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Original Article

Lentivirus-mediated RNAi knockdown of VEGFA in RKO colorectal cancer cells decreases tumor formation and growth *in vitro* and *in vivo*

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Abstract: Vascular endothelial growth factors (VEGF) play important roles in angiogenesis, vasculogenesis and endothelial cell growth. In endothelial cancers, secreted VEGF proteins induce endothelial cell proliferation, promote cell migration, inhibit apoptosis and induce blood vessel permeabilization. VEGFA is frequently overexpressed in human colorectal cancers (CRC) and its expression correlates with tumor progression and invasiveness. In this study we examine the effect of knocking down VEGFA expression by infecting RKO colorectal cancer cells with lentiviral particles containing VEGFA-targeting RNAi constructs. We found that suppressing VEGFA dramatically decreased RKO cell proliferation, colony formation, invasion, migration and tumor growth. Furthermore, VEGFA knock-down reduced MAPK pathway signaling and Smac/DIABLO expression. These results suggest that lentivirus-mediated RNAi knock-down of VEGFA could be an effective therapy for the treatment of CRC.

Keywords: vascular endothelial growth factor A, shRNA, knock-down, colorectal cancer, lentivirus

Introduction

Surgical treatment combined with chemotherapy or radiotherapy has significantly improved clinical outcomes for patients with colorectal carcinoma (CRC). In spite of these advancements, better treatment options are needed because CRC is still associated with a high rate of mortality in men and women worldwide [1].

Vascular endothelial growth factor A (VEGFA) plays a critical role in the growth of primary tumors and tumor-related angiogenesis. VEGFA is secreted by most solid tumors [2-4] and, via its interaction with its receptor VEGFR-2, promotes endothelial cell proliferation, invasion, migration, and survival, as well as vessel permeability. In human CRC, high levels of VEGFA correlate with tumor progression and invasiveness [5], and VEGFA expression in CRC has been found to be a strong prognostic indicator of metastasis and survival [6].

Bevacizumab, a monoclonal antibody against VEGFA, has been approved in combination with platinum-based chemotherapy for the treatment of patients with advanced CRC [7]. However, clinical trial results have shown that while bevacizumab may be effective as adjuvant therapy, many tumors fail to respond from the outset or after a few months of treatment and tumor regression occurs in some cases. Furthermore, some investigators have observed resistance to this therapy owing to the ability of tumor cells to up-regulate the expression of VEGFA and thereby overcome the anti-angiogenic effect [8].

As an alternative to immunologic suppression of VEGFA activity, a potential and promising strategy is to silence VEGFA gene expression at the messenger RNA (mRNA) level. The purpose of the current study was to use RNA interference (RNAi) technology in order to explore the effects of VEGFA knock-down on RKO cell proliferation, tumor formation and tumor cell signaling. Our results demonstrate that lentivirus-mediated delivery of short hairpin RNA (shRNA) in RKO cells significantly down-regulates the expression of VEGFA and dramatically decreases tumor cell

proliferation, colony formation, invasion and migration. Furthermore, our results suggest that knocking down VEGFA suppresses tumor progression in part by reducing the activity of the MEK/ERK-Smac/DIABLO signaling pathway in RKO cells.

Materials and methods

Patients and tissue samples

Sixteen colorectal tumor samples were obtained with informed consent from patients at the Department of Surgical Oncology, First Affiliated Hospital of Xiamen University (Xiamen, China). For RNA extraction, tissues were immediately snap-frozen in liquid nitrogen and stored at −80°C. The Surgical Oncology Ethics Committee approved this study.

Cell cultures

Human RKO cells were obtained from the ATCC Cell Biology Collection and cultured in DMEM (Dulbecco's modified Eagle medium) supplemented with 4.5 g/l glucose, 10% FBS, 4 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. RKO cells were seeded at 1×10^5 cells per well in 6-well plates and incubated overnight at 37°C with 5% CO_2.

VEGF siRNA lentivirus package

Two short hairpin RNAs (shRNA-1, 5'- GACAAGAAAAUCCCUGUGG -3' and shRNA-2, 5'-AAGUGGUGAAGUUCAUGGAUG -3') targeting the VEGFA ORF (Genbank no. NM_001025368) and a non-targeting RNA sequence serving as a negative control (Scr-shRNA) were cloned into the pENTR/U6 entry vector (Invitrogen). A shRNA-expressing lentivector was then constructed following the manufacturer's instructions (BLOCK-iT™ U6 RNAi Entry Vector Kit, Invitrogen) and packaged using the Virapower packaging mix (Invitrogen). Virus particles were titered using 293FT cells, as described in the Invitrogen Instruction manual for Virapower Lentiviral Expression Systems (Version E).

Lentiviral vector transduction

RKO cells were transduced with shRNA-expressing lentivirus at a multiplicity of infection (MOI) of 20 particles/cell in serum-free growth medium containing 6 μg/mL polybrene at 37°C, 5% CO_2 in 96-well or 6-well plates. GFP expression was observed by fluorescent microscopy three to six days post-transduction, and cells were harvested 7 days for RT-PCR or 10 days for Western blot post-transduction.

RNA extraction and real-time RT-PCR

RNA from tissue samples or cell cultures was extracted using Trizol reagent (Gibco) according to the manufacturer's instructions. First strand cDNA was synthesized from 5 μg of total RNA using SuperScript II RT 200 U/μl (Invitrogen). VEGFA and Smac/Diablo mRNA expression was evaluated by RT-PCR on an ABI 7500 (Applied Biosystems) with SYBR Green PCR core reagents. Actin was used as the input reference. Primers used were: VEGFA, 5'- CAACATCACCATGCAGATTATGC -3' (forward) and 5'-TCGGCTTGTCACATTTTTCTTGT-3' (reverse); and actin 5'-GGCAGTCCACAGCAAGAAC-3' (forward) and 5'-CACCCTGTTGCTGTAGCCAAA-3' (reverse). RT-PCR analysis was performed in triplicate and results are presented as Ct values, defined as the threshold PCR cycle number at which an amplified product is first detected. The mean Ct was calculated, and ΔCt was determined as the mean Ct for the target gene minus the mean Ct for actin.

Western blot and ELISA

RKO cells were collected, washed with PBS and then lysed in lysis buffer containing 100 mM Tris−HCl, pH 7.5, 0.5% NP-40 and protease inhibitor cocktail. The supernatant was collected after centrifugation and protein concentrations were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL). Cell lysate samples (40 μg) were separated by SDS-PAGE, blotted onto a nitrocellulose membrane and incubated with a primary antibody for 4 hours, including monoclonal antibodies against Smac/Diablo and p-ERK. Samples were then incubated with a secondary antibody for an hour and detected using an enhanced chemiluminescence kit (Pierce, Rockford, IL). Beta-actin was used as a loading control. VEGFA ELISA was performed using a VEGFA-specific ELISA kit (R&D Systems) following the manufacturer's instructions. Each experiment was performed in triplicate.

Cell proliferation and colony formation assays

Briefly, cells were seeded at an initial density of

3x10^4 cells/ml in a 96-well plate for 24h. After lentivirus infection, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added into each well to a final concentration of 0.5 mg/ml. The formazan precipitate was collected, dissolved in dimethylsulfoxide and measured using an ELISA reader (Bio-Rad, U.S.A.) at a wavelength of 490 nm. Analysis of colony formation in Matrigel was performed following the manufacturer's instructions (BD Biosciences). RKO cells were seeded at a density of 0.5 × 10^5 cells/well in growth medium and incubated for 24 h at 37°C, after which images were captured. All studies were performed in triplicate.

Cell migration assays

Cell migration was evaluated using QCM-Collagen I Quantitative cell migration assay kit (ChemiconA). Cells from the various treatment groups were added to the top compartment and cell migration assays were carried out at 37°C for 12 hours. Cells that remained in the top compartment were removed with cotton swabs and insert filters were fixed with 3% paraformaldehyde and stained with 1% (v/v) crystal violet in acetic acid. The number of migrated cells was determined using Image Pro-Plus 3D Imaging System (Apparatus Co., Ardmore, PA).

Cell cycle analysis

Cells were treated as indicated. Cell cycles were analyzed using Multicycle-DNA Cell Cycle Analyzing Software (BD Biosciences). In brief, the uninfected and infected cells were collected and washed with PBS. After centrifuge, the cells were fixed in 70% ethanol at 4°C for half an hour. Prior to analysis, Cells were collected by centrifugation and washed with PBS, then resuspended in PBS containing 20 μg/ml RNase A and 50 μg/ml PI, and then incubated at 4°C for 30 min in the dark. Cells were analyzed by flow cytometry and the data were analyzed with FlowJo 7.6.1 software.

In vivo tumor formation

Cultured RKO cells were harvested with trypsin-EDTA, washed and resuspended in PBS. C57Bl/6 nude mice (10 weeks old) were subcutaneously injected along the dorsal midline with 5 × 10^6 RKO cells (in 100 ml PBS) per site. Tumor sizes were measured using calipers up to 27 days after injection.

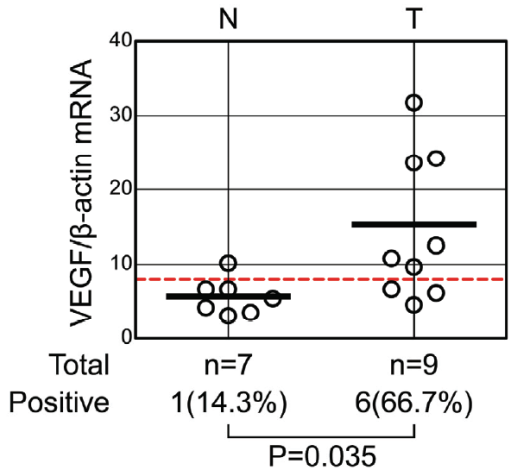

Figure 1. VEGFA expression in malignant colon tumor tissues. Graph shows VEGFA mRNA levels relative to β-actin in total RNA samples prepared from normal (N) or tumour (T) colon tissue from patients with colorectal cancer. The cut-off point was determined using ROC (receiver operating characteristics) curve-based statistical analysis; the number of samples above the ROC cut-off in each group is indicated (Positive).

Statistical analysis

Statistical analyses were performed using SPSS-12.0 software. All results are expressed as mean ± standard error. Each experiment was repeated 3 times, unless otherwise indicated. Comparisons between groups were done using the unpaired Student's *t*-test. A p-value of < 0.05 was considered statistically significant for all analyses.

Results

Elevated expression of VEGFA in CRC tumor samples

To explore the role of VEGFA in human colorectal tumors, we began by analyzing the expression of VEGFA in human colorectal cancer tissues. Colorectal tumor samples and healthy tissue were obtained from 16 node-positive patients, and VEGFA expression levels were examined by RT-PCR. We observed elevated levels of VEGFA mRNA in tumors compared with corresponding healthy colon tissues (P = 0.035, **Figure 1**), confirming the positive relationship between VEGFA expression levels and tumor malignancy in colon cancer.

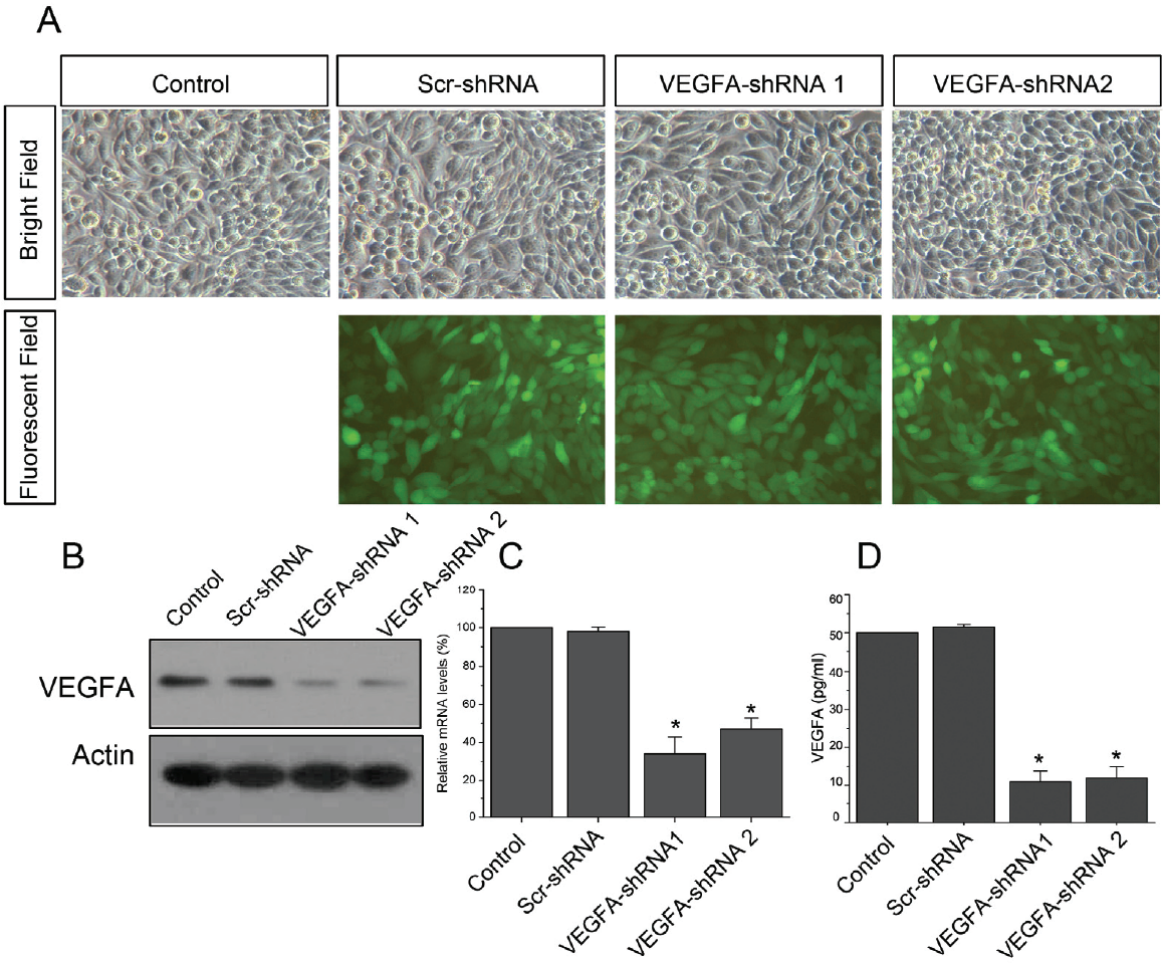

Figure 2. Knock-down of VEGFA in RKO cells by lentivirus-mediated RNAi silencing. (A) RKO cell cultures 4 days after transduction with lentivirus containing either negative-control shRNA (Scr-shRNA) or VEGFA-targeting shRNA constructs. GFP expression (bottom panels) indicates that the cell has been infected with shRNA. (B) Western blot showing VEGFA protein levels in cultures treated as in (A). (C) VEGFA mRNA levels in cultures treated as in (A) measured by RT-PCR. (D) Concentrations of secreted VEGFA in the medium of cultures treated as in (A) as determined by ELISA. *p<0.05 relative to control; n=3 per group.

Effect of VEGFA knockdown on cell proliferation and tumor growth

RKO is a poorly differentiated colon carcinoma cell line that constitutively expresses high levels of VEGFA. To suppress VEGFA expression, RKO cells were infected with lentivirus containing shRNAs directed against VEGFA (VEGFA-shRNA1 and VEGFA-shRNA2) or a non-targeting negative control shRNA (Scr-shRNA). The lentivirus infection rate was quite high as measured by GFP fluorescence (**Figure 2A**). VEGF mRNA expression was then measured by RT-PCR. As shown in **Figure 2C**, endogenous VEGFA mRNA was significantly reduced in VEGFA-shRNA expressing cultures compared with control cultures. We further analyzed the degree of suppression by measuring VEGFA protein levels by Western blot and ELISA. The results showed that cellular levels of VEGFA protein (**Figure 2B**) and the concentration of secreted VEGFA (**Figure 2D**) were significantly reduced by VEGFA-shRNA lentivirus treatments. As determined by RT-PCR, the expression of the VEGFA receptor VEGFR-2 was not affected by VEGFA-shRNA expression (data not shown).

We next investigated cancer cell proliferation and invasiveness, both hallmarks of tumor progression, in RKO cells treated with VEGFA-

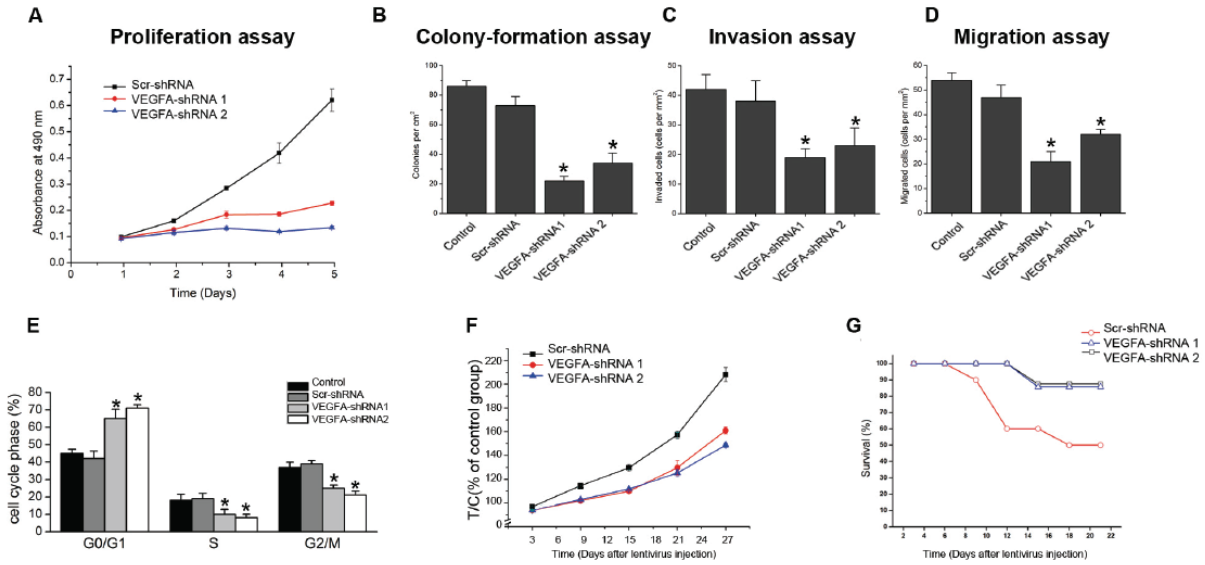

Figure 3. Effect of VEGFA suppression on RKO cell growth *in vitro* and *in vivo*. (A) Growth rates of control and VEGFA-shRNA infected cells measured by MTT assay. (B) Formation of control and VEGFA-shRNA expressing RKO cell colonies on soft agar plates. Four weeks after seeding, colonies with a diameter of > 100 μm were counted under a microscope. (C) Quantification of the volume of control and VEGFA-shRNA expressing RKO cells in the matrigel matrix-coated upper chamber of 96-well plates 24 h after being seeded with RKO cells on filters with 8 μm pores. (D) Quantification of the volume of control and VEGFA-shRNA expressing RKO cells in the uncoated upper chamber of 96-well plates 24 h after being seeded with RKO cells on filters with 8 μm pores. (E) FACS analysis showing the proportion of control and VEGFA-shRNA expressing RKO cells in different phases of the cell cycle. (F) Tumor growth curves in nude mice injected with RKO cell masses. Tumor sizes are expressed as the percentage of the mean control tumor size on day 3. (G) Survival of nude mice treated as in (F). *p<0.05 relative to control; n=3 per group.

shRNA. Under normal culture conditions, VEGFA down-regulation inhibited cell proliferation rates, as indicated by a lower growth rate in VEGFA-shRNA groups compared to control cells (**Figure 3A**). The capacity to form colonies in soft agar was also inhibited in cells with reduced levels of VEGFA (**Figure 3B**), indicating that VEGFA down-regulation inhibits cancer cell growth.

Next, to examine the invasive and metastatic potential of these cells, invasion assays showed that VEGFA knock-down significantly decreased the number of cells that penetrate Matrigel-coated membranes (**Figure 3C**). We also found that VEGFA knock-down decreased the migratory ability of RKO cells (**Figure 3D**). The results of these assays indicate that the proliferation rate and metastatic potential of the cancer cells was significantly inhibited by VEGFA knock-down.

We then studied the effects of VEGFA down-regulation on cell cycle rates by fluorescence activated cell sorter analysis (FACS). The results showed that almost 70% of VEGFA-shRNA expressing cells were arrested in G0/G1-phase compared to 45% of control cells ($P < 0.05$, **Figure 3E**) with an associated decrease in the percentage of cells in S and G2/M phases, indicating that suppressing VEGFA levels reduces the rate of cell proliferation.

To further confirm the suppressive effect of VEGFA knock-down on tumor progression *in vivo*, we stably transfected RKO cells with VEGF-shRNA-1 or -2 and subcutaneuously xenografted cell masses (5×10^6 cells) into nude mice and measured tumor sizes up to 27 days after implantation. Consistent with our *in vitro* proliferation data, significantly less tumor growth was observed in mice injected with cells expressing VEGFA-shRNA-1 or -2 compared to control and Scr-shRNA tumors. As shown in **Figure 3F**, VEGFA-shRNA expressing tumors had a 41% T/C ratio (mean treated tumor volume to mean control tumor volume) after 27 days. Furthermore, mice injected with tumors expressing VEGFA-shRNA had a significantly higher survival rate than controls (**Figure 3G**). These results

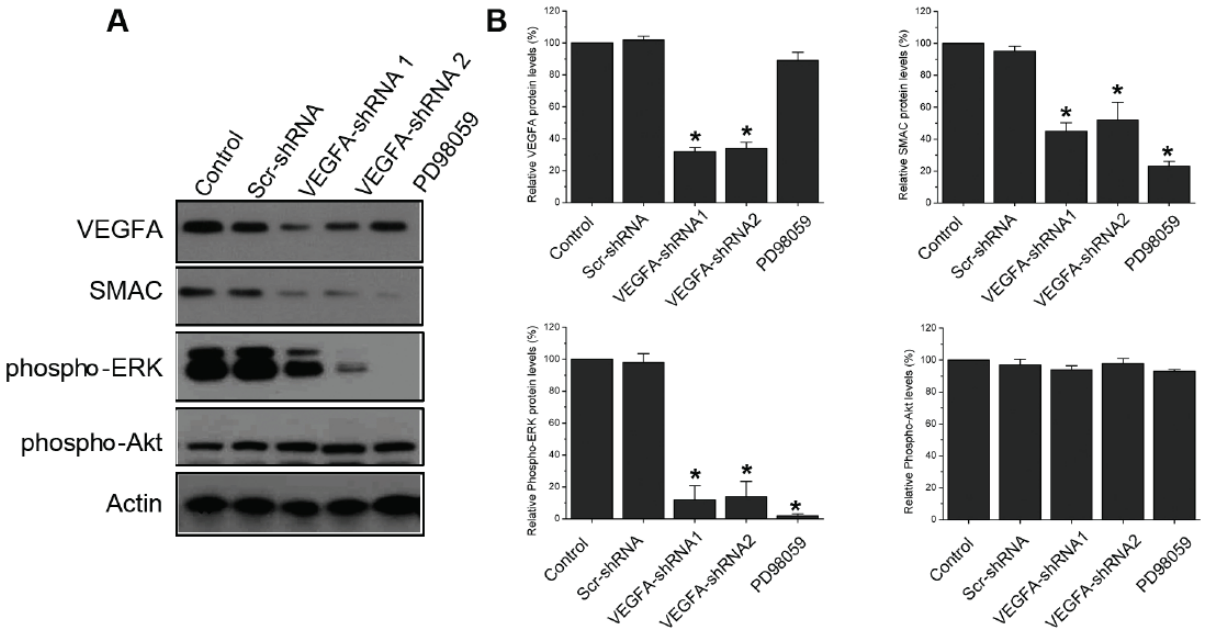

Figure 4. Effect of VEGFA suppression on the expression of downstream signaling factors. (A) Representative Western blot showing levels of VEGFA, Smac/DIABLO, phosphorylated ERK and phosphorylated Akt in RKO cells treated as indicated. Actin was included as a loading control. (B) Quantification of results in (A). Values are normalized to control. *p<0.05 relative to control; n=3 per group.

suggest that knocking down VEGFA suppresses tumor progression both *in vitro* and *in vivo*.

Effect of VEGF knockdown on downstream signaling

To investigate the mechanisms underlying the growth suppression effect of VEGFA knock-down, we analyzed the expression of factors involved in angiogenesis and cell growth. VEGFA is an important promoter of angiogenesis in cancers and is known to activate several mitochondrial apoptosis pathway associated proteins, such as Bcl-2 and Mcl-1. We investigated whether VEGF could also regulate Smac/Diablo, another important mitochondrial protein. In VEGFA-depleted RKO cells, Smac/Diablo mRNA (data not shown) and protein (**Figure 4A** and **B**) levels were significantly down-regulated. Since the MEK/ERK pathway is tightly associated with cell growth [9, 10] and VEGFA has been reported to activate and phosphorylate MEK and ERK [11], we further investigated whether the MEK/ERK pathway was involved in the down-regulation of Smac/Diablo. As shown in **Figure 4A** and B, levels of phosphorylated ERK were reduced following VEGFA knock-down, correlating with reduced levels of Smac/Diablo. Specifically inhibiting MEK and ERK activity with PD98059 also reduced Smac/Diablo protein levels without affecting VEGFA. We also measured the level of phospho-Akt to assess PI3K-Akt pathway signaling and found that Akt activation is not affected by VEGFA suppression. These results demonstrated that VEGFA acts upstream to promote Smac/Diablo expression via activation of the MEK/ERK pathway. To evaluate the specificity of the VEGFA-shRNA-1 and -2 knock-down, and to examine the effect upon downstream pathways, we analyzed protein levels of four other genes related to cell growth (survivin, cyclinE, cyclinD1 and c-myc). The results showed that knocking down VEGFA did not affect the expression of these genes in RKO cells (data not shown). Taken together, these data suggest that suppressing VEGFA with short interfering RNA constructs in RKO colorectal cancer cells decreases tumor formation and proliferation potential by suppressing the activity of the MEK/ERK-Smac/Diablo pathway.

Discussion

Although surgical treatment combined with chemotherapy or radiotherapy has significantly improved clinical outcomes for patients with CRC,

the disease is still associated with a high rate of mortality in men and women worldwide [1]. VEGFA is a critical effector of tumor angiogenesis and vasodilatation, and is expressed in many solid tumors [15-16]. However, the role of VEGFA in those solid tumors is poorly understood. Our findings revealed that VEGFA is expressed at high levels in colon cancer tissues from patients, which led us to hypothesize that VEGFA stimulates tumor growth not only via its actions on endothelial cells but also by directly stimulating its receptor VEGFR-2 on tumor cells [17-18].

Using lentivirus-mediated shRNA silencing, we stably suppressed the expression of VEGFA both at the mRNA and protein levels in cultured RKO colorectal cancer cells and significantly decreased the amount of secreted VEGFA proteins, as shown by ELISA. Treating RKO colorectal cancer cells with VEGFA shRNA decreased cell cycle progression, proliferation and invasiveness *in vitro*, demonstrating that autocrine VEGFA signaling plays a critical role in colon cancer cell growth. Furthermore, we show that when injected into nude mice, VEGF-shRNA expressing RKO cells fail to grow as quickly as control RKO cell masses, leading to higher survival rates. These results provide a foundation for further study into the clinical potential of lentiviral-mediated delivery of VEGFA RNAi therapies for the treatment of CRC.

The advantage of using lentivirus-mediated RNAi as an anti-angiogenic therapeutic tool is its ability to specifically suppress target genes long term. This is in contrast to other methods for targeting VEGFA, such as neutralizing antibodies, which do not stably reduce the expression and which the tumor may overcome by up-regulating VEGFA transcription and translation [8],[19]. Furthermore, lentiviral-mediated RNAi has advantages over other RNAi delivery methods, such as injecting naked siRNA, including a higher infection rate and stability, and duplication-defective lentivirus particles are bio-safe and are currently in use in clinical trials. Thus, lentivirus-mediated RNAi VEGFA therapy may provide the greatest therapeutic benefit for the treatment of CRC [20]. However, these treatments, like most current gene therapy experiments, show promise but are yet to be established as safe and effective in controlled human studies. Furthermore, the use of lentivirus in humans is in black future due to critical safety concerns such as the generation of replication competent virus through recombination with related primate viruses. Therefore, a gene transfer system based on non-primate lentiviruses, such as feline immunodeficiency virus (FIV) or adeno-associated viruses, may circumvent such concerns.

In this study we also show that VEGFA knockdown down-regulates the expression of the pro-apoptotic protein Smac/DIABLO by reducing MEK/ERK pathway activity. Furthermore, although VEGF-shRNA arrested the cell cycle in G0/G1 phase, this was not due to changes in cyclin D1 and c-Myc transcription. Smac/DIABLO functions as an endogenous inhibitor of IAP genes (Inhibitors of Apoptosis), and is believed to neutralize IAP function, liberating caspase activity and promoting apoptosis. Thus, the down-regulation of Smac/DIABLO downstream of VEGFA suppression would be expected to inhibit caspase activity and promote apoptosis.

In conclusion, this study further highlights the crucial role of VEGFA in promoting colorectal cancer cell proliferation and tumor growth and demonstrates that suppressing VEGFA with targeted shRNA constructs delivered to tumor cells via lentivirus is an effective method to reduce VEGFA activity long term. The production of VEGFA is associated with tumor progression and poor prognosis in CRC [23-26], and strategies targeting VEGFA activity improve survival in colon and lung cancer [27]. Therefore, lentivirus-mediated VEGFA knock-down could prove to be an effective and targeted anti-angiogenic therapy.

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References

[1] Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. CA Cancer J Clin 2007;57:43-66.

[2] Tomisawa M, Tokunaga T, Oshika Y, Tsuchida T,

Fukushima Y, Sato H, Kijima H, Yamazaki H, Ueyama Y, Tamaoki N, Nakamura M. Expression pattern of vascular endothelial growth factor isoform is closely correlated with tumour stage and vascularization in renal cell carcinoma. Eur J Cancer 1999;35:133-137.

[3] Volm M, Koomagi R, Mattern J. Prognostic value of vascular endothelial growth factor and its receptor Flt-1 in squamous cell lung cancer. Int J Cancer 1997; 74:64-68.

[4] Yoshiji H, Gomez DE, Shibuya M, Thorgeirsson UP. Expression of vascular endothelial growth factor, its receptor, and other angiogenic factors in human breast cancer. Cancer Res 1996;56:2013-2016.

[5] Cascinu S, Staccioli MP, Gasparini G, Giordani P, Catalano V, Ghiselli R, Rossi C, Baldelli AM, Graziano F, Saba V, Muretto P, Catalano G. Expression of vascular endothelial growth factor can predict event-free survival in stage II colon cancer. Clin Cancer Res 2000;6:2803-2807.

[6] Ishigami SI, Arii S, Furutani M, Niwano M, Harada T, Mizumoto M, Mori A, Onodera H, Imamura M. Predictive value of vascular endothelial growth factor (VEGF) in metastasis and prognosis of human colorectal cancer. Br J Cancer 1998;78:1379-1384.

[7] Ortega J, Vigil CE, Chodkiewicz C. Current progress in targeted therapy for colorectal cancer. Cancer Control 2010,17:7-15.

[8] Viloria-Petit A, Crombet T, Jothy S, Hicklin D, Bohlen P, Schlaeppi JM, Rak J, Kerbel RS. Acquired resistance to the antitumor effect of epidermal growth factor receptor-blocking antibodies in vivo: a role for altered tumor angiogenesis. Cancer Res 2001;61:5090-5101.

[9] Chai J, Du C, Wu JW, Kyin S, Wang X, Shi Y. Structural and biochemical basis of apoptotic activation by Smac/DIABLO. Nature 2000;406:855-62.

[10] Chang F, Steelman LS, Shelton JG, Lee JT, Navolanic PM, Blalock WL, et al. Regulation of cell cycle progression and apoptosis by the Ras/Raf/MEK/ERK pathway (Review). Int J Oncol 2003;22:469-80.

[11] Gupta K, Kshirsagar S, Li W, Gui L, Ramakrishnan S, Gupta P, Law PY, Hebbel RP. VEGF prevents apoptosis of human microvascular endothelial cells via opposing effects on MAPK/ERK and SAPK/JNK signaling. Exp cell Res 1999;247:495-504.

[12] Ferrara N, Alitalo K. Clinical applications of angiogenic growth factors and their inhibitors. Nat Med 1999;5:1359-64.

[13] Ria R, Roccaro AM, Merchionne F, Vacca A, Dammacco F, Ribatti D. Vascular endothelial growth factor and its receptors in multiple myeloma. Leukemia 2003;17:1961-68.

[14] Ohta Y, Endo Y, Tanaka M, Shimizu J, Oda M, Hayashi Y, Watanabe Y, Sasaki T. Significance of vascular endothelial growth factor messenger RNA expression in primary lung cancer. Clin Cancer Res 1996;2:1411-1416.

[15] Tian X, Song SM, Wu J, Meng L, Dong ZW, Shou CC. Vascular endothelial growth factor: acting as an autocrine growth factor for human gastric adenocarcinoma cell MGC803. Biochem Biophys Res Commun 2001;286:505-512.

[16] Price DJ, Miralem T, Jiang SX, Steinberg R, Avraham H. Role of vascular endothelial growth factor in the stimulation of cellular invasion and signaling of breast cancer cells. Cell Growth Differ 2001;12:129-135.

[17] Jackson MW, Roberts JS, Heckford SE, Ricciardelli C, Stahl J, Choong C, Horsfall DJ, Tilley WD. A potential autocrine role for vascular endothelial growth factor in prostate cancer. Cancer Res 2002;62:854-859.

[18] Masood R, Cai J, Zheng T, Smith DL, Hinton DR, Gill PS. Vascular endothelial growth factor (VEGF) is an autocrine growth factor for VEGF receptor–positive human tumors. Blood 2001;98:1904-1913.

[19] Duxbury MS, Matros E, Ito H, Zinner MJ, Ashley SW, Whang EE. Systemic siRNAmediated gene silencing: a new approach to targeted therapy of cancer. Ann Surg 2004;240:667-674.

[20] iscornia G, Singer O, Ikawa M, Verma IM. A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. Proc Natl Acad Sci U S A 2003;100:1844-8.

[21] Carroll VA, Ashcroft M. Role of hypoxia-inducible factor (HIF)-1alpha versus HIF-2alpha in the regulation of HIF target genes in response to hypoxia, insulin-like growth factor-I, or loss of von Hippel-Lindau function: implications for targeting the HIF pathway. Cancer Res 2006;66:6264-70.

[22] Oda Y, Yamamoto H, Tamiya S, Matsuda S, Tanaka K, Yokoyama R, et al. CXCR4 and VEGF expression in the primary site and the metastatic site of human osteosarcoma: analysis within a group of patients, all of whom developed lung metastasis. Mod Pathol 2006;19:738-45.

[23] Ishigami SI, Arii S, Furutani M, Niwano M, Harada T, Mizumoto M, Mori A, Onodera H, Imamura M. Predictive value of vascular endothelial growth factor (VEGF) in metastasis and prognosis of human colorectal cancer. Br J Cancer 1998;78:1379-1384.

[24] De Vita F, Orditura M, Lieto E, Infusino S, Morgillo F, Martinelli E, Castellano P, Romano C, Ciardiello F, Catalano G, Pignatelli C, Galizia G. Elevated perioperative serum vascular endothelial growth factor levels in patients with colon carcinoma. Cancer 2004;100:270-278.

[25] Lee JC, Chow NH, Wang ST, Huang SM. Prognostic value of vascular endothelial growth factor expression in colorectal cancer patients. Eur J Cancer 2000;36:748-753.

[26] Takahashi Y, Kitadai Y, Bucana CD, Cleary KR,

Ellis LM. Expression of vascular endothelial growth-factor and its receptor, KDR, correlates with vascularity,metastasis, and proliferation of human colon-cancer. Cancer Res 1995;55:3964-3968.

[27] Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004; 350:2335-2342.