RNAi microarray by reverse transfection of siRNA and shRNA for functional genomics

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Abstract:

RNA interference (RNAi) is a widely used method for gene function analyses in a variety of organisms. Establishment of a large-scale, high-throughput platform for analysing gene function may require development of an RNAi microarray system using reverse transfection. Here, we show that shRNA is more effective in microarray gene silencing compared to siRNA. Endogenous mammalian genes are suppressed efficiently if highly functional class I sequences were used for siRNAs produced via shRNAs. Furthermore, the method appears applicable for functional genomics. Indeed, apoptotic cell death induced by staurosporine and actinomycin D was found to be blocked by shRNA against apoptotic protease-activating factor-1 and caspase-3, both of which are required in the cytochrome c-dependent apoptosis cascade.

1. INTRODUCTION

Double-stranded RNA (dsRNA) directs the sequence-specific degradation of mRNA through a gene silencing process known as RNA interference (RNAi) [1,2]. In mammalian cells, RNAi is triggered by the transfection of short-interfering RNA (siRNA), a dsRNA 19-bp in length with 2-nt 3′ overhangs [3], or by short hairpin RNA (shRNA) produced within cells [4] through sequence-specific cleavage of a cognate transcript. Thus, siRNA is usually chemically synthesized, whereas shRNA is transcribed from a DNA plasmid containing an appropriate promoter such as the H1 or U6 RNA polymerase III promoter. In the latter case, RNA produced within cells possesses a hairpin structure containing a stem sequence of approximately 20-bp that matches the target RNA sequence precisely. This hairpin RNA is thought to be processed by Dicer and other enzymes to generate active siRNA *in vivo* [5]. RNAi has been rapidly adopted as a technology for the analysis of gene function, together with ongoing efforts for constructing large-scale human siRNA libraries.

Cell microarray is a tool that allows the functional analysis of many genes in a systematic and high-throughput fashion [6–9]. A gelatin solution containing plasmids and transfection reagents is spotted onto a glass slide using a robotic microarray system, and mammalian cells are then applied across the surface of the entire slide. Cells growing on gelatin spots, each of which may have a different set of plasmids, undergo reverse transfection by incorporating that locally applied plasmids, whereas cells growing outside the gelatin spots are non-transfectants. Thus, the cell microarray system makes it possible to introduce many different plasmids separately into mammalian cells. Several groups have already attempted to establish an RNAi microarray system but initial attempts have not been very successful, particularly with the regard to endogenous gene silencing.

Here, we report improvements in the cell microarray system combined with RNAi technology and reverse transfection that can be used for large-scale analysis of gene function by high-throughput RNAi. Our results show that the efficiency of gene silencing by shRNA is much higher than that of siRNA when functional class I sequences [10] are used. Endogenous genes were inactivated by our reverse transfection system depending on shRNA, indicating that shRNA-mediated RNAi microarray may be applicable to the functional RNAi.

2. METHODS

2.1 siRNA preparation and construction of the shRNA expression plasmid

RNA oligonucleotides were purchased from Proligo and siRNA was prepared as described previously [10]. The passenger and guide strand sequences of siRNAs are as follows:
5’-GCCACAACGUCUAUAUCAUGG-3’/5’-AUGAUAUAGACGUUGUGGCUG-3’(siEGFP),
5’-CCGACAUCCCCGACUACAAGA-3’/5’-UUGUAGUCGGGGAUGUCGGUG-3’(siDsRed), and
5’-UAAAGAAAGGCCCGGCGCCAU-3’/5’-GGCGCCGGGCCUUUCUUUAUG-3’(siLuc). The hairpin shRNA expression vectors were constructed by inserting the annealed synthetic oligonucleotides into *Bam*HI/*Hind*III sites in the pSilencer3.1-H1 puro (Ambion) or pSUPER.retro.puro (OligoEngine) vector. The inserted oligonucleotide sequences are as follows: pEGFP441:
5’-GATCCCGCCACAACGTCTATATCATGGGCTTCCTGTCACCCATGATATAGACGTTGTGGCTTTTTTGGAAA-3’ and
5’-AGCTTTTTCCAAAAAAGCCACAACGTCTATATCATGGGTGACAGGAAGCCCATGATATAGACGTTGTGGGG-3’. pYes270:
5’-GATCCCAGGTGGTGTTACTATATTTGTGCTTCCTGTCACACAAATATAGTAACACCACCTTTTTTTGGAAA-3’ and
5’-AGCTTTTTCCAAAAAAAGGTGGTGTTACTATATTTGTGTGACAGGAAGCACAAATATAGTAACACCACCTGG-3’. pYes430:
5’-GATCCCAGCAATTATGTAGCGCCTGCAGCTTCCT

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GTCACTGCAGGCGCTACATAATTGCTTTTTTTGGAA
A-3’ and
5’-AGCTTTTTCCAAAAAAAGCAATTATGTAGCGCCT
GCAGTGACAGGAAGCTGCAGGCGCTACATAATTGC
TGG-3’. pVim812:
5’-GATCCCGTACGTCAGCAATATGAAAGTGCTTCCT
GTCACACTTTCATATTGCTGACGTACTTTTTTGGAA
A-3’ and
5’-AGCTTTTTCCAAAAAAGTACGTCAGCAATATGAA
AGTGTGACAGGAAGCACTTTCATATTGCTGACGTA
CGG-3’. pVim33:
5’-GATCCCGCAGGATGTTCGGCGGCCCGGGCTTCCT
GTCACCCGGGCCGCCGAACATCCTGCTTTTTTGGAA
A-3’ and
5’-AGCTTTTTCCAAAAAAGCAGGATGTTCGGCGGCC
CGGGTGACAGGAAGCCCGGGCCGCCGAACATCCTG
CGG-3’. pFyn444:
5’-GATCCCGTGGTACTTTGGAAAACTTGGGCTTCCT
GTCACCCAAGTTTTCCAAAGTACCACTTTTTTGGAA
A-3’ and
5’-AGCTTTTTCCAAAAAAGTGGTACTTTGGAAAACT
TGGGTGACAGGAAGCCCAAGTTTTCCAAAGTACCA
CGG-3’. pFyn89:
5’-GATCCCATGGCACAGACCCCACCCCTGCTTCCTG
TCACGAGGGGTGGGGTCTGTGCCATTTTTTTGGAAA
-3’ and
5’-AGCTTTTTCCAAAAAAATGGCACAGACCCCACCC
CTGGTGACAGGAAGCAGGGGTGGGGTCTGTGCCAT
GG-3’. pCaspase-3:
5’-GATCCCGACATGGCGTGTCATAAAATAGCTTCCT
GTCACTATTTTATGACACGCCATGTCTTTTTTGGAA
A-3’ and
5’-AGCTTTTTCCAAAAAAGACATGGCGTGTCATAAA
ATAGTGACAGGAAGCTATTTTATGACACGCCATGT
CGG-3’. pApaf-1:
5’-GATCCCGAAAGTCGTTTCGTTATTATTGCTTCCTG
TCACAATAATAACGAAACGACTTTCTTTTTTGGAAA
-3’ and
5’-AGCTTTTTCCAAAAAAGAAAGTCGTTTCGTTATT
ATTGTGACAGGAAGCAATAATAACGAAACGACTTT
CGG-3’. Two expression constructs, pCx-DsRed encoding DsRed and pCx-EGFP encoding EGFP [11], were used as reporters.

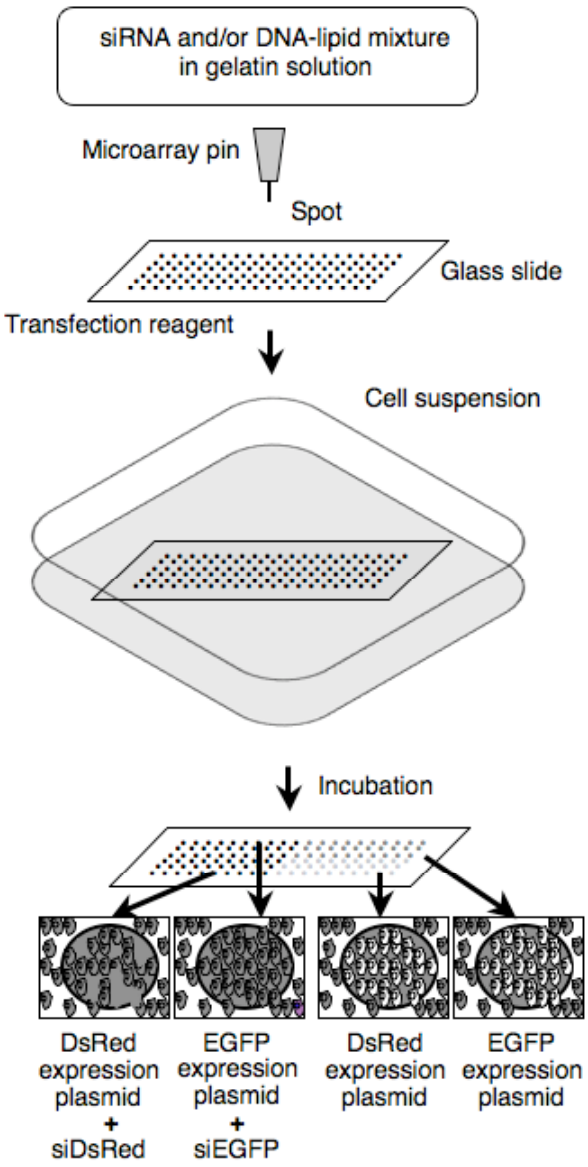

Fig. 1. Outline of the protocol used for RNAi microarray. The mixture of transfection reagent and reporter plasmids are spotted onto GAPSII glass slides using an SPBIO robotic microarray system. The glass slide was then placed inside of a culture dish and cells were poured into the dish. After incubation, the expression of reporter plasmids expressing DsRed or EGFP were detected via fluorescent microscopy.

2.2 Microarray printing and reverse transfection

Microarray printing was performed using a modified procedure of lipid-DNA method developed originally by Ziauddin and Sabatini [5]. The siRNA or shRNA expression plasmid with or without target plasmid DNA was suspended in 15 µl of DNA-condensation buffer (EC buffer) containing 0.35 M sucrose. After incubation with 1.8 µl of Enhancer solution and 6 µl of Effectene transfection reagent, one volume of 0.2 % gelatin was added and re-mixed. A resultant solution (20 µl) was collected into a well of 96-well plate for subsequent printing. A robotic arrayer spotting system (SPBIO^{TM}, Hitachi Soft) equipped with stealth pins was used to print siRNA or shRNA plasmid solution onto CMT GAPS glass slides (2549, Corning). For RT-PCR, Western blotting and immunohistochemistry, a siRNA-DNA-lipid mixture in gelatin solution was directly printed onto a glass slide and stored at room temperature for 16–24 h in a vacuum desiccator with anhydrous calcium sulphate pellets.

2.3 Reverse transfection

HEK293 or HeLa cells were cultured at 37 ºC under 5 % CO_2 in Dulbecco’s modified Eagle’s medium (DMEM;

Gibco-BRL) supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Mitsubishi Kagaku) and antibiotics (10 U/ml penicillin and 50 μg/ml streptomycin; Meiji). Reverse transfection was carried out using an Effecte™ transfection kit (301425, Qiagen). Enhancer solution (16 μl) was added to 150 μl of EC buffer, mixed, and allowed to sit at room temperature for about 5 min followed by the addition of 25 μl of Effectene transfection reagent (25 μl). The total volume of the mixture was used to coat the surface of a printed glass slide, sealed using the Hybriwell sealing system (Molecular Probes), and then inbubated at room temperature for 20 min. After removing the Hybriwell slip, the printed slide was placed printed side up in a 100×100×10 mm square culture dish (Corning). The cell suspension (1×10^7 cells in 25 ml of medium) was gently poured into the dish, after which the dish was placed in a humidified incubator under 5 % CO_2 at 37 ºC for 3 days.

2.4 Laser scanning

The microarrays were imaged using a laser fluorescence scanner (GenePix4000B; Axon Instruments) at a 5 μm resolution. EGFP and Cy3 emissions were measured separately after sequential excitation of the FITC and Cy3 fluorophores, respectively.

2.5 RT-PCR

Puromycin (3 μg/ml) was added to the medium 1 day after reverse transfection with shRNA expression vectors and pCAGIPuro-EGFP [10], which encodes the puromycin resistant gene and EGFP, and cells were incubated for 2 additional days to select the pCAGIPuro-EGFP-transfected HeLa cells. Total RNA was extracted from cells using RNeasy 96 (Qiagen) according to the manufacturer's protocol. After cDNA was synthesized with Oligo(dT) primers, the following primers were used for amplification of human genes. yes; 5'-CACCATTTGGAGGATCCTCAG and 5'-CCTCGTTGATTTCCAGGATTC, Fyn; 5'-CTCAGCACTACCCCAGCTTC and 5'-CATCTTCTGTCCGTGCTTCA, vimentin; 5'-GATGTTGACAATGCGTCTCTG and 5'-TTAAGGGCATCCACTTCACAG, β-actin; 5'-CACACTGTGCCCATCTACGA and 5'-GCCATCTCTTGCTCGAAGTC.

2.6 Western blotting

Cells were lysed in 1 % NP-40 lysis buffer (20 mM HEPES, [pH 7.3], 1mM $CaCl_2$, 1 mM $Mg(OAc)_2$, 125 mM KOAc, 5 mM EGTA, 1 mM dithiothreitol, and complete protease inhibitor [Roche]. Cell extracts (20 μg) were fractionated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Immunobilon-P; Millipore). The blots were treated with rabbit anti-human Fyn antibody (Upstate Biotechnology), rabbit anti-human Yes antibody (Upstate Biotechnology) or mouse anti-porcine vimentin antibody (Oncogene Research Products). The same membrane was also treated with mouse anti-β-actin antibody (Sigma). The resultant membrane was treated with alkaline phosphatase-conjugated anti-rabbit or anti-mouse secondary antibody (Amersham), and visualised by chemiluminescence CDP-Star detection reagent (Amersham).

2.7 Immunostaining and immunofluorescence microscopy

Three days after reverse transfection, cells were washed in phosphate-buffered saline (PBS) and fixed in 3.7 % formaldehyde in PBS for subsequent immunofluorescence staining. After permeabilisation in 0.1 % Triton X-100 in PBS for 30 min and blocking with 5 % goat whole serum for 1 h, cells were incubated with primary antibodies for 12 h at 4 ºC. Rabbit anti-human Fyn antibody (Upstate Biotechnology) and rabbit anti-human Yes antibody (Upstate Biotechnology) were diluted to 1/150 in 1 % whole goat serum in PBS and used as primary antibodies. The cells were washed three times with PBS and incubated with the secondary antibody, Cy3-conjugated anti-rabbit IgG (Jackson Immuno Research), at a dilution of 1/300 for 30 min at room temperature. After washing, cells were observed under a fluorescence/phase-contrast inverted microscope (Axiovert 200, Carl Zeiss) equipped with an AxioCam HRC digital camera.

2.8 Hoechst33342 staining

Apoptotic cell death was detected by staining with the DNA-binding fluorescent dye, Hoechst 33342 (Molecular Probes). After 24 h treatment with staurosporine (0.3 μM) or actinomycin D (0.5 μg/ml), cells were washed twice in PBS and incubated with 10 μg/ml Hoechst 33342 (Molecular Probes) at room temperature for 30 min. Apoptotic cell death was determined by the presence of brightly staining condensed chromatin or nuclear fragmentation.

3. RESULTS AND DISCUSSION

3.1 RNAi microarray using reporter genes

As shown in **Fig. 1**, siRNA and/or DNA-lipid mixtures in gelatin solution were spotted onto a GAPS glass slide using the SPBIO™ Robotic arrayer. After drying, the glass slides were treated with transfection reagents and cells suspended in culture medium were applied to a printed glass slide in a square culture dish. After an appropriate culture period, gene silencing effects were examined.

Using visible EGFP and DsRed genes as reporters, gene silencing efficiency by reverse transfection was examined in HEK293 cells 3 days after reverse transfection. As shown in the first and seventh rows, labelled with siRNA concentration = 0, in **Fig. 2A**, clear signals indicative of DsRed and EGFP transfection were observed in cells situated within six spotted areas of about 500–600 μm in diameter, in which pCx-EGFP or pCx-DsRed plasmids (32 ng/μl) had been printed. Transfected gene activity was detected using a laser fluorescence scanner. When siRNAs

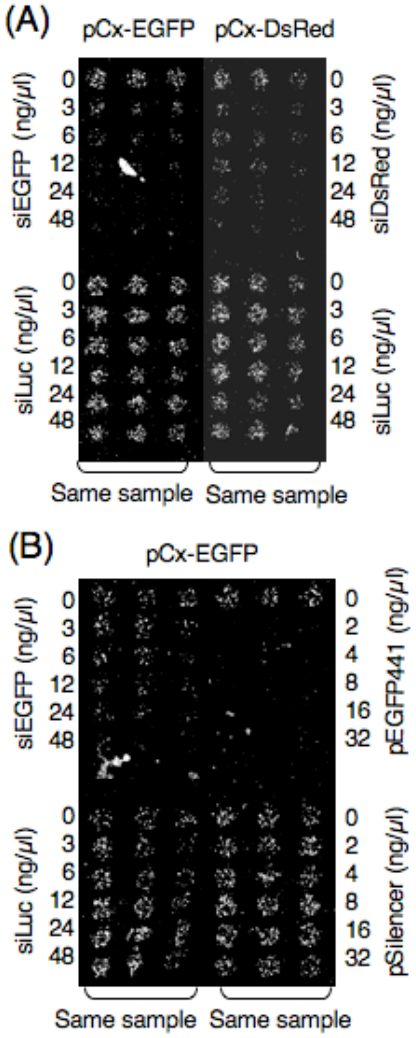

Fig. 2. Dose-dependent silencing effects of siRNA and shRNA for reporter genes. (A) Effects of siRNAs against EGFP and DsRed. The reporter plasmid, pCx-DsRed or pCx-EGFP (32 ng/μl), was introduced into HEK293 cells with siEGFP or siDsRed (0-48 ng/μl), and the silencing effects were observed via fluorescent microscopy after 3 days. siLuc was used as a control. (B) Comparison of siRNA versus shRNA expression plasmid on silencing of EGFP. pCx-EGFP (2 ng/μl) was introduced into HEK293 cells with siEGFP (0-48 ng/μl) or pEGFP441 (0-32 ng/μl) and EGFP silencing effects were observed via fluorescent microscopy after 3 days. siLuc and pSilencer vector were used as siRNA and plasmid controls, respectively.

against EGFP or DsRed were co-transfected, EGFP or DsRed signals, respectively, decreased with increasing siRNA concentration (**Fig. 2A**, rows 2–6). In contrast, no change was detected when a control siRNA, siLuc, was used (rows 8–12). Next, we compared the silencing efficiencies of siRNA and shRNA treatment using EGFP as a target (**Fig. 2B**, rows 1–6). The pEGFP441 vector expresses a shRNA, whose target sequence is very similar, if not identical, to that of the siRNA used. Both siRNA and shRNA inhibited EGFP expression, and no change in EGFP expression was induced by non-cognate siRNA or control shRNA-encoding DNA treatment (siLuc). However, a difference in target knockdown efficiency between cognate siRNA and shRNA-encoding DNA treatment was evident. Although >12 ng/μl (>1000 nM) of siRNA was necessary to almost completely knockdown the EGFP signal, >2 ng/μl (>1nM) of shRNA-encoding plasmid was sufficient to suppress EGFP expression, indicating that shRNA-encoding DNA treatment is much more effective than siRNA treatment at least in human cells.

3.2 Identification of shRNA sequence for highly effective reverse transfection-dependent endogenous gene silencing

Although efficient silencing of reporter genes has been demonstrated by other groups [6,7], successful studies targeting endogenous genes are limited [8]. Our previous experiment [10] indicated that the efficiency of siRNA-dependent gene silencing varies considerably depending on the siRNA sequence used. Thus, the siRNA sequence dependency of reverse transfection-mediated gene silencing in HeLa cells was examined.

SiRNAs may be classified into three groups, class I, II and III [10]. Class I siRNAs are highly functional in typical gene silencing and satisfy the following conditions simultaneously: A/U residues at the 5′ end of the guide strand; G/C at the 5′ end of the passenger strand; at least four A/U residues in the 5′ terminal 7 bp of the guide strand. In contrast, siRNAs opposite in these features give rise to little gene silencing in mammalian cells, and are grouped as class III. In addition, a G/C stretch, more than 9 bp in length is absent in class I siRNAs. The siRNAs other than those of class I and III are defined as class II. To confirm whether our algorithm for siRNA classification is applicable for reverse transfection, the effects of the plasmids encoding either class I or class III siRNA as shRNA were examined. Three endogenous genes (the human src family kinase genes, Yes and Fyn, and the intermediate filament protein gene, vimentin) were used as targets. pYes270, pVim812 and pFyn444 are shRNA plasmids capable of producing class I siRNA, whereas pYes430, pVim33 and pFyn89 are shRNA plasmids producing class III siRNA. These shRNA plasmids were printed separately on a glass slide with pCAGIPuro-EGFP, and reverse transfection was carried out. After selecting pCAGIPuro-EGFP transfectants after a 2-day treatment with puromycin (3 μg/ml), cells were recovered from the glass slide and gene silencing effects were examined by RT-PCR, Western blotting and immunohistochemical analyses (**Fig. 3**). pSilencer was used as a negative control. In all cases examined, shRNA plasmids capable of producing class I siRNAs markedly suppressed target gene expression, whereas little target silencing was observed in cells treated with shRNA plasmids producing class III siRNA. We conclude that our algorithm for classifying siRNAs for standard RNAi is also applicable for a selection of shRNA sequences for effective reverse

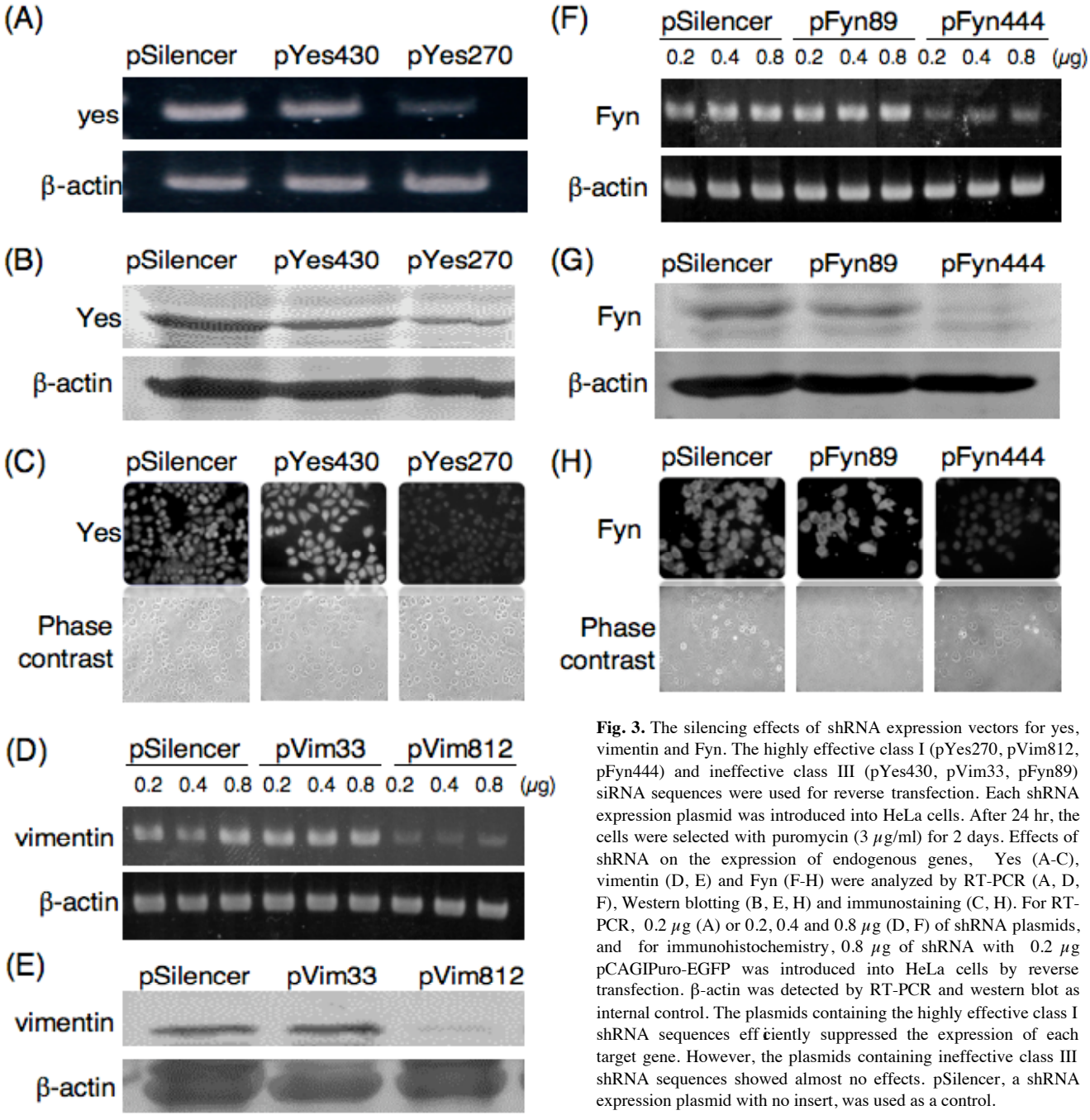

Fig. 3. The silencing effects of shRNA expression vectors for yes, vimentin and Fyn. The highly effective class I (pYes270, pVim812, pFyn444) and ineffective class III (pYes430, pVim33, pFyn89) siRNA sequences were used for reverse transfection. Each shRNA expression plasmid was introduced into HeLa cells. After 24 hr, the cells were selected with puromycin (3 μg/ml) for 2 days. Effects of shRNA on the expression of endogenous genes, Yes (A-C), vimentin (D, E) and Fyn (F-H) were analyzed by RT-PCR (A, D, F), Western blotting (B, E, H) and immunostaining (C, H). For RT-PCR, 0.2 μg (A) or 0.2, 0.4 and 0.8 μg (D, F) of shRNA plasmids, and for immunohistochemistry, 0.8 μg of shRNA with 0.2 μg pCAGIPuro-EGFP was introduced into HeLa cells by reverse transfection. β-actin was detected by RT-PCR and western blot as internal control. The plasmids containing the highly effective class I shRNA sequences efficiently suppressed the expression of each target gene. However, the plasmids containing ineffective class III shRNA sequences showed almost no effects. pSilencer, a shRNA expression plasmid with no insert, was used as a control.

transfection.

3.3 Functional analysis of drug-induced apoptosis by reverse transfection

To demonstrate the efficacy of reverse transfection for functional genomics, we used a model system involving drug-induced apoptosis. Staurosporine and actinomycin D are known to trigger cytochrome c release from mitochondria [13]. Cytochrome c/Apaf-1/procaspase-9-containing apoptosomes formed in the cytosol activate downstream caspases, caspase-3, -6 and -7, leading to cell death [14,15]. If reverse transfection is effective in endogenous gene phenotype analysis, shRNA plasmids expressing human caspase-3 and Apaf-1 would be expected to suppress drug-induced apoptosis. Highly functional class I shRNA expression plasmids for caspase-3 and Apaf-1 were introduced into HeLa cells by reverse transfection, and the cells were treated with staurosporine and actinomycin-D. As shown in **Fig. 4**, apoptosis was significantly inhibited in cells co-transfected with shRNA

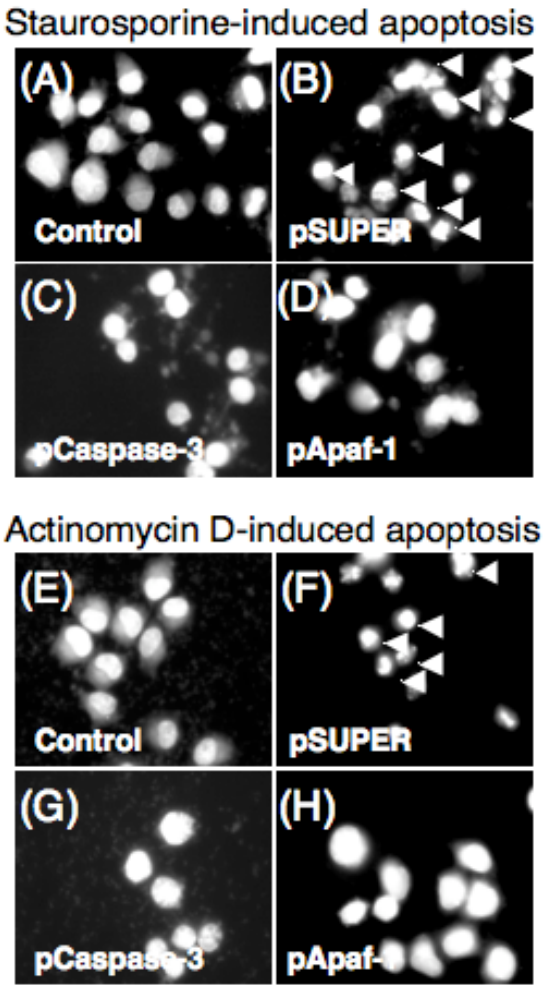

Fig. 4. Functional analysis using reverse transfection. Highly effective class I shRNA expression plasmids against human caspase-3 (C, G) and Apaf-1 (D, H) were introduced into HeLa cells, respectively, using reverse transfection with pCAGIPuro-EGFP. After 24 h, puromycin (3 μg/ml) was added to the culture and incubated for 2 days. At 3 days after transfection, the cells were treated with staurosporine (0.3 μM) for 6 hr (B-D) and actinomycin D (0.5 μg/ml) for 12 hr (F-H), and stained with Hoechst 33342. Arrowheads in (B) and (F) indicate the condensed or fragmented nuclei in the cells undergoing apoptosis. Control cells were no treated cells (A, E). pSUPER, a shRNA expression plasmid with no insert, was used as a control vector (B, F).

plasmids against caspase-3 or Apaf-1. In contrast, cells transfected with control vector (pSUPER) underwent apoptotic cell death.

4. CONCLUSION

In summary, we established an efficient shRNA-mediated RNAi microarray system. The shRNA appeared to induce reverse transfection much more effectively than did siRNA, at least in HEK293 cells. As with standard siRNA-mediated gene silencing, class I siRNA generated via shRNA triggered reverse transfection-mediated gene silencing most effectively, which may facilitate genome-wide scale analyses of gene function.

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REFERENCES

[1] Hannon, G.J. (2002) RNA interference. *Nature,* **418,** 244-251.
[2] Tomari, Y. and Zamore, P.D. (2005) Perspective: machines for RNAi. *Genes Dev*., **19,** 517-529.
[3] Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature,* **411,** 494-498.
[4] Shi, Y. (2003) Mammalian RNAi for the masses. *Trends Genet.*, **19,** 9-12.
[5] Paddison, F.J., Caudy, A.A. and Hannon, G.J. (2002) Stable suppression of gene expression by RNAi in mammalian cells. *Proc. Natl. Acad. Sci, USA*, **99**, 1443-1448.
[6] Ziauddin, J. and Sabatini, D.M. (2001) Microarrays of cells expressing defined cDNAs. *Nature*, **411,** 107-110.
[7] Silva, J.M, Mizuno, H., Bragy, A., Lucito, R. and Hannon, G.J. (2004) RNA interference microarrays: High-throughput loss-of-function genetics in mammalian cells. *Proc. Natl. Acad. Sci. USA*, **101,** 6548-6552.
[8] Mousses, S., Caplen, N.J., Cornelison, R., Weaver, D., Basik, M., Hautaniemi, S., Elkahloun, A.G., Lotufo, R.A., Choudary, A., Dougherty, E.R., Suh, E. and Kallioniemi, O. (2003) RNAi microarray analysis in cultured mammalian cells. *Genome Research*, **13,** 2341-2347.
[9] Kumar, R., Conklin, D.S. and Mittal, V. (2007) High-throughput selection of effective RNAi probes for gene silencing. *Genome Research*, **13**, 2333-2340.
[10] Ui-Tei, K., Naito, Y., Takahashi, F., Haraguchi, T., Ohki-Hamazaki, H., Juni, A., Ueda, R. and Saigo, K. (2004) Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA. *Nucleic Acids Res*., **32**, 936-948.
[11] Doi, N., Zenno, S., Ueda, R., Ohki-Hamazaki, H., Ui-Tei, K. and Saigo, K. (2003) Short-interfering-RNA-mediated gene silencing in mammalian cells requires Dicer and eIF2C translation initiation factors. *Curr. Biol*., **8**, 41-46.
[12] Yu, J.Y., DeRuiter, S.L. and Turner, D.L. (2002) RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc. Natl. Acad. Sci. USA*, **99**, 6047-6052.
[13] Andreau, K., Castedo, M., Perfettini, J.L., Roumier, T., Pichart, E., Souqere, S., Vivet, S., Larochette, N., Kroemer, G. (2004) Preapoptotic chromatin condensation upstream of the mitochondrial checkpoint. *J. Biol.Chem*., **279**, 55937-55945.
[14] Kumar, S. (1999) Mechanisms mediating caspase activation in the cell death. *Cell Death Differ*., **6**, 1060-1066
[15] Nicholson, D.W. (1999) Caspase structure, proteolytic substrate, and function during apoptotic cell death. *Cell Death Differ.*, **6**, 1028-1042.