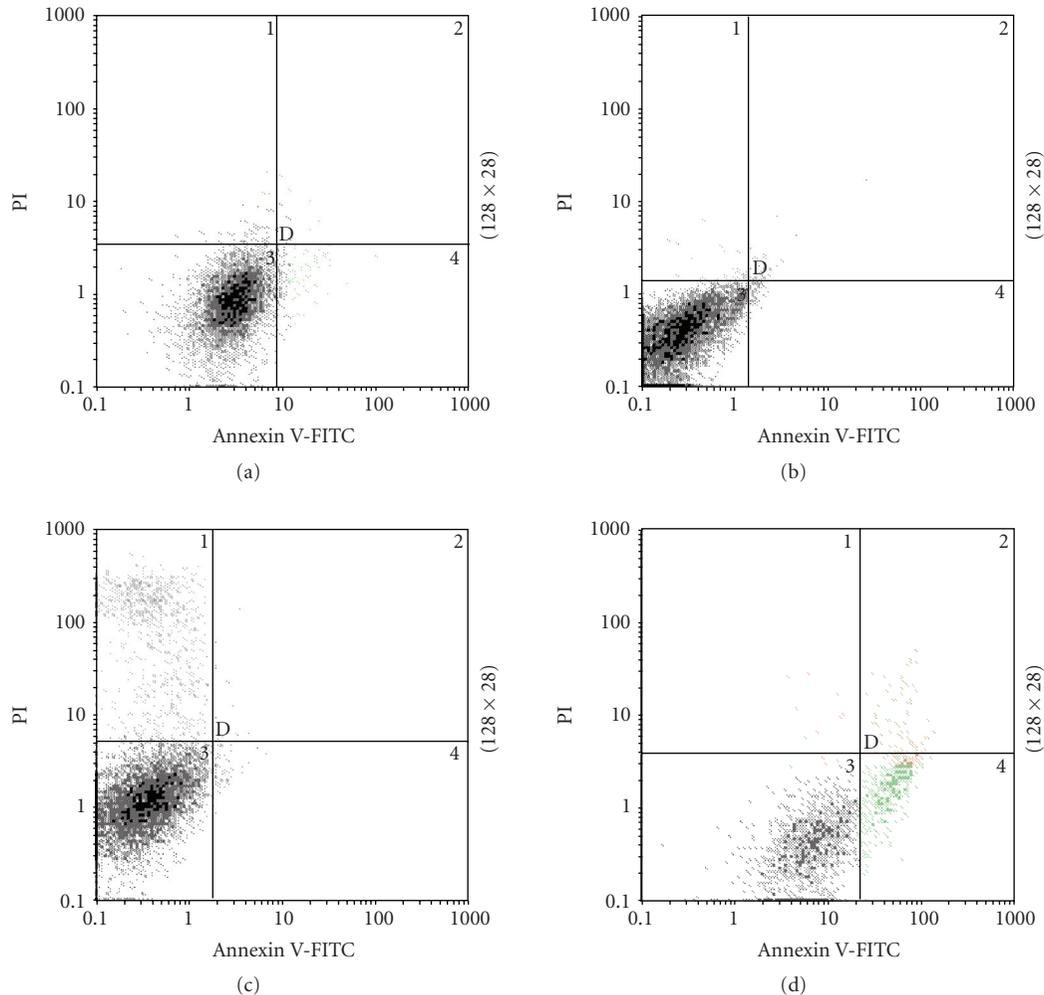


FIGURE 6: Flow cytometric determination of apoptosis. (a) Unstained normal MCF-7 cells (b) EP3-transfected cells stained with both PI and Annexin V-FITC (c) ET-transfected cells stained with both PI and Annexin V-FITC (d) Cisplatin-treated cells stained with both PI and Annexin V-FITC. Note that some cells transfected with PT were dead as a result of high cell density. Results are representative of two replicates.

the proliferation rates of MCF-7 were analyzed. As shown in Figure 5, MCF-7 cells transfected with EP3, in which Pokemon levels were reduced significantly both on mRNA and protein scale, proliferated much more slowly than cells transfected with ET, which was served as a negative control. The reduction of proliferation rate is believed to be at least partially due to the decrease of Pokemon protein level in MCF-7 cells.

Recent research has suggested that the inhibition of Pokemon might result in the apoptosis or the cell cycle arrest [23]. However, in the present study, no typical apoptosis was observed by Flow Cytometry in MCF-7 cells transfected by EP3 and ET, while cells treated with cisplatin did show apoptosis (Figure 6).

4. Discussions

In the present study, we found that CMV enhancer, one of the widely used enhancers, could increase the efficiency of tRNA^{lys} promoter. It has been reported that the com-

munication between the enhancer and the promoter could induce gene activation [26], while the disruption of the communication leads to the inhibition of gene expression [27]. Therefore, enhancers may activate the transcription of downstream shRNA, resulting in the increase of RNAi efficiency.

Pokemon, also known as LRF, OCZF, and FBI-1, is a POK protein family member, a specific ARF suppressor, whose expression level is a critical determinant of cellular response to oncogenic transformation. So Pokemon acts as a genuine proto-oncogene when over-expressed [23]. It is thought that downregulated Pokemon results in an increase in p53 expression, and p53's activation may lead to apoptosis [24]. In our study, no apoptosis but the slower proliferation rate is observed in the inhibition of Pokemon in MCF-7 cells induced by shRNA controlled by CMV enhancer with tRNA^{lys} promoter. However, as the ARF/p53 pathway is frequently abrogated in human cancers, if MCF-7 cells do lose this path, this apparent discrepancy could be solved. What is more, current evidences show that Pokemon null

mouse resulted in embryonic lethal due to severe anemia and profoundly impaired differential cellular in multiple tissues [23]. But MCF-7 cells are already differentiated cells; thereby they may avoid apoptosis for that reason. However, more investigation should be made to confirm our findings.

In conclusion, the shRNA expression vector controlled by CMV enhancer-tRNA^{lys} promoter is powerful in silencing Pokemon and may be a useful tool for scientific research and therapy use with RNAi.

Acknowledgments

The authors gratefully acknowledge the National Natural Science Foundation of China (NSFC20132020, 20572060) and the Ministry of Science and Technology of China (2007AA02Z160) for financial support of this work. The pBS/U6 plasmid was kindly provided by Yang Shi of Harvard University. they also thank Dr. Tan Chuanyan and Mr. Yan Dongdong for the manuscript.

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