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Anti-angiogenesis and anticancer effects of a plasmid expressing both ENDO-VEGI$_{151}$ and small interfering RNA against survivin

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Abstract. We have previously reported that the overexpression of the endostatin-vascular endothelial cell growth inhibitor (VEGI) fusion protein inhibits angiogenesis and achieves a strong anticancer effect. In this study, we constructed the dual-function expression plasmid pCDNA3.1-ENDO-VEGI$_{151}$/survivin-small interfering RNA (siRNA) (pEV/si-survivin), and evaluated the anti-angiogenesis and anticancer effects of this plasmid. Efficient siRNA sequences against survivin were identified; and the pEV/si-survivin expression vector was constructed and transfected into MDA-MB-231 cells and human umbilical vein endothelial cells (HUVECs). The expression levels of ENDO-VEGI$_{151}$ and survivin were detected by RT-PCR and Western blotting analysis. MTT assay was used to detect the proliferation of cancer cell lines. Flow cytometry was used to detect cell cycle states and apoptosis. The expression of both ENDO-VEGI$_{151}$ and survivin-siRNA were detected in MDA-MB-231 and HUEVC cells transfected with pEV/si-survivin. The expression of survivin was reduced in cells transfected with pEV/si-survivin. Furthermore, pEV/si-survivin inhibited proliferation and promoted apoptosis in MDA-MB-231 and HUEVC cells. It also caused cell cycle arrest in both cell lines. In conclusion, the dual-function expression plasmid pEV/si-survivin is involved in inhibition of angiogenesis and promoting tumor cell apoptosis *in vitro*. Therefore, it is also expected to improve the treatment of tumors by exerting synergistic effects *in vivo*.

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Abbreviations: DR3, death receptor-3; TAI, tumor angiogenesis inhibitor; VEGI, vascular endothelial cell growth inhibitor

Key words: breast cancer, survivin, anti-angiogenesis, gene therapy

Introduction

Malignant tumor is one of the major diseases threatening human health and leads to high mortality. However, traditional therapeutic drugs targeting tumor angiogenesis have limited effect on the treatment of malignant tumors. Combination of tumor angiogenesis inhibitor (TAI) with other traditional anti-cancer methods causes unexpected toxicity and side-effect. Therefore, the exploration of novel combinational strategies has received wide attentions.

Endostatin, a C-terminal fragment of type XVIII collagen, has been reported to be an efficient anti-angiogenesis agent. It reduces angiogenesis from both inhibiting the proliferation and migration and stimulating apoptosis of endothelial cells specifically (1). Previous studies revealed that endostatin treatment could inhibit tumor growth significantly (2). Vascular endothelial cell growth inhibitor (VEGI), a member of tumor necrosis factor (TNF) superfamily, has been shown to inhibit cancer through three different ways: directly sacrificing tumor cells; indirectly inhibiting the proliferation of endothelial cells; and stimulating the maturation of dendritic cells (3). Our previous studies showed the overexpression of endostatin-VEGI fusion protein inhibited angiogenesis and achieved strong anti-cancer effect (4,5).

Survivin has been considered as the most effective inhibitor of cell apoptosis so far and widely expressed in human tumors. It is regarded as an ideal target for tumor gene therapy (6). In this study, we constructed the dual function expressional plasmid pCDNA3.1-ENDO-VEGI$_{151}$/survivin-small interfering RNA (siRNA) (pEV/si-survivin), to promote tumor cell apoptosis and angiogenesis inhibition simultaneously in order to provide a novel strategy for tumor gene therapy.

Materials and methods

Materials and reagents. Adenovirus vectors pCA13-ENDO-VEGI$_{151}$, pSilencer™ 2.1-U6 neo and pCDNA3.1 with ENDO-VEGI$_{151}$ fusion gene were constructed in our laboratory; pMD18-T, restriction enzymes, reverse transcription kit and real-time PCR kit were purchased from Takara; TRIzol and Lipofectamine™ 2000 were from Invitrogen; RPMI-1640 medium and fetal bovine serum (FBS) were from Hyclone; siRNA were synthesized by Shanghai GenePharma Co. Ltd.;

Table I. Sequence of siRNAs and PCR primers.

Symbol	Forward 5'-3'	Reverse 5'-3'
siRNA-1	GGACCACCGCAUCUCUACAtt	UGUAGAGAUGCGGUGGUCCtt
siRNA-2	GCAUUCGUCCGGUUGCGCUtt	AGCGCAACCGGACGAAUGCtt
siRNA-3	CUGGACAGAGAAAGAGCCAtt	UGGCUCUUUCUCUGUCCAGtt
Scramble-siRNA	UUCUCCGAACGUGUCACGUtt	ACGUGACACGUUCGGAGAAtt
U6/survivin-shRNA	ccatgcatCCCCAGTGGAAAGACGC	cggtcgacTTCCAAAAAAGCATTCGTCC
Survivin	TTCTCAAGGACCACCGCATCT	GCGCACTTTCTTCGCAGTTTC
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
ENDO-VEGI$_{151}$	CCATCTACCTCGGAGCCATGT	ACTGACGTTCACCATTAGCTTGTC

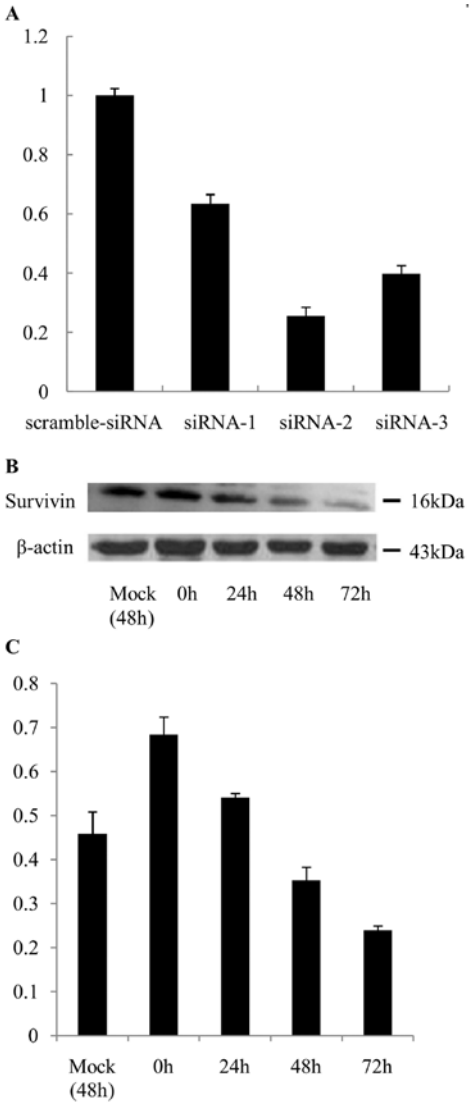

Figure 1. The changes of the expression levels of survivin mRNA and protein in MDA-MB-231 cells by siRNA treatment. (A) The expression of survivin mRNA are examined by real-time PCR. (B) The expression of survivin protein are examined by Western blot analysis. (C) Densitometric quantifications of the survivin levels normalized against β-actin levels.

PCR primers and primers used for survivin-short hairpin RNA (shRNA) were synthesized in Generay (Shanghai), primers used in this study are listed in Table I. Rabbit anti-human survivin polyclonal antibody was purchased from Santa Cruz Biotechnology, β-actin was purchased from Sigma. Rabbit anti-human VEGI$_{151}$ polyclonal antibody was a gift of Dr Min Zhang (Department of Microbiology, Second Military Medical University, Shanghai).

Cell culture and transfection. Human breast cancer cell line (MDA-MB-231) and human umbilical vein endothelial cell line (HUEVC) were purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Both cell lines were grown in RPMI-1640 medium supplemented with 10% FBS in a humidified incubator with 5% CO_2 at 37˚C; Transfection was done with Lipofectamine 2000 according to the manufacturer's instructions. Briefly, cells were seeded at 1x10^6 cells per 6-well plate and incubated for 24 h. The medium was replaced with serum-free DMEM and cells were cultured for another 2 h prior transfection. Mixture was prepared with a ratio of siRNA or DNA to Lipofectamine 2000 of 4 μg: 8 μl, added to the culture drop by drop. Cells were left in transfection medium for 4 h, the medium was replaced with fresh medium.

Screening for effective survivin-siRNA fragment. siRNA target finder software online (http://www.ambion.com/techlib/misc/siRNA_finder.html) was used for survivin siRNA design following the principles given. Three pairs of primers were picked up and synthesized. These survivin-siRNAs and one pair of scramble-siRNA as a negative control were transfected into MDA-MB-231 cells, respectively. Cells were cultured for 36 h and then collected for RNA extraction. Survivin expression was determined by real-time PCR method. Western blotting was used for the detection of protein levels in cells transfected with distinct surviving-siRNAs. Sequences of siRNAs and primers for real-time PCR are listed in Table I.

Construction of the dual function expressional plasmid pCDNA3.1-ENDO-VEGI$_{151}$/survivin-siRNA (pEV/si-survivin). The cDNA sequences of survivin-shRNA was designed in accordance with the pre-selected survivin-siRNA and synthesized with *Bgl*II and *Hin*dIII sites on 5' site: 5'-*gatct*GCATTCGT CCGGTTGCGCTTTCAAGACGAGCGCAACCGGACGAT GCTTTTTTGGAA*a*-3' and 5'-*agctt*TTCCAAAAAAGCATT CGTCCGGTTGCGCTCGTCTTGAAAGCGCAACCGGAC GAATGC*a*-3'; it was inserted into pSilencer™ 2.1-U6 neo plasmid after annealing. The fragment of U6-survivin-shRNA

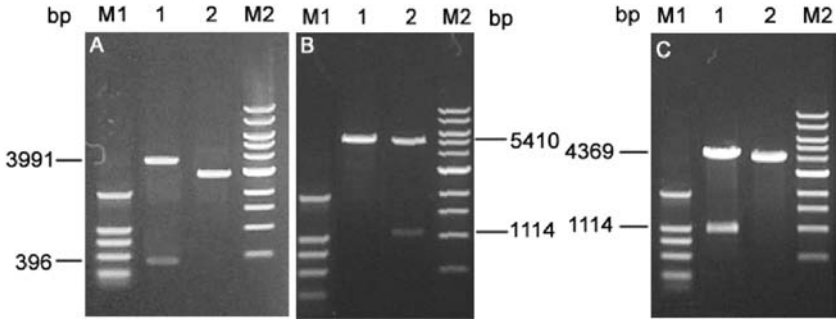

Figure 2. Identification of the recombinant plasmids by restriction enzyme digestion. M1, DL2000; M2, 1 kb DNA ladder. (A) psi-survivin digested by *Nsi*I and *Sal*I got 3991 and 396 bp fragments (U6/si-survivin). (B) pEV digested by *Hin*dIII and *Bam*HI got 5410 and 1114 bp fragments (ENDO/VEGI$_{151}$). (C) pEV/si-survivin digested by *Hin*dIII and *Bam*HI got 4369 and 1114 bp fragments (ENDO/VEGI$_{151}$).

(396 bp) was amplified by PCR with this plasmid as the template. PCR primers were designed with *Nsi*I and *Sal*I sites. PCR fragment was inserted into pMD18-T vector (primers are listed in Table I). U6/surviving-shRNA fragment was purified after *Nsi*I and *Sal*I digestion and inserted into pcDNA3.1 vector to generate the pcDNA-U6/survivin-shRNA (psi-survivin) plasmid.

ENDO-VEGI$_{151}$ fragment was purified after *Hin*dIII and *Bam*HI double digestion and inserted into pcDNA3.1 and psi-survivin vectors to generate pcDNA-ENDO/VEGI$_{151}$ (pEV) and pcDNA-ENDO-VEGI$_{151}$/U6/survivin-shRNA (pEV/si-survivin) plasmids.

Psi-survivin, pEV, pEV/si-survivin and pcDNA3.1 plasmids constructed above were transfected into MDA-MB-231 cells, respectively. Total RNAs and proteins were extracted at 48 h. The expression pattern of ENDO-VEGI$_{151}$ and survivin were determined with real-time PCR and Western blotting. Densitometry and quantification were performed by using National Institutes of Health ImageJ software version 1.44. PCR primers are listed in Table I.

MTT method for the analysis of MDA-MB-231 and HUEVC proliferation after transfection with pEV/si-survivin. MDA-MB-231 and HUEVC cells in logarithmic growth phase were seeded in 96-well plate with 0.5x10^5 cells/well. Transfection was done with cells at 80% confluency; the experiment was carried out in four groups: transfected with pcDNA3.1, psi-survivin, pEV or pEV/si-survivin with five replications. MTT of 20 μl (5 mg/ml) was added into wells at 48 h and 72 h, respectively. Plates were incubated in dark for 4 h at 37˚C. Medium was discarded and 100 μl DMSO/well was added and vibrated for 10 min in dark. The OD value of the samples was measured at a wavelength of 570 with 655 nm as a reference wavelength. Experiments were repeated three times independently. Inhibition ratio was calculated as follows inhibition ratio = ($OD_{control\ group}$-$OD_{experimental\ group}$)/$OD_{control\ group}$ x 100%.

Flow cytometry for cell cycle analysis of MDA-MB-231 and HUEVC cells. MDA-MB-231 and HUEVC cells in logarithmic growth phase were seeded in 6-well plate with 2x10^5 cells per well. Transfection was done with cells at 80% confluency. Forty-eight hours after transfection, cells were trypsinized and washed twice with PBS, fixed with -20˚C pre-cooled 70% ethanol and incubated at 4˚C overnight, and then washed twice with PBS and centrifuged to collect cells. Propidium iodide (PI) and RNase (final con. 50 μg/ml) were added and cells were incubated in dark for 30 min before analysis with flow cytometry. After incubation, the flow cytometry was done on a Becton-Dickinson FACScalibur flow cytometer and data were analyzed by using flowjo analysis software (Version 5.7.2).

Flow cytometry for analysis of MDA-MB-231 and HUEVC apoptosis. MDA-MB-231 and HUEVC cells were treated as described above. All cells were collected and washed twice with PBS, and then suspended with 500 μl buffer; 5 μl Annexin V-FITC and 5 μl PI were added and mixed thoroughly, and cells were incubated in dark for 10 min at room time before analysis with FACS.

Statistical analysis. SPSS14.0 software was used for statistical analysis. All values are presented as the mean ± SEM and statistical significance was determined by analysis of variance with Dunnett's test. The differences were accepted as significant at $P<0.05$.

Results

Screening of high effective survivin-siRNA. Scramble-siRNA, siRNA-1, siRNA-2 and siRNA-3 were transfected into MDA-MB-231 cells, respectively. Total-RNA and proteins were extracted at 36 h post transfection. The mRNA levels of survivin were determined by real-time PCR and normalized to GAPDH. Results showed that the siRNA-2 had the most effective inhibition effects, which reduced the expression of survivin mRNA by 75% (Fig. 1A). Therefore, siRNA-2 was chosen for the following experiments. The level of survivin protein was also reduced in MDA-MB-231 cells after transfection with siRNA-2 (Fig. 1B).

Identification of the dual function expressional plasmid with restriction enzyme digestion. Three recombinant plasmids were digested with corresponding restriction enzymes and digestion results were examined with agarose gel electrophoresis. As predicted, psi-survivin digested with *Nsi*I and *Sal*I got 3991 and 396 bp fragments; pEV digested with *Hin*dIII and *Bam*HI got 5410 and 1114 bp fragments; and pEV/si-survivin digested with *Hin*dIII and *Bam*HI got 4369 and 1114 bp fragments (Fig. 2).

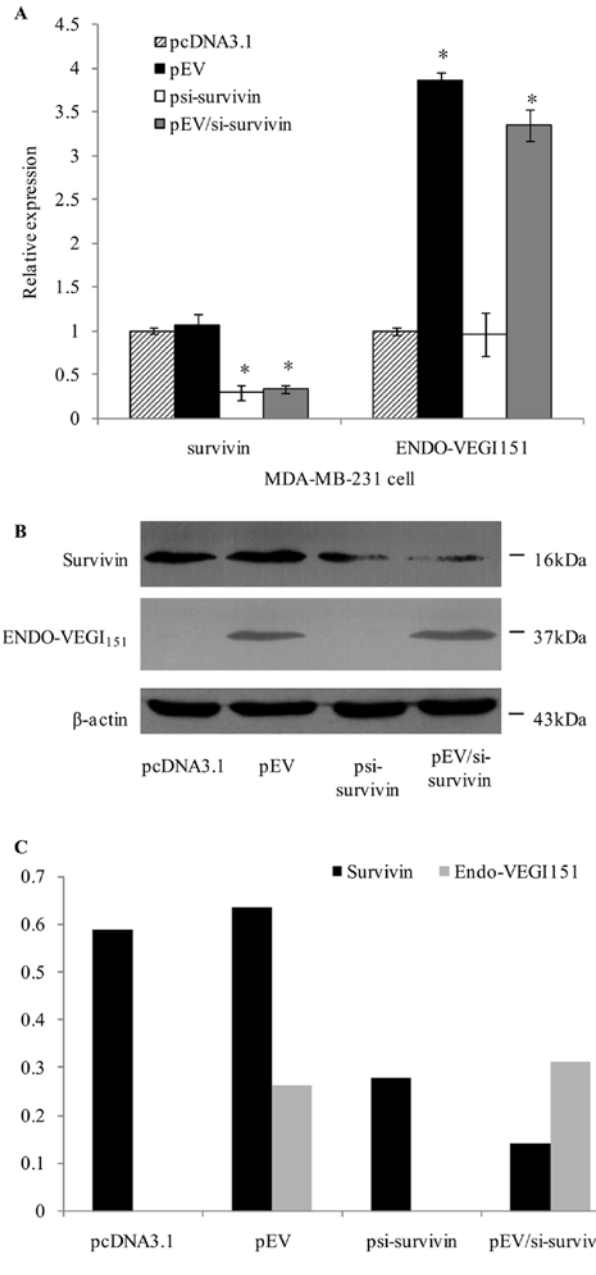

Figure 3. Expression of survivin and ENDO-VEGI$_{151}$ in MDA-MB-231 cell transfected with different recombinant plasmids for 48 h. (A) Real-time PCR analysis of survivin and ENDO-VEGI$_{151}$ on mRNA level. *P<0.05 vs. pcDNA3.1 group (ANOVA and Dunnett's test). (B) Western blotting analysis of survivin and ENDO-VEGI$_{151}$ on protein level. (C) Densitometric quantifications of the survivin and ENDO-VEGI$_{151}$ levels normalized against β-actin levels.

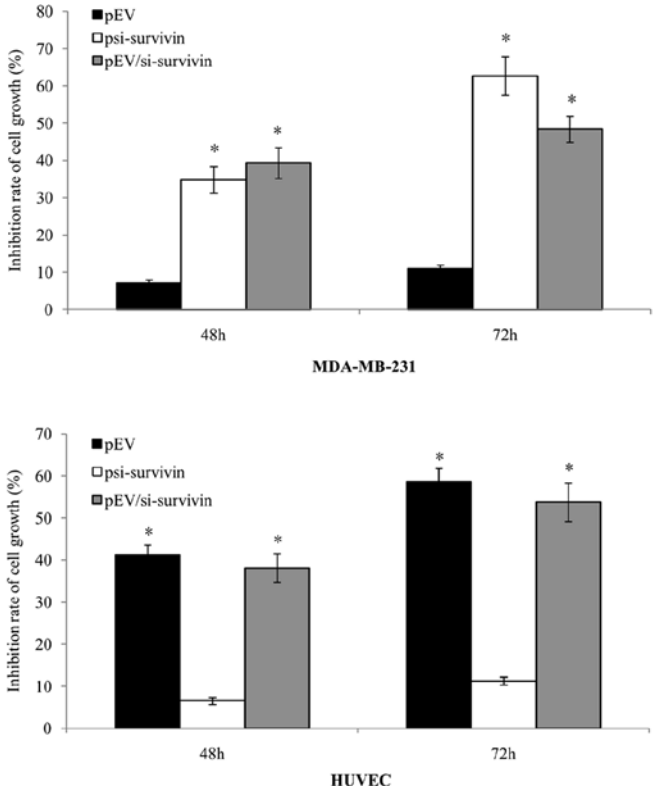

Figure 4. Growth inhibitory rate for MDA-MB-231 and HUVEC cells transfected with psi-survivin, pEV and pEV/si-survivin. *P<0.05 vs. pcDNA3.1 group (ANOVA and Dunnett's test).

Expression level of the dual function expressional plasmid. MDA-MB-231 cells were transfected with plasmid containing pcDNA3.1 (negative control), psi-survivin, pEV, and pEV/si-survivin, respectively, and incubated for 48 h. Real-time PCR results indicated that the mRNA level of survivin was significantly higher in cells transfected with psi-survivin or pEV/si-survivin than that in cells transfected with pcDNA3.1 or pEV (P<0.05); while the mRNA level of ENDO-VEGI$_{151}$ was higher in cells transfected with pEV or pEV/si-survivin than that in cells transfected with pcDNA3.1 or psi-survivin (P<0.05) (Fig. 3A). Protein level was detected with Western blot assay. The expression of survivin in MDA-MB-231 cells was inhibited by the transfection of psi-survivin or pEV/si-survivin, and ENDO-VEGI$_{151}$ protein was only detected in cells transfected with pEV or pEV/si-survivin (Fig. 3B).

Effects of the dual function expressional plasmid pEV/si-survivin on cell proliferation. The proliferation of MDA-MB-231 and HUVEC cells transfected with different plasmids were examined with MTT method. The psi-survivin and pEV/si-survivin transfections significantly inhibited the proliferation of MDA-MB-231 breast cancer cells, and pEV and pEV/si-survivin transfections inhibited the proliferation of HUVEC endothelial cells (P<0.05) (Fig. 4).

Effects of the dual function expressional plasmid pEV/si-survivin on the cell cycle. The cell cycle of MDA-MB-231 and HUVEC cells transfected with different plasmids were examined with flow cytometry. Following transfection with psi-survivin or pEV/si-survivin for 48 h, the percentage of MDA-MB-231 cells at G1 phase (76.35±1.01% and 87.89±0.81% for cells transfected with psi-survivin and pEV/si-survivin, respectively) was higher than that of cells transfected with pcDNA3.1 (54.69±3.50%, P<0.05) (Fig. 5), indicating that survivin-siRNA inhibits the proliferation of MDA-MB-231 cells by inducing G1 phase arrest. Similar results were obtained from HUVEC cell groups (51.21±7.25%, 65.41±2.38% and 7.41±3.46% for cells transfected with pcDNA3.1, pEV and pEV/si-survivin, respectively, P<0.05, (Fig. 5), indicating that ENDO-VEGI$_{151}$ caused G1 phase arrest in HUVEC cells.

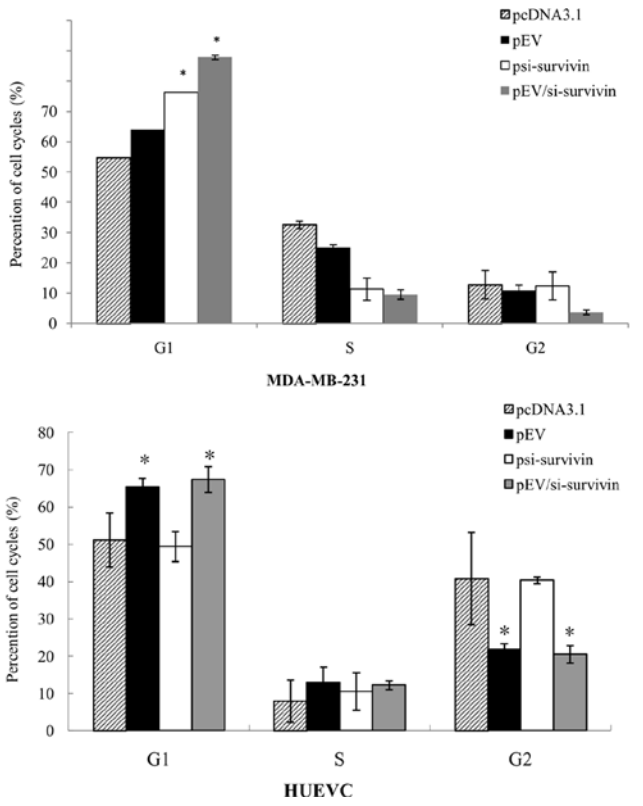

Figure 5. Cell cycle analysis of MDA-MB-231 and HUVEC cells transfected with pcDNA3.1, psi-survivin, pEV or pEV/si-survivin for 48 h. *P<0.05 versus pcDNA3.1 group (ANOVA and Dunnett's test).

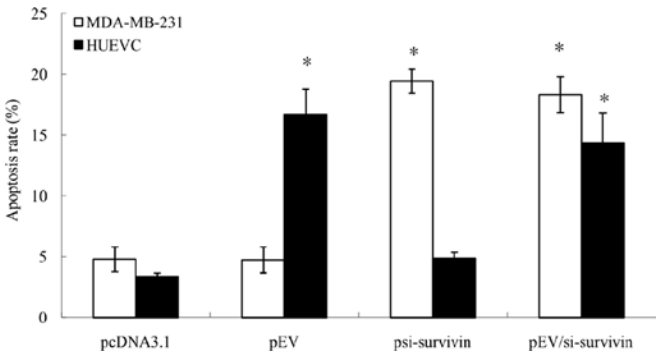

Figure 6. Apoptosis rates of MDA-MB-231 and HUVEC cells transfected with pcDNA3.1, psi-survivin, pEV or pEV/si-survivin for 48 h. *P<0.05 vs. pcDNA3.1 group (ANOVA and Dunnett's test).

Effects of the dual function expressional plasmid pEV/si-survivin on cell apoptosis. Apoptosis of MDA-MB-231 and HUVEC transfected with different plasmids were examined with flow cytometry. It is shown that the apoptotic rate of MDA-MB-231 cells transfected with psi-survivin or pEV/si-survivin were higher than that of cells transfected with pcDNA3.1 (P<0.05), and the apoptotic rate of HUVEC cells transfected with pEV or pEV/si-survivin were higher than that of cells transfected with pcDNA3.1 (P<0.05) (Fig. 6).

Discussion

Thirty years have passed since Folkman proposed that a tumor would die by cutting off its blood supply. The role of angiogenesis in tumor growth and metastasis has been accepted widely. Anti-angiogenesis treatment has attracted research to develop useful approaches for tumor therapy (7). However, theoretically, anti-angiogenesis therapy just inhibits the growth of tumor and could not clear the micrometastases independent of blood supply (8,9). Combination of anti-angiogenesis agents with a direct tumor cell killer has been regarded as an ideal strategy for tumor therapy because of the interdependency between tumor cells and endothelial cells. However, clinical trials have shown that combination of TAI with other methods could cause unexpected toxicity and side-effects (10). Therefore, the exploration of novel combinational strategies has received more and more attentions. It has been known that hypoxia induced by anti-angiogenesis could enhance the sensitivity of tumor cells to apoptosis, so a strategy of combining anti-angiogenesis with apoptosis-induction may achieve synergistic anti-tumor efficiency.

Survivin has been considered as the most effective cell apoptosis inhibitor. Therefore, it serves as an ideal target for anti-tumor therapy (6). It has been reported that survivin is abundantly expressed in neovascular endothelial cells and involved in tumor angiogenesis (11). Endostatin and VEGI are angiogenesis inhibitors that could suppress tumor growth *in vivo* (12). As we have previously constructed an ENDO-$VEGI_{151}$ fusion gene for anti-angiogenesis therapy, survivin was chosen, and the dual function expressional plasmid pCDNA3.1-ENDO-$VEGI_{151}$/survivin-siRNA (pEV/si-survivin) was developed in this study. We screened out an efficient siRNA and synthesized short hairpin RNA (shRNA) sequences to guarantee the specificity and efficiency of siRNA; we then inserted CMV/ENDO-$VEGI_{151}$ and U6/survivin siRNA with BGH poly A between them into pcDNA3.1 vector. Cell transfection experiments proved that the dual function expression plasmid produced functional fusion protein and effective siRNA in breast cancer cells, indicating that the conformation of proteins and the secondary structure of siRNA were not affected by this strategy suggesting such a dual function plasmid was feasible for the preliminary gene therapy.

The choice of target for gene therapy is crucial for the effect of anti-angiogenesis. The limited success of TAI on market indicates that angiogenesis is a multistep and complex regulated process, with distinct factors involved in different stages (13,14); the therapy effect of TAI is quite limited because TAI is a drug which only inhibits one target directly (15); blocking angiogenesis indirectly by inhibiting angiogenesis signal pathways such as VEGF could result in compensatory responses for tumor growth and lead to secondary drug resistance (16). It is important to develop multi-target TAI or combinational TAI with distinct mechanisms to achieve robust effect of anti-angiogenesis (17,18). However, limited progress has been achieved in this field. To improve and replace combinational therapy, modifying anti-angiogenesis gene by genetic engineering to create novel fusion gene with higher activity, more powerful function and stability is receiving more and more attentions (19,20). ENDO-$VEGI_{151}$ is a novel anti-angiogenesis gene from our laboratory (4,5,21,22). Although current understanding of its mechanism is incomplete, we have proved that fusion protein could be detected by anti-endostatin and anti-$VEGI_{151}$, indicating that fusion protein may preserve the biological activities of both proteins. Previous research indicates that endostatin

and $VEGI_{151}$ have distinct mechanisms (23-26). Therefore, fusion protein could block the angiogenesis by promoting apoptosis of endothelial cells, inhibiting the migration and blocking the signals for angiogenesis enhancement. VEGI belongs to TNF superfamily and involves three isoforms: VEGI174, 192, 251; $VEGI_{151}$ is a shared C-terminus segment of all three isoforms with biological activity. It could interact with death receptor-3 (DR3) in cell membranes to sacrifice tumor cells directly, blocking the proliferation of endothelial cells and stimulating the maturation of dendritic cells. It is possible that fusion protein could function as VEGI to sacrifice tumor cells and modulate the immune response. As the normal activity of VEGI may require involvement of the immune system, cytotoxicity and immune modulation has not been observed *in vitro*. We plan to explore the function of VEGI *in vivo* in the future.

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