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Full Length Research Paper

Construction of lentiviral shRNA expression vector targeting phospholipase D2 (PLD2) gene△

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The aim of this research was to construct a lentiviral shRNA vectors targeting phospholipase D2 (PLD2) gene, thus providing information for further study of the biological functions of PLD2 and clinical treatments of leukemia. Specific siRNA targets with short hairpin frame were designed using the siDESIGN software and synthesized according to cDNA sequence of PLD2 (GenBank accession number: NM_002663). DNA oligo was cloned into lentiviral expression vector, and then polymerase chain reaction (PCR) and sequencing analyses were conducted to verify the constructs. The verified vectors were co-transfected into 293FT cells that could produce lentiviral. shRNA lentiviruses from the selected constructs were propagated and harvested with a virus packaging system, and the virus titers were determined by flow cytometry. After the lentiviral packing with PLD2-shRNA2 transfecting 293FT cells, using fluorescence microscope, we found the lentiviral interference vectors, which have GFP report genes expression accompanied with the host genes in 293FT cells. The optimal interfering target was then selected, while the titer of lentiviral packing PLD2-shRNA was 3.47 × 10^4 TU/ml and the virus was successfully packaged. PCR and sequencing analyses revealed that lentiviral shRNA vectors of three targeting PLD2 gene were successfully constructed.

Key words: RNA interference (RNAi), phospholipase D2 (PLD2), lentiviral vectors.

INTRODUCTION

The essence of cancer development and growth is that mutant cells unlimitedly proliferate and cannot be removed by the immune system in the body. Compared with other tumors, leukemia has a peculiarity with more changes in chromosomes at molecular level (Thomas et al., 2006). For example, chromosome translocation can activate specified oncogenes or lead to production or activation of new fusion genes. These genes are often selected as targets for gene silencing because they can cause leukemia directly or maintain pathologic status after their activation.

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It has been well known that PLD2 is closely related to carcinogenesis and apoptosis in cancer cells. Many phospholipase can hydrolyze phospholipid in living bodies, for example the phospholipase D (PLD). PLD is a big enzyme family that can act on ester bonds between phosphoglyceric acetic substituents and hydrolyze phosphatidyl choline (PC) for phosphatidic acid (PA) and choline. PA can yield diacylglycerol (DAG) when hydrolyzed by phosphatidic acid phosphohydrolase (PAP) and yield lysophosphatidic acid (LPA) by phospholipase A2 (PLA2). Thus, the activation of human PLD is closely related with many important cellular functions, including phagocytosis, respiratory burst, mitogenic signaling via cross-talk with the ras/ERK signaling cascade and the secretion regulation (Exton, 2002). Many of these functions are vital in disease states, such as inflammation, tumor, hypertension, etc. Some tumor cells can express PLD2 genes for example, Daudi cells in Burkitt's lymphoma cell line express high PLD2 gene (Cataldi et al.,1992). The relationship of PLD2 and leukemia also has attracted great attention recently (Di Fulvio and

Table 1. Design of shRNA targeting PLD2 gene.

Primer Name	Sequence
NM_002663_2066_top	5'-TGCTGTCAGGATTCTGTCCACAATCTGTTTTGGCCACTGACTGACAGATTGTGCAGAATCCTGA-3'
NM_002663_2066_bottom	5'-CCTGTCAGGATTCTGCACAATCTGTCAGTCAGTGGCCAAAACAGATTGTGGACAGAATCCTGAC-3
NM_002663_2383_top	5'-TGCTGTGCAGAACCAATGATGACTGTGTTTTGGCCACTGACTGACACAGTCATTTGGTTCTGCA-3
NM_002663_2383_bottom	5'-CCTGTGCAGAACCAAATGACTGTGTCAGTCAGTGGCCAAAACACAGTCATCATTGGTTCTGCAC-3
NM_002663_2600_top	5'-TGCTGACTGGAAGAAGTCATCACAGAGTTTTGGCCACTGACTGACTCTGTGATCTTCTTCCAGT-3
NM_002663_2600_bottom	5'-CCTGACTGGAAGAAGATCACAGAGTCAGTCAGTGGCCAAAACTCTGTGATGACTTCTTCCAGTC-3

Table 2. Constructed reaction system.

Reagent	DSPLD$_2$-shRNA (µl)	Positive control (µl)	Negative control (µl)
5×ligase buffer	4	4	4
pcDNA™6.2-GW/EmGFP-miR (5 ng µl)	2	2	2
DSPLD$_2$-shRNA (10 nM)	4	——	——
DSmiR-LacZ positive control (10 nM)	——	4	——
DEPC water	9	9	13
T4 DNA ligase (1 U/µl)	1	1	1
Total volume	20	20	20

Gomez-Cambronero, 2005).

The most effective way to investigate the relationship between the PLD2 genes expression and leukemia, is knocking out PLD2 gene of leukemia cells, and then determining the related effect on apoptosis (Thomas et al., 2006). In our laboratory, a site-specific mutant of human phospholipase D2 named recombinant human phospholipase D2 (rhPLD2) was constructed (Ling et al., 2003). We found that rhPLD2 exhibited a higher basal activity compared to PLD2. Our previous work showed that 24 h after combination treatment with various concentrations of rhPLD2 (10, 20 and 40 µg/ml) and 10 µg/ml of camptothecin, there was a significant interactive relationship between rhPLD2 and camptothecin ($P<0.01$), indicating that both rhPLD2 was able to enhance camptothecin-induced apoptosis (Ling et al., 2008). Therefore, we silenced the expression of PLD$_2$ genes by RNAi in this study. We also designed the corresponding small interfering RNA (siRNA) oligonucleotide template according to cDNA sequence of PLD2 (GenBank accession no: NM_002663) and with the template, we synthesized oligonucleotide fragments *in vitro*. Finally, we constructed the expression vectors pcDNATM6.2-GW/EmGFP-PLD2-miR and lentiviral interference vectors pLenti6/V5-GW/EmGFP-PLD2-miR. This data thus provides new information for investigation of the biological functions of PLD2 and clinical treatments of leukemia.

MATERIALS AND METHODS

Reagents and cells

293FT cells, Lipofectamine™ 2000, Trizol, Midiprep Plasmid HIPure Kit 25 reaction, Library Efficiency DB3.1 Competent Cells, WesternBreeze Chemiluminescent Western Blot Immunodetection Kit, and BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System were purchased from Invitrogen Ltd (U.S.A). AccessQuick™ RT-PCR System was from Promega Corporation (U.S.A), bovine serum albumin was from Bovogen Biologicals Pty Ltd. Australia, while PLD$_2$ monoclonal antibody was purchased from Abnova Company (Taiwan).

Designed and synthesized PLD$_2$ targeted shRNA

According to cDNA sequence of PLD2 (GenBank accession number: NM_002663), we designed shRNA to direct at PLD2 by using RNAi Designer (Invitrogen, U.S.A.). The shRNA of PLD2 was abbreviated PLD2-shRNA. Oligonucleotide sequence was synthesized by Shanghai Yingjun Biotechnology Company, China. The sequence of PLD2-shRNA is shown in Table 1.

Construction of pcDNATM6.2-GW/EmGFP- PLD2-miR expression vectors

The construction of double strands PLD$_2$-shRNA and expression vectors pcDNA™6.2-GW/EmGFP-miR

The pcDNA™6.2-GW/EmGFP-miR expression vectors kit (K493800) was purchased from Invitrogen company (U.S.A.). Following the test kit instructions, we mixed the reaction system thoroughly (Table 2). The mixture was then incubated at ambient temperature for 1 h, after which 2 µl of it was taken for the next transformation experiment, and the remains were stored at -20°C.

The ligation products were transformed into One Shot® TOP10 *Escherichia coli* and verification was done by sequencing

One Shot® TOP10 *E. coli* was thawed on ice, and then 2 µl of the

connected products was added to it, while 1 µl of pUC19 plasmids was added to another tube with the same *E. coli* for a positive control. These were incubated for 20 min on the ice, heat-shock for 30 s and then incubated on ice again for 5 min. 250 µl of S.O.C medium was later added and shook for 1 h at 250 rmp, 37°C, then 25, 50 and 75 µl positive control solution respectively were spread on the LB/Spectinomycin solid plates overnight at 37°C. Monoclonal colonies were picked into 5 ml LB/Spectinomycin fluid medium at 37°C, for 20 h. Finally, positive clones that were successfully connected were subjected to sequencing analysis (Shanghai Yingjun Biotechnology Co.,China) (Figure 2).

Construction of entry vectors

PcDNATM6.2-GW/EmGFP-PLD2 miR expression plasmids were extracted using small plasmid extractive kit (Takara, Japan). The absorbance and concentrations of the plasmids in λ230, λ260 and λ280 was measured using ND1000 ultraviolet-visible light detector. The concentration of the plasmids was 128.4 ng/µl. The plasmids were used for the BP reaction, and the remains were stored at -20°C.

2 µl of the BP reaction clonal enzyme was added into the reaction system as shown in Table 3 at 25°C for 3 h in water bath box, then 1 µl protein kinase K was added to the system at 37°C for 10 min in water bath box. Some of these were used for the transformation experiment, while the remains were kept at -20°C.

BP reaction products were transformed into One Shot® TOP10 *E. coli* and confirmed by sequencing. The operations were the same as the positive clones that were successfully connected and subjected to sequencing analysis (Shanghai Yingjun Biotechnology Company, China) (Figure 3).

Construction of pLenti6/V5-GW/EmGFP-PLD$_2$-miR lentiviral interference vectors

First, the plasmids were extracted, and then the bacterial liquid with the right sequencing was inoculated into 10 ml LB liquid medium with Kan cells added. The solution was left on a shaker overnight at 250 rpm, centrifuged (12000 *g*) at 4°C for 5 min after which the supernatant was removed and the precipitate of bacterium collected. The concentration of the extracted plasmids was 104.2 ng/µl.

2 µl of the LR reaction clonal enzyme was added to the reaction system as shown in Table 4, at 25°C for 3 h in water bath box. Then, 1 µl protein kinase K was added to the reaction at 37°C for 10 min in water bath box. LR reaction products were transformed into One Shot® Stbl3™ *E. coli* and confirmed by sequencing. The operations were the same as the positive clones that were successfully connected and subjected to sequencing analysis (Shanghai Yingjun Biotechnology Co., China) (Figure 4). The LR reaction plasmids sequenced correctly were lentiviral interference vectors: pLenti6/V5-GW/EmGFP- PLD2-miR.

Lentiviral packaging and titer testing

We used four plasmid package systems; the working plasmids were lentiviral interference vectors pLenti6/V5-GW/EmGFP-PLD2-miR (pLenti6/V5-GW /EmGFP-miR-PLD$_2$) and package plasmids were PLP /VSVG. The extraction of working plasmids was pLenti6/V5-GW/EmGFP-miR-PLD2. The bacterial liquid that its sequencing was right was inoculated into 10 ml LB liquid medium and Kan was added. The solution was left on a shaker overnight at 250 rpm and then centrifuged (12, 000 *g*) at 4°C for 5 min and the supernatant was removed while the precipitate of bacterium was collected. The concentration of the extracted plasmids was 173.1 ng/µl. 293FT cells in logarithmic phase were seeded into a 25 ml dish. Once cell fusion reached 70%, recombinant lentiviral frame plasmid and packaging helper plasmid were co-transfected into 293FT cells (virus packaging cells) with the aid of calcium phosphate and the concentration of 293FT cells to 1.2×10^6 /Ml was adjusted accordingly.

The packed virus is described thus: the preparation of solution A: 9 µg of the packed virus mixture (ViraPower Packaging Mi) and 3 µg pLenti6/V5-GW/EmGFP-miR-PLD$_2$ were dissolved in 1.5 ml Opti-MEM non blood serum medium and were thoroughly mixed with caution. The preparation of solution B: 36 µg lipofectamine 2000 was dissolved in 1.5 ml Opti-MEM non blood serum medium, mixed uniformly and incubated at room temperature for 5 min. Solutions A and B were mixed and incubated at room temperature for 20 min to ensure the DNA- lipofectamine 2000 complex form completely. The 293FT cells were digested by pancreatin and counted when the lipofectamine 2000 complex was formed and the cells were suspended in Opti-MEM culture fluid with 10% fetal calf serum (FBS) with the concentration adjusted to 1.2×10^6/ml. 5 ml Opti-MEM medium with 10% fetal bovine serum (FBS) was added into 10 cm aseptic culture plate, then the complex of solution A and solution B (DNA- lipofectamine 2000 complex) and 5 ml 293FT cells after been suspended were added. The mixture was mixed uniformly and cultured at 37°C in the atmosphere with 5% CO_2 in RPMI-1640 complete medium for 48 h. The supernatant of 293FT cell culture was collected and centrifuged (4000 *g*) at 4°C for 10 min to remove cell debris after 72 h transfection. Plasmids were transformed into 293FT cells in a definite percentage and the viral titer was calculated by the determination of GFP fluorescence on a lentiviral vector 48 h after transfection (Figures 5 and 6).

RESULTS

Gel electrophoresis of PLD$_2$-shRNA

A clear double strands shRNA strip is shown in Figure 1, which was 64 bp and was annealed successfully. The weaker strip above it was annealed unsuccessfully.

The sequence alignment result of PLD2-shRNA

Figure 2 shows the alignment result of pcDNA^{TM}6.2-GW/EmGFP-PLD$_2$ miR expression vector and PLD$_2$-shRNA after annealing by BLAST, indicating that the sequence of expression vector was consistent with PLD$_2$-shRNA completely, and the recombinant plasmid was constructed successfully for amplification by *E coli*. The plasmid amplified by *E. coli* after ligation reaction not only showed PLD$_2$-shRNA but also attB site, suggesting it can ligate attP site with entry vetcor for recombination reaction (BP reaction).

Figure 3 shows the alignment result of entry vector and PLD$_2$-shRNA after BP reaction by BLAST, suggesting that the sequence of entry vector was consistent with PLD$_2$-shRNA completely. PLD$_2$-shRNA has been cloned in entry vector correctly and completely, and the product of BP reaction could be amplified by *E. coli*.

In the BP reaction, attB site in expression vector and attP site in intermediate vetcor can recombinate a new site called attL, which is in entry vector. The entry vector with attL site can recombinate the target vector with attR site

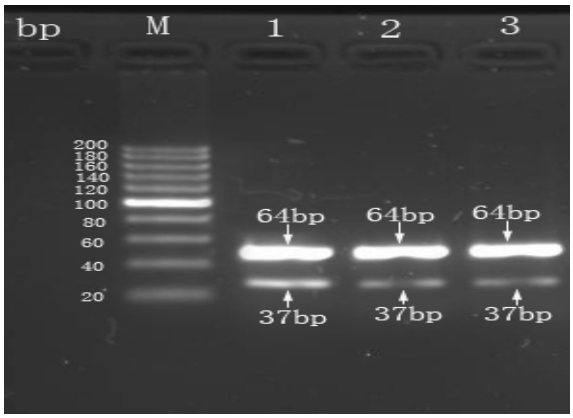


Figure 1. The three PLD2-shRNA electropherogram after annealing. 1, PLD2-shRNA1 electrophoresis strip after annealing; 2, PLD2-shRNA2 electrophoresis strip after annealing; 3, PLD2-shRNA3 electrophoresis strip after annealing; M, DNA marker.

Table 3. PCR reaction.

Reagent	Sample tube (µl)	Positive control (µl)	Negative control (µl)
Expression plasmids vectors	5	——	5
pDONR™221 vectors	1	1	1
pEXP7-tet positive control	——	2	——
TE buffer	3	5	3
Total volume	8	8	8

```
Score =  123 bits (64),  Expect = 1e-24
Identities = 64/64 (100%), Gaps = 0/64 (0%)
Strand=Plus/Plus

Query  68   TGCTGTGCAGAACCAATGATGACTGTGTTTTGGCCACTGACTGACACAGTCATTTGGTTC  127
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TGCTGTGCAGAACCAATGATGACTGTGTTTTGGCCACTGACTGACACAGTCATTTGGTTC  60

Query  128  TGCA  131
            ||||
Sbjct  61   TGCA  64
```

Figure 2. The sequence alignment result of PLD2-shRNA2 after ligation reaction.

specifically; LR reaction. In this step, PLD_2-shRNA was cloned in entry vector correctly and completely, and the entry vector obtained attL site. Thus, this step of experiment was successful.

Figure 4 shows the alignment result of lentiviral pLenti6/V5-GW/EmGFP-PLD2-miR vector and PLD2-shRNA vector after LR reaction by BLAST. This sequence of lentiviral pLenti6/V5-GW/EmGFP-PLD_2-miR vector was consistent with PLD_2-shRNA completely. PLD_2-shRNA was cloned in lentiviral vector correctly and completely, and the product of LR reaction can be amplified by *E. coli*.

PLD_2-shRNA2 vector transfected into 293FT cells

Figure 5 shows the lentiviral packing with PLD2-shRNA2 transfected 293FT cells. Using fluorescence microscope,

```
Score =  123 bits (64),  Expect = 1e-24
Identities = 64/64 (100%), Gaps = 0/64 (0%)
Strand=Plus/Plus

Query  907  TGCTGTGCAGAACCAATGATGACTGTGTTTTGGCCACTGACTGACACAGTCATTTGGTTC  966
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TGCTGTGCAGAACCAATGATGACTGTGTTTTGGCCACTGACTGACACAGTCATTTGGTTC  60

Query  967  TGCA  970
            ||||
Sbjct  61   TGCA  64
```

Figure 3. The sequence alignment result of PLD2-shRNA2 after BP reaction.

```
Score =  123 bits (64),  Expect = 5e-26
Identities = 64/64 (100%), Gaps = 0/64 (0%)
Strand=Plus/Minus

Query  1    TGCTGTGCAGAACCAATGATGACTGTGTTTTGGCCACTGACTGACACAGTCATTTGGTTC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  200  TGCTGTGCAGAACCAATGATGACTGTGTTTTGGCCACTGACTGACACAGTCATTTGGTTC  141

Query  61   TGCA  64
            ||||
Sbjct  140  TGCA  137


CPU time:     0.03 user secs.          0.01 sys. secs     0.04 total secs.
```

Figure 4. The sequence alignment result of PLD2-shRNA2 after LR reaction.

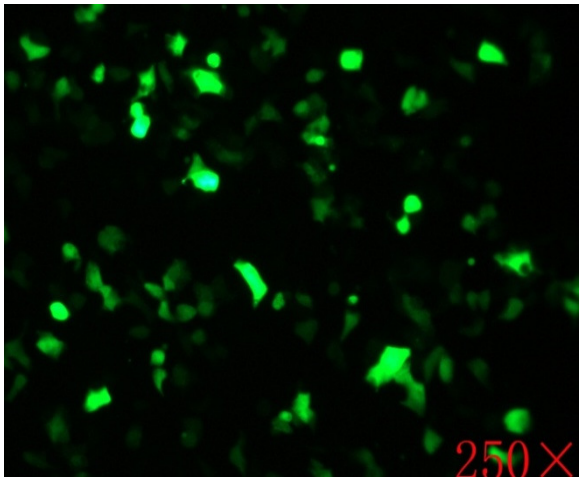
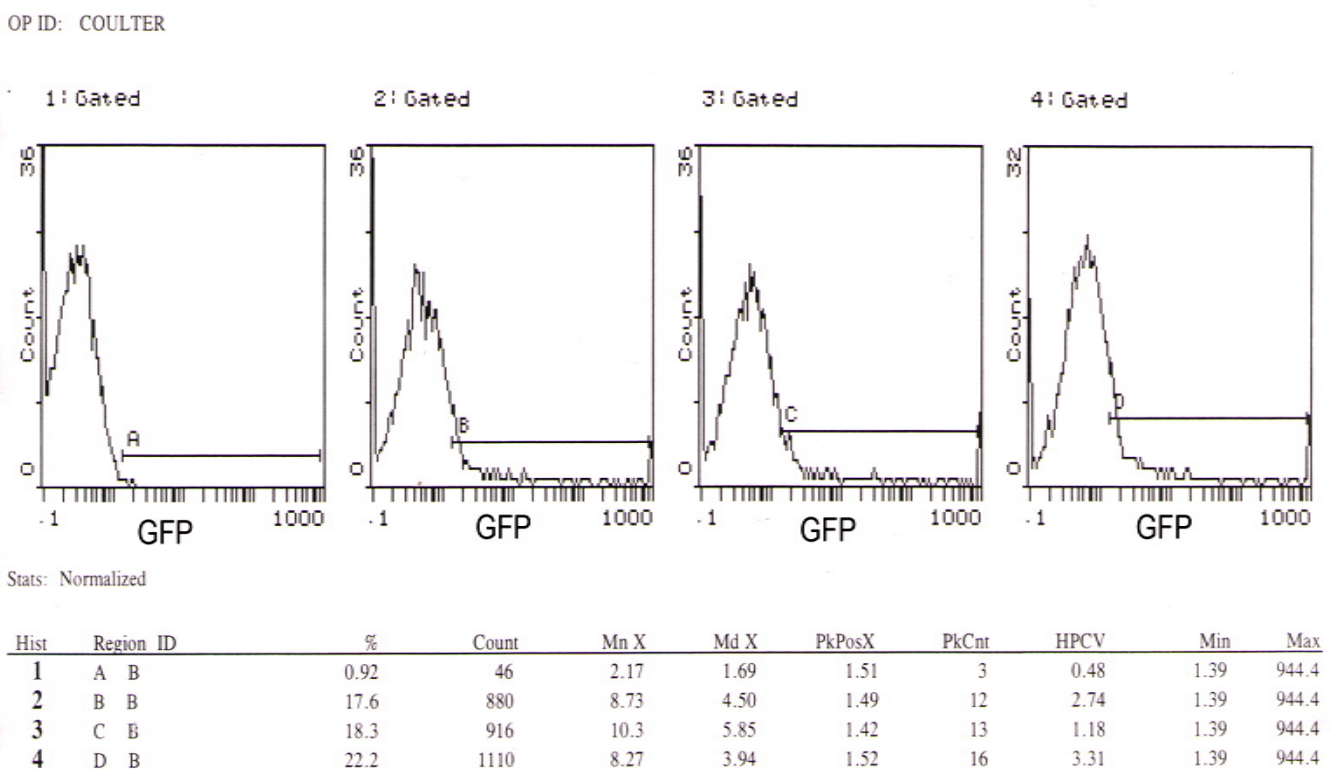


Figure 5. The lentiviral packing with PLD2-shRNA2 transfected 293FT cells.



Hist	Region ID	%	Count	Mn X	Md X	PkPosX	PkCnt	HPCV	Min	Max
1	A B	0.92	46	2.17	1.69	1.51	3	0.48	1.39	944.4
2	B B	17.6	880	8.73	4.50	1.49	12	2.74	1.39	944.4
3	C B	18.3	916	10.3	5.85	1.42	13	1.18	1.39	944.4
4	D B	22.2	1110	8.27	3.94	1.52	16	3.31	1.39	944.4

Figure 6. The result of flow cytometry detecting GFP (+) 293FT cells. The Gated 1, 2, 3, and 4, respectively, represented the results of 293FT cells transfected by negative control and the lentiviral PLD_2-shRNA1/PLD_2-shRNA2/PLD_2-shRNA3 vectors.

we found the lentiviral vectors, which have GFP report genes expression accompanied with the host genes in 293FT cells at 487 nm.

After the lentiviral was transfected into 293FT cells, the expression of GFP protein was observed under a fluorescence microscope and the percentage of GFP $^{(+)}$ 293FT cells was counted using flow cytometry (Figure 6).

The total number of 293FT cells transfected was 2×10^5, the volume of virus was 1 ml, and the virus was not diluted. The titer of lentiviral packing PLD_2-shRNA was 3.47×10^4 TU/ml.

DISCUSSION

In this study, we designed three PLD_2-shRNAs plasmids targeting human PLD_2 gene using BLOCK-iT Lentiviral PolII miR RNAi expression system. The lentiviral were packed and transfected the cells.

There is a number of confirmed silence of PLD_2 expression by RNAi. Padrón et al. (2006) transfected the siRNA into Hela cells and down-regulated the PLD_2 expression by the use of Oligofectamine reagent. Lee et al. (2006) and Du et al. (2004) transfected the siRNA into mast cells by electroporation and silenced the PLD_2 expression successfully. The lentiviral vector we chose was one of the most widely used siRNA delivery systems. In comparison with others, it shows some advantages such as: transfecting cells not only in mitotic active phase but also in mitotic delay or terminal differentiation in hematopoietic stem cells (Seggewiss and Dunbar, 2005), neurons (Hioki et al., 2007), parenchymal liver cells (Kang et al., 2005) etc; secondly, the target gene carried by lentiviral and integrated into host genome shows strong resistance to transcription silence and could be expressed in host cells chronically and steadily; thirdly, the lentiviral vector was compatible with many transcription promoters, including cell specific promoters and the housekeeping gene promoters widely used in genome. In addition, the lentiviral vector was rebuilt to accommodate about 10 kb exogenous genes thus, most of cDNA can be cloned into it. The major deficiency of the lentiviral vector however is the concern of biological safety (He and Falo, 2007).

The CMV promoter in pcDNATM6.2-GW/EmGFP-miRNA can start expression in a highly efficient and persistent manner, and can be activated in most of the mammalian cells. The pcDNATM6.2-GW/EmGFP-miRNA plasmid also has the double resistance to spectinomycin and blasticidin that can be used in the screening of eucaryotic or prokaryotic cells. The EmGFP green fluorescent protein expression gene in it can be used in

subsequent detection of transfection efficiency. We designed and synthesized three groups of shRNA sequences using the online miRNA design software of Invitrogen Company, and integrated them into the pcDNATM6.2-GW/EmGFP-miRNA vector. Due to the possibility of the bases mispairing in the procedure, synthetic shRNA was integrated and inserted into the destination vector, which may lead to the inability to obtain mature miRNA after cut by endogenous Dicer enzyme. For ensuring the sequence before inserting, we sequenced the destination plasmid. PCR, and the sequencing analyses revealed that lentiviral shRNA vectors of three targeting PLD2 gene were successfully constructed.

In a follow-up study, we interfered with the lentiviral shRNA vectors of targeting PLD2 gene that were constructed, and effectively reduced PLD2 gene expression level in the HL-60 cell lines. This means that though RNAi specifically silence PLD2 gene expression in HL-60 cells, it could cause an increase of apoptosis increase in the HL-60 cell (data not shown).

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