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Lentivirus-mediated siRNA targeting VEGF inhibits gastric cancer growth *in vivo*

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Abstract. Vascular endothelial growth factor (VEGF), a crucial promoter of blood vessel growth, not only stimulates endothelial cell proliferation, migration and survival, but also increases vascular permeability. The promotion of angiogenesis is a well-known prerequisite for tumor growth, invasion and metastasis. Evidence has shown that VEGF is overexpressed in many types of tumor tissues. Small interfering RNA (siRNA) targeting VEGF may effectively suppress cell proliferation and induce apoptosis in tumor cells. In this study, we aimed to evaluate whether lentivirus-mediated siRNA targeting VEGF inhibits gastric cancer growth *in vivo*. The transfection of VEGF siRNA into SGC7901 human gastric cancer cells downregulated the expression of VEGF and Bcl-2, but upregulated the expression of p21. In a nude mouse model of subcutaneous xenografts, 24 days after VEGF siRNA treatment, the tumor volume and weight were significantly smaller in the VEGF siRNA group compared to the control scrambled siRNA group. Furthermore, the expression of VEGF, sirtuin 1 (SIRT1), survivin and Bcl-2 was downregulated, whereas the expression of p53 and p21 was upregulated in the tumor cells, indicating that VEGF siRNA induced apoptosis in gastric cancer cells by inhibiting SIRT1 expression, leading to p53 transcriptional upregulation and the activation of downstream p21, while suppressing Bcl-2 and survivin expression. Our results demonstrate that lentivirus-mediated siRNA targeting VEGF offers a potential strategy to prevent the growth of gastric cancer.

Introduction

Gastric cancer is one of the most common types of malignant tumor and is the second leading cause of cancer-related mortality worldwide, with an overall survival of approximately 10 months (1). Despite advances in the treatment of gastric cancer, the prognosis of gastric cancer remains poor. It has been acknowledged that gastric carcinogenesis is a multistep process, involving numerous genetic and epigenetic alterations, such as abnormalities in growth factors/receptors, angiogenic factors and cell cycle regulators. These abnormalities also define the biological characteristics of gastric cancer cells, which may serve as therapeutic targets for gastric cancer (2). Thus, additional novel targets for therapeutic development have been identified and are being explored.

A previous study demonstrated that vascular endothelial growth factor (VEGF) is a potent pro-angiogenic factor which not only stimulates endothelial cell proliferation, migration and survival, but also increases vascular permeability. The promotion of angiogenesis is a well-known prerequisite for tumor growth, invasion and metastasis (3). It has been reported that tumor cells secrete VEGF, and this process is necessary for tumor growth (4). Evidence has shown that solid tumors do not grow beyond the volume of 2-3 mm^3 in the absence of neoangiogenesis due to the insufficient diffusion of oxygen and nutrients from the blood vessels. VEGF directly activates the VEGF receptor expressed in tumor cells, leading to an autocrine activation of primary cancer growth and acts as a survival factor for the VEGF receptor-expressing tumor cells (5,6). The inhibition of VEGF has shown promising results in reducing tumor metastasis and/or primary tumor growth in a number of models (7). Blocking of VEGF-D by a mouse monoclonal anti-human-VEGF-D antibody has been shown to be effective in halting primary tumor growth and suppressing local tumor metastasis in a mouse xenograft tumor model. Similarly, neutralizing antibodies against VEGF-receptor (R)3 have been shown to inhibit lymph node metastasis and soluble VEGF-R3, trapping both VEGF-C and VEGF-D, and thus blocking lymphangiogenesis and lymph node metastasis in several models (7). In addition, VEGF-Trap (8), antisense oligonucleotides (9) and RNA interference (RNAi) have also been used to inhibit VEGF in tumor therapy studies. As a result, VEGF may be an important target for tumor therapy.

Small interfering RNA (siRNA) has emerged as a powerful strategy for the investigation of gene expression and function compared with antisense oligonucleotides and neutralizing antibodies. siRNA leads to greater specificity and efficiency when targetting genes, and it may be designed in diverse ways due to its versatility. In addition, siRNA is becoming an

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important tool for the study of biological processes and has the potential for therapeutic applications in human cancer diseases.

In our previous studies (10), we constructed lentivirus-mediated VEGF siRNA, and proved that it can suppress gastric cancer cell growth and induce apoptosis in SGC7901 human gastric cancer cells. The aim of this study was to evaluate whether VEGF siRNA can inhibit gastric cancer growth *in vivo* and elucidate the mechanism of apoptosis induced by VEGF siRNA in SGC7901 cells.

Materials and methods

Cell culture. The SGC7901 human gastric cancer cell line was obtained from the Chinese Type Culture Collection (Shanghai, China). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were incubated at 37˚C in a humidified incubator with 5% CO_2.

Construction of lentiviral vectors and transfection. Lentiviral vectors for human VEGF siRNA encoding a green fluorescent protein (GFP) sequence were constructed by GeneChem Co. (Shanghai, China). The target siRNA sequence was CAGGAGT ACCCTGATGAGATC (GenBank accession no. NM003376). The lentiviral vectors containing VEGF-C siRNA were constructed by ligating the *Hpa*I/*Xho*I digests of pGCL-GFP and the VEGF siRNA PCR products were confirmed by DNA sequencing. The negative control siRNA was provided by GeneChem. The lentivirus-encoded siRNA targeting VEGF and the control were prepared and titered to $2x10^9$ (TU/ml) as previously described (10).

The SGC7901 cells ($2x10^5$) were seeded in 6-well plates overnight before transfection. The virus [multiplicity of infection (MOI)=10] was added to each well containing an enhanced infection solution (EIS; Genechem) and incubated for 8-12 h at 37˚C, followed by incubation for 96 h in complete RPMI-1640 medium. The cells were then harvested for subsequent studies.

Human gastric tumor xenograft mouse model. Four-week-old male Balb/c nude mice were purchased from the Nanjing Peng Sheng Biotechnology Development Company, Nanjing, China (certification no. 2007-004). The mice were housed in a pathogen-free animal facility and were randomly divided into the following 3 groups with 6 mice/group: the blank control, negative control lentivirus (Nc-Lv) and VEGF-RNAi-Lv group. Cells ($2x10^6$/0.2 ml) were subcutaneously injected into the right axillary fossa of each mouse. Once the tumors had emerged, tumor growth was monitored every 3 days and measured in 2 dimensions. Tumor volume was calculated using the formula $V = W^2 \times L/2$, where 'L' and 'W' are the longest and shortest diameters, respectively, and the tumor growth curve was drawn. After a 2-week treatment, the mice were sacrificed, the tumor weight and inhibition rate were evaluated and the tumors were obtained for RT-PCR, western blot analysis and pathological examination. All experimental procedures were carried out according to the National Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee.

Histological sections and staining. For the histological analysis, the tumor tissues were immediately fixed in 4% neutral-buffered formalin and embedded in paraffin after the nude mice were sacrificed and the slides were prepared for hematoxylin and eosin (H&E) staining. After deparaffinization and redehydration the slides were stained with H&E.

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA from tumor cells and tissues was isolated with a total RNA extraction kit (Sangon, Shanghai, China) according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed into cDNA with the PrimeScript RT-PCR kit (Takara Bio, Inc., Dalian, China). Subsequently, 2 μl of cDNA product were subjected to PCR amplification with TaqDNA polymerase (Bioer Technology, Beijing, China) on a thermal cycler. The PCR primers used in this study are shown in Table I. Human GAPDH (hGAPDH) was used as the internal control. The PCR conditions were as follows: 1 cycle of denaturation at 94˚C for 10 min, followed by 30 cycles at 94˚C for 1 min, 55˚C for 1 min and 72˚C for 1 min, before a final extension at 72˚C for 10 min. The PCR products were loaded onto 2% agarose gels and visualized with ethidium bromide under UV light. This experiment was performed 3 times and representative data are shown.

Western blot analysis. Whole-cell protein extracts from SGC7901 cells and the tumor xenografts were prepared with the Tissue and Cell Lysis Solution (BIOS, Beijing, China), according to the manufacturer's instructions. Protein concentrations were determined using a BCA assay kit (BIOS). Samples were adjusted to equal protein concentrations and volume and subjected to SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Following blocking, the membranes were incubated with primary antibodies against SIRT1, p53, p21, Bcl-2 and survivin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and then incubated with HRP-conjugated secondary antibodies. The specific protein was detected using a SuperSignal protein detection kit (Pierce, Rockford, IL, USA).

ELISA. Ninty-six hours post lentivirus infection, the SGC7901 cell supernatants were collected to detect the VEGF levels. The absorbance at 450 nm was measured using a human VEGF ELISA kit (Jingmei Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions and the concentration of VEGF in the supernatants was calculated.

Statistical analysis. The data are expressed as the means ± SD. The significance of the data was determined by one-way ANOVA analysis. A P-value <0.05 was considered to indicate a statistically significant difference. All the statistical analyses were performed with SPSS 13.0 software.

Results

Effect of VEGF siRNA on the expression of VEGF in SGC7901 cells. The silencing effects of VEGF siRNA in SGC7901 cells were evaluated by RT-PCR and ELISA. The RT-PCR results showed that the VEGF mRNA expression in the VEGF siRNA group was significantly suppressed compared to the control groups (Fig. 1). In accordance with this, ELISA indicated

Table I. hGAPDH and VEGF primers used in this study.

Gene		Primer sequence	PCR product length (bp)
hGAPDH	Sense:	5'-GGCTCTCCAGAACATCAT-3'	240
	Antisense:	5'-CACCTGGTGCTCAGTGTA-3'	
VEGF	Sense:	5'-CTACCTCCACCATGCCAAGT-3'	411
	Antisense:	5'-AAATGCTTTCTCCGCTCTGA-3'	

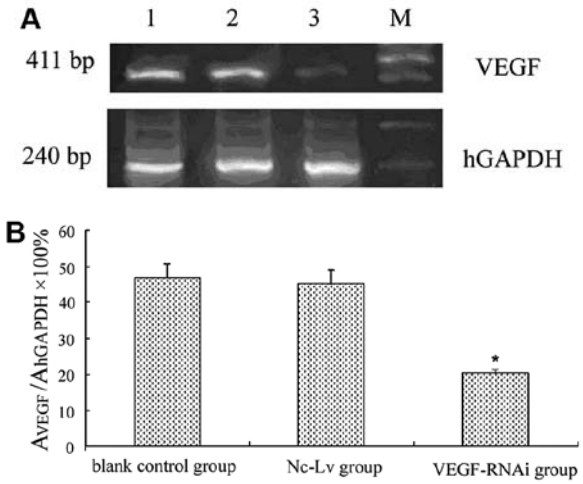

Figure 1. Downregulation of VEGF expression by lentivirus-mediated VEGF siRNA in SGC7901 cells. SGC7901 cells were infected with lentivirus-based VEGF siRNA (VEGF-RNAi-Lv group), negative control siRNA (Nc-Lv group), or mock siRNA (blank control group). (A) RT-PCR analysis demonstrating that the expression of VEGF mRNA was significantly suppressed in the VEGF-RNAi-Lv group (lane 3), compared with the Nc-Lv group (lane 2) or the blank control group (lane 1). (B) Densitometric analysis indicated the decreased VEGF expression in the VEGF-RNAi-Lv group, compared with the Nc-Lv or blank control group (*P<0.05).

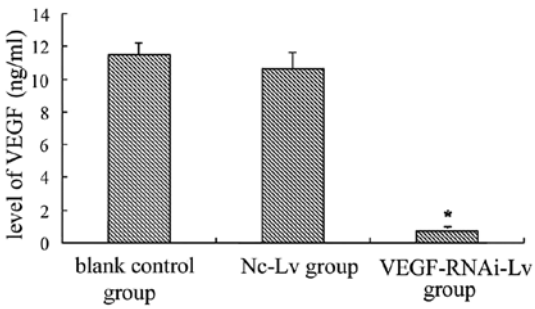

Figure 2. The secretion of VEGF was determined by ELISA in the 3 groups 96 h after VEGF siRNA treatment. The VEGF protein levels in the VEGF-RNAi-Lv group supernatants were decreased compared to those in the control group (*P<0.05).

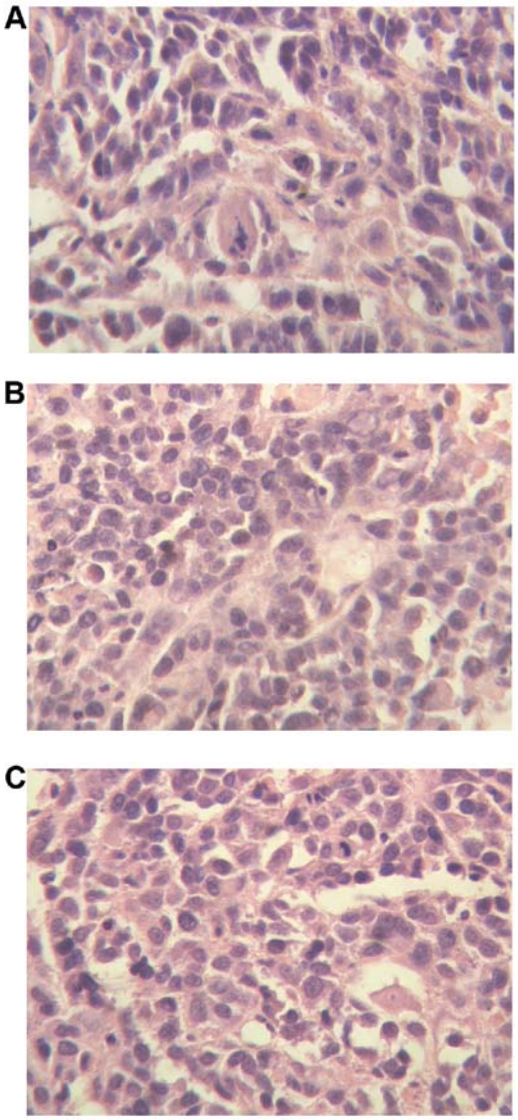

Figure 3. Tumor sections were stained with hematoxylin and eosin (H&E). SGC7901 gatric tumors were established subcutaneously in mice. On day 24, the mice were sacrificed and the tumors were obtained for H&E staining. H&E staining indicated that the xenograft tumors in the 3 groups were poorly differentiated carcinomas, with large areas of necrosis in the (A) blank control group and (B) negative control group. (C) No necrosis was observed in the siRNA-treated group.

that the VEGF protein level in the VEGF siRNA group was decreased by 93.6% (0.699±0.054 ng/ml) compared to the blank control group (11.459±0.782 ng/ml) and the negative control group (10.642±0.981 ng/ml) (Fig. 2).

Histological staining results. To confirm the biological characteristics of the xenograft tumors, H&E staining was performed. The results indicated that the xenograft tumors in the 3 groups were poorly differentiated carcinoma, with large areas of necrosis in the blank control and negative control groups. However, there was no necrosis in the siRNA-treated group (Fig. 3).

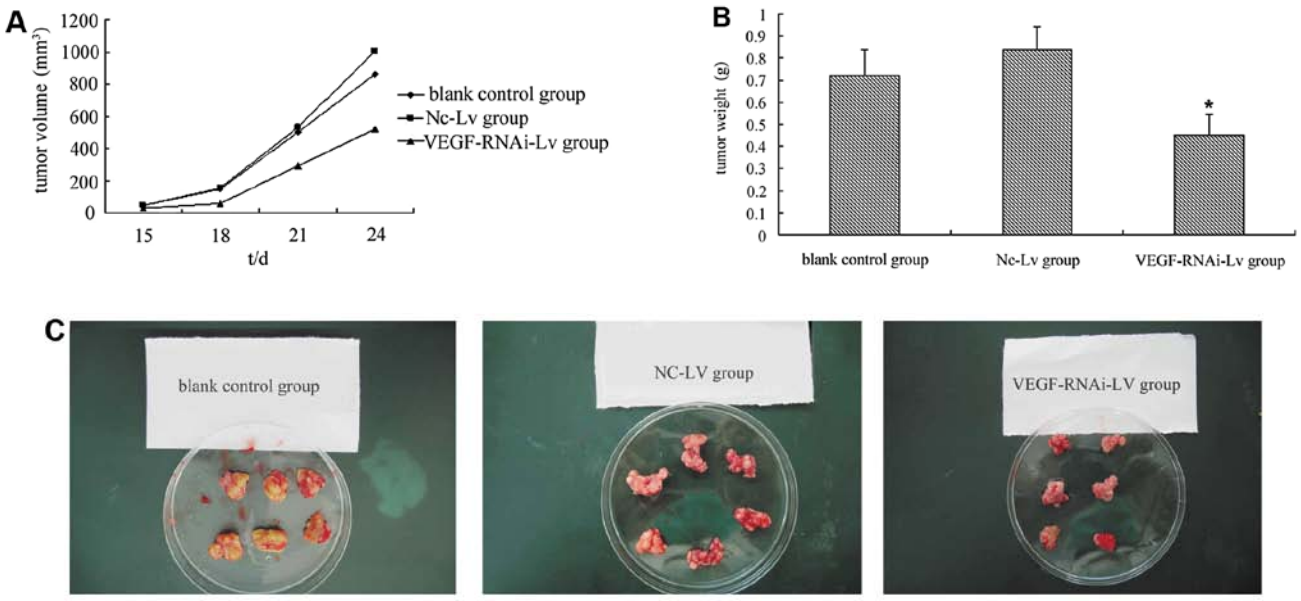

Figure 4. Antitumor effect of VEGF siRNA in the SGC7901 xenografts. SGC7901 gatric tumors were established subcutaneously in mice. Once the tumors were emerged, tumor growth was monitored every 3 days and measured in 2 dimensions. Tumor volume was calculated using the formula $V = W^2 \times L/2$, where 'L' and 'W' are the longest and shortest diameters, respectively. (A) The tumor growth curves were drawn. (B) After a 2-week treatment, the mice were sacrificed and tumor weights were evaluated. *Significantly different from the negative control siRNA ($P<0.05$). (C) The images of the tumors from the 3 groups.

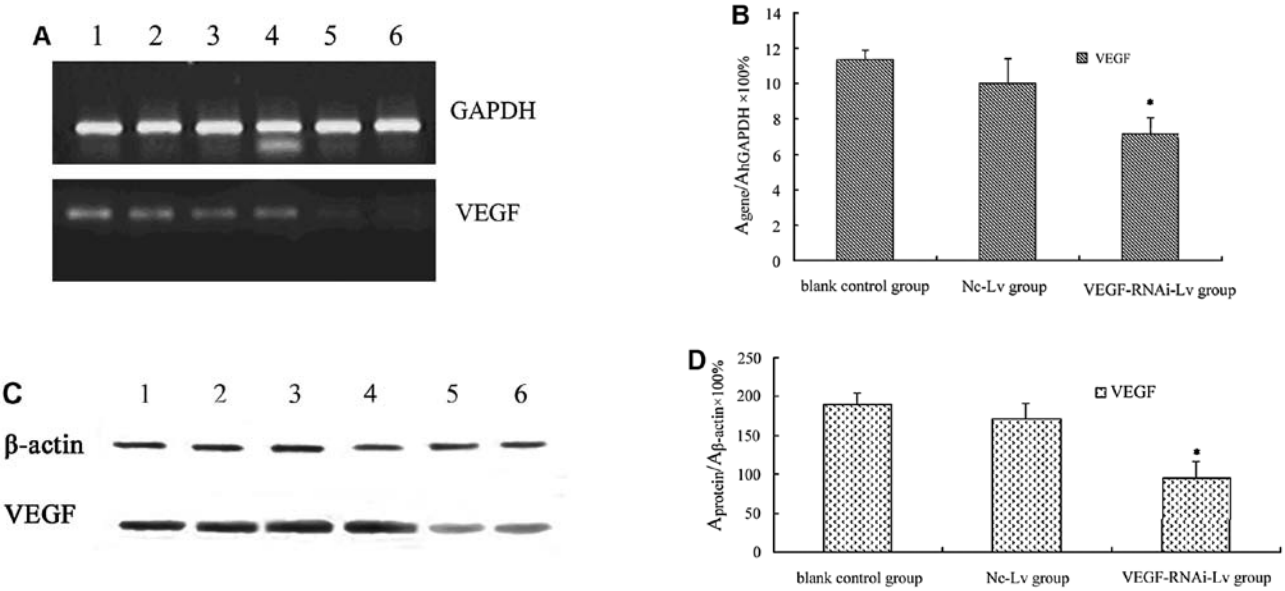

Figure 5. Suppression of VEGF by lentivirus-mediated VEGF siRNA in SGC7901 cell xenografts. Tumors from mice in the blank control (lanes 1 and 2), negative control (lanes 3 and 4) and VEGF siRNA-treated groups (lanes 5 and 6) were homogenized and (A) subjected to RT-PCR analysis to detect VEGF mRNA expression and (C) western blot analysis to detect VEGF protein expression. GAPDH was used as the loading control and β-actin served as the internal control. (B and D) Data shown are the mean values of 3 different experiments from each group. *Significantly different from the negative control siRNA ($P<0.05$).

Influence of VEGF siRNA on gastric tumor growth in xenografts. We then investigated the possibility of using VEGF as a target gene for gastric tumor therapy in the nude mouse tumor xenograft model. The data showed that on day 10, all mice formed a palpable tumor at the sites of injection. Animals were sacrificed on day 24. The mean tumor size of the blank control group was 856.84±89.39 mm^3; the mean tumor size of the Nc-Lv negative control group was 1002.01±142.07 mm^3 and that of the VEGF-RNAi-Lv group was 518.01±67.98 mm^3. There were no significant differences between the blank control group and the Nc-Lv negative control group ($P>0.05$) (Fig. 4). However, the VEGF-RNAi-Lv group showed significant tumor growth suppression compared to the blank control group ($P<0.05$). No toxicity was observed in the mice, as assessed by changes in behavior, appearance or weight.

VEGF siRNA suppresses VEGF expression in tumor xenografts. We then examined the effect of VEGF siRNA on VEGF mRNA *in vivo* by RT-PCR. The VEGF mRNA expression was significantly decreased in the VEGF-RNAi-Lv group compared to the blank control group (Fig. 5A and B). However, the difference between the Nc-Lv group and the blank control group was not

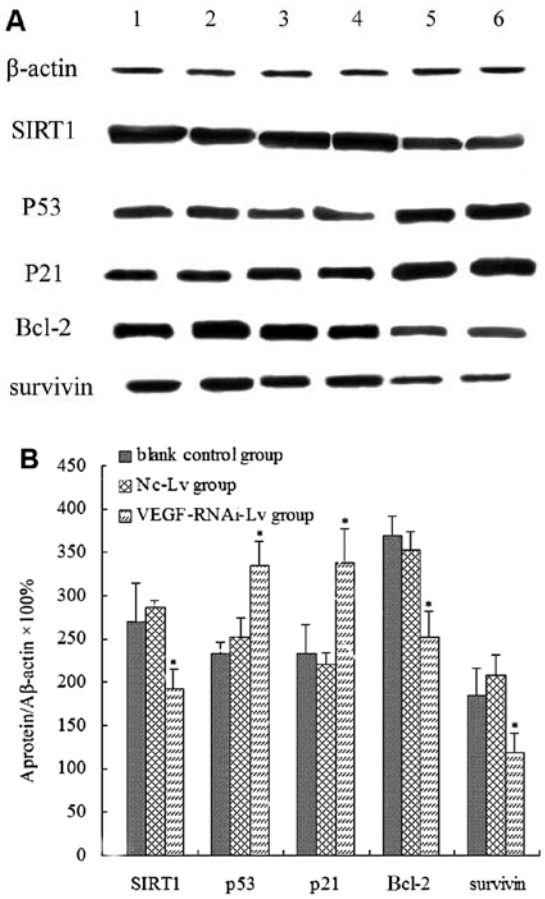

Figure 6. Expression of apoptosis-associated proteins in tumor xenografts. (A) Tumors from mice in the blank control (lanes 1 and 2), negative control (lanes 3 and 4) and VEGF siRNA-treated groups (lanes 5 and 6) were homogenized and subjected to western blot analysis to detect the expression of SIRT1, p53, p21, Bcl-2 and survivin. β-actin served as the internal control. (B) Data shown are the mean values of 3 different experiments from each group. *Significantly different from the negative control siRNA (P<0.05)

statistically significant (P>0.05). We also detected the VEGF protein level of these 3 groups. The results showed that the expression of the VEGF protein in the VEGF-RNAi-Lv group was suppressed significantly. Similar resutls were obsereved with the VEGF mRNA expression (Fig. 5C and D).

VEGF siRNA affects the expression of apoptosis-associated proteins. We show that the downregulation of VEGF by RNAi induces apoptosis in SGC7901 cells. It is known that SIRT1, p53, Bcl-2, survivin and p21 are important proteins associated with apoptosis. Therefore, we detected the levels of these apoptosis-associated proteins by western blot analysis. The results indicated that the expression of SIRT1, Bcl-2 and survivin was downregulated (P<0.05) in the VEGF-RNAi-Lv group; however, the expression of p53 and p21 was upregulated (P<0.05) in the VEGF-RNAi-Lv group. There was no significant difference between the Nc-Lv control group and the blank control group (Fig. 6).

Discussion

The VEGF system is essential for angiogenesis, and VEGF overexpression frequently correlates with increased microvascularity and metastasis and decreased spontaneous apoptosis (11). VEGF is expressed in a variety of cells, including smooth muscle, endothelial, epithelial and a number of cancer cells (12). It has been reported that VEGF is overexpressed in many types of human cancer and cell lines, such as ovarian, breast, lung and gastric cancer. Evidence has shown that VEGF is associated with migration and proliferation and prolongs cell survival in gastric cancer. The effects of VEGF on gastric cancer cell proliferation, differentiation, migration and survival predicates VEGF for potential therapeutic antitumor strategies.

RNA interference is a naturally occurring endogenous regulatory process where short double-stranded RNA (dsRNA) causes sequence-specific post-transcriptional gene silencing. In the cytoplasm, long dsRNAs are cleaved by the endoribonuclease Dicer into siRNA and loaded onto a RNA-induced silencing complex (RISC), causing mRNA degradation and gene silencing (13). Compared with the traditional gene silencing techniques, RNAi is a highly efficient and specific regulatory process. At present, RNAi is widely used to inhibit genes involved in signaling transduction, angiogenesis, drug resistance and regulating apoptosis and cell cycle changes in cancer studies (14).

It has been reported that the downregulation of survivin expression by siRNA may induce apoptosis in pancreatic cancer cells (PC-2 cell line) and enhance its sensitivity to radiotherapy (15). Yao *et al* (16) transfected COX-2 siRNA into the human gastric cancer cell line SGC7901, and showed that the downregulation of COX-2 can significantly inhibit the growth of gastric cancer cells *in vitro* and *in vivo* and suppress the migration and tube formation of human umbilical vein endothelial cells.

Recent studies (17-20) have demonstrated that siRNA targeting VEGF may specifically suppress VEGF expression in breast, prostate, colorectal cancer and a number of other cancer cells. By contrast, data on cancer treatments with lentivirus-mediated VEGF siRNA are rare.

In this study, we applied lentivius-mediated siRNA to inhibit VEGF expression in SGC7901 cells and in a nude mouse model of subcutaneous xenografts. We demonstrate that the silencing of VEGF may suppress the growth of xenograft tumors. Compared to the controls, the VEGF siRNA-treated mice showed a significant suppression of tumor growth. This antitumor efficacy may be attributed to the inhibition of tumor angiogenesis as suggested by a reduced VEGF expression, demonstrating that siRNA targeting VEGF may be an effective pathway for inhibiting gastric tumor growth, and may represent a novel treatment for VEGF overexpression in gastric cancer. This strategy may also have great potential for use in clinical trials for the treatment of gastric tumors.

In a previous study (21), we demonstrated that VEGF silencing induced apoptosis in SGC7901 cells. However, the precise mechanism involved remains unclear. Since p53 is important in cell apoptosis, it is interesting to investigate whether VEGF siRNA has an effect on p53, Therefore, we studied the correlation between p53 and apoptosis induced by VEGF silencing. Our results showed that the expression of the p53 protein increased with siRNA targeting VEGF, which indicated that p53 was involved in the process of SGC7901 cell apoptosis induced by VEGF silencing. It has been acknowledged that p53 translocates into the nucleus to activate p21

while suppressing Bcl-2 and survivin gene expression, leading to cell apoptosis (22,23). Therefore, we detected the expression levels of p21, Bcl-2 and survivin, and the results showed that in the VEGF-RNAi-Lv-treated group, p21 expression was upregulated, while the expression of Bcl-2 and survivin was downregulated, suggesting that in principle, VEGF siRNA efficiently inhibited intracellular signal transduction.

SIRT1, a proto member of the sirtuin family, not only modifies histones through deacetylation but also deacetylates many non-histone proteins that are involved in cell growth, apoptosis, neuronal protection, cell senescence and tumorigenesis (24). p53 has been shown to be a downstream target of SIRT1. SIRT1 physically interacts with p53 and deacetylates the p53 protein with a specificity at its C-terminal Lys382 residue, decreases p53-mediated transcriptional activation and reduces the downstream protein (p21) level (25). It has also been reported that the inhibition of SIRT1 causes p53 hyperacetylation and increases p53-dependent transcriptional activity (23), causing a decrease in cell growth, cell viability and the colony-forming ability of prostate cancer cells (26). Therefore, we further analyzed the expression pattern of the p53 and SIRT1 proteins. Our data showed that SIRT1 was downregulated significantly, while p53 was upregulated significantly after siRNA silencing. The reason may be that SIRT1 blocks the nuclear translocation of p53 via its deacetylation, resulting in the inhibition of p53 function as a transcriptional regulator (27). p53 transcriptional activity increases when SIRT1 is inhibited. The mechanism of VEGF siRNA-induced apoptosis in gastric cancer cells is as follows: siRNA decreases VEGF expression, which inhibits SIRT1 expression, leading to the p53 transcriptional upregulation, the activation of downstream p21 and the suppression of Bcl-2 and survivin. Although VEGF silencing did not reach 100% knockdown in the SGC7901 cells, signal transduction was efficiently inhibited, supporting the effective antitumor response *in vivo*.

Taken together, the results from our study demonstrated that lentivirus-mediated VEGF siRNA inhibited tumor growth in SGC7901 cell xenografts. These data indicate that lentivirus-mediated siRNA targeting VEGF offers a future option against gastric cancer.

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