

VEGF-siRNA SILENCING INDUCES APOPTOSIS, INHIBITS PROLIFERATION AND SUPPRESSES VASCULOGENIC MIMICRY IN OSTEOSARCOMA *IN VITRO*

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Aim: To inhibit the gene expression of vascular endothelial growth factor (VEGF) with the RNA interference (RNAi) technique and explore its influence on the apoptosis, proliferation, vasculogenic mimicry of osteosarcoma *in vitro*. **Methods:** pSilencer-VEGF plasmid transfected into osteosarcoma cell line of MG63 was recombinated by gene cloning, and VEGF expression was examined by Western blotting after transfection. The biological features of transfected MG63 including proliferation, apoptosis and vasculogenic mimicry, were assessed by MTT assay, flowcytometer, annexin V-FITC/PI, light microscopy and HE staining. **Results:** The sequence of pSilencer-VEGF plasmid was confirmed by DNA sequencing. Sequence-specific siRNA targeting VEGF significantly decreases its expression in MG63 cells. Cell growth was inhibited and early apoptosis was induced compared with non-siRNA transfected cells. Decreased VEGF expression was associated with reduced vasculogenic mimicry of MG63 cells. **Conclusions:** VEGF gene might be closely related to the apoptosis and proliferation of osteosarcoma cells, and appears to be crucial for formation of vasculogenic mimicry in osteosarcoma. Inhibition of VEGF expression by sequence-specific siRNA may become a novel approach for the treatment of VEGF-enriched neoplasma such as osteosarcoma.

Key Words: vascular endothelial growth factor, osteosarcoma, vasculogenic mimicry, RNA interference.

Osteosarcoma poses serious hazard to children's health. It affects 5.6 per million children younger than 15 years annually, with a peak incidence occurring in the second decade of life [1]. It arises from mesenchymal bone-forming cells and most commonly affects the metaphyseal growth plate of long bones. The formation of new blood vessels is a fundamental biological process that plays a critical role in the pathogenesis of osteosarcoma.

Vascular endothelial growth factor (VEGF) has been demonstrated to be a key mediator of angiogenesis in many models. Previous studies showed that high indexes of VEGF expression were significantly associated with osteosarcoma recurrence, metastasis and death. But most of the researches, who studied VEGF, focused only on endothelial cells/angiogenesis.

RNA interference (RNAi), as commonly defined, is a phenomenon leading to posttranscriptional gene silencing (PTGS) after endogenous production or artificial introduction into a cell of small interfering double strand RNA (siRNA) with sequences complementary to the targeted gene. Compared with those traditionally used for antisense DNA-mediated suppression of gene expression, RNAi has many superiorities such as its efficiency, precision, versatility, speed and cost-effectiveness [2–4]. Studies have shown that synthetic 21–22 bp-long siRNAs can be used to silence both exogenous and endogenous gene expression in mammalian cells [5–6]. Zhang et al. [7] pointed out

that RNAi provides a novel tool to study the function of various VEGF isoforms and may contribute to VEGF isoform-specific treatment in cancer.

In this study, we inhibited the vascular endothelial growth factor gene expression using RNAi technique and explored its influence on the apoptosis, proliferation and vasculogenic mimicry of osteosarcoma cell *in vitro*.

MATERIALS AND METHODS

Reagents. In the study Dulbecco's minimal essential medium (DMEM), 0.25% trypsin, 10% fetal bovine serum (GIBCO, USA), Matrigel–collagen, Annexin V-FITC Apoptosis Detection Kit, Type I collagen (BD, USA), Effectene Transfection Reagent (Qiagen, Germany), DNA polymerase, restriction endonuclease, T4 DNA ligase (TaKaRa, Japan), secondary peroxidase-conjugated goat antimouse antibodies (Beijing Dingguo, China), primary anti-VEGF antibodies (R&D, USA), MTT, acrylamide, TEMED, ammonium persulfate (Sigma, USA), pSilencer neo (Ambion, USA), E. coli XLI-Blue (Department of Biochemistry, Fudan University, China) were used.

Construction of specific siRNA targeting VEGF. According to optimization principle of siRNA, we designed 19nt sequence-specific siRNA targeting VEGF [5]. All chemically synthesized oligonucleotides were obtained from Shanghai Shenyou Company. DNA sequences of the double-stranded siRNAs are as follows:

sense: 'GATCCCGGCCAGCACATAGGAGAGATTCA-AGAGATCTCTCCTATGTGCTGGCCCTTTTTGGAAA-3';
antisense: 'GCCGGTCGTGTATCCTCTCTAAGTTCTCTAGAGAGGATACACGACCGGAAAAAACCTTTTCGA-5'.

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Abbreviations used: Annexin-V-FITC – fluorescein-conjugated annexin-V; HE – hematoxylin and eosin; PTGS – posttranscriptional gene silencing; RNAi – RNA interference; VEGF – vascular endothelial growth factor.

According to the manufacturer directions, the products were phosphorylated, annealed, ligated with T4DNA ligase to DNA plasmid and transformed to bacteria. The colonies were amplified and purified using a miniprep purification kit after transformation. First, plasmid DNAs were incised and verified on an 15% nondenaturing polyacrylamide gel. Then, the correctly sized plasmid DNAs were sequenced. After those proof-tests, the plasmid DNAs with correct sequences were purified with QIAGEN Maxi Plasmid DNA kit and used to transfect MG63 cells in the following experiments.

Cell culture. Osteosarcoma cell line of MG63 (American Typical Culture Collection) was cultured in DMEM medium, containing 10% (v/v) heat-inactivated fetal calf serum and maintained at 37 °C in an incubator containing 5% CO₂. The cells were harvested at monolayer confluence with 0.25% trypsin — 1 mM EDTA, and subcultured for following experiments.

siRNA transfection. MG63 cells (7.5×10^5) were seeded in 35 mm plates with 1 mL DMEM and MG63 cells (1×10^4) were seeded in 96 well plates with 100 μ L DMEM. After incubation to achieve 40–80% confluence, MG63 cells were transfected with siRNA according to the manufacturer's instructions.

Three-dimensional culture of osteosarcoma cells. As amended by Montesano [7], type I collagen was prepared. For all experiments, 500 μ L of Matrigel or type I collagen (Collaborative Biomedical) were dropped onto glass coverslips and allowed to polymerize for 1 h at 37 °C. After the transfection, MG63 cells (7.5×10^5 /ml) were seeded on the top of the gels respectively and supplemented with DMEM medium, containing 10% (v/v) of heat-inactivated fetal calf serum. Fresh medium was replaced every day and cultured MG63 cells were observed during the 1st week.

Hematoxylin and eosin (HE) staining. Glass coverslips with sample of three-dimensional culture were taken out after one-week culture. The samples were fixed with buffered formalin, embed into paraffin and thin sections were cut. Sections were stained with hematoxylin and eosin and analyzed using light microscopy.

Western blotting. Osteosarcoma cells and the medium, in which they were harvested, were placed in 100 μ L buffer (Tris, NaCl, 20% Triton-X-100, and protease inhibitors)/60 mm plate. Immediately afterwards, cell samples were sonicated on ice and incubated for 5 min at 95 °C before centrifugation. The protein content was measured, and samples applied to 15% PAGE, followed by transfer to polyvinylidene difluoride (PVDF) membrane. Membranes were blocked by 5% milk in PBST for 1 h at room temperature, followed by overnight incubation with VEGF primary antibody at 4 °C in a concentration 1 : 1000. The incubation with secondary antibody was done for 2 h at RT, after which the membranes was washed in PBST. Membranes were exposed to a chemiluminescence reagent and the chemiluminescence was captured on X-ray film

over exposure times to determine the optimal exposure time. Protein concentrations were analyzed by densitometry.

MTT. MG63 cells (1×10^4) were seeded in 96 well plates with 1 mL of DMEM and incubated to 40–80% confluence. After transfection, cells were cultured for 48 h, 72 h, and 96 h. 5 mg/ml of MTT was added to each well, and incubation was continued at 37 °C for 4 h. The content of each well was removed, and 150 μ L of DMSO was added to extract the dye. After gentle agitation, the optical density (OD) was measured with a enzyme-linked immune instrument. Cell culture experiments were performed in triplicate. Inhibitory effect of pSilencer-VEGF on proliferation of osteosarcoma cells was calculated by following equation: inhibitory rate of cells = { (OD control wells – OD treated wells) / OD control wells} \times 100%.

Apoptosis assays. To analyze the cells for apoptosis, osteosarcoma cells (2×10^6) including the cells in the medium were harvested on the third day after transfection. The cells were trypsinized, pelleted, washed in PBS, and resuspended in 1x binding buffer containing conjugated annexin-V (Annexin-V-FITC) antibody and propidium iodide (PI) according to the manufacturer's protocol. The cells without Annexin-V-FITC and PI were used as negative controls. The foregoing samples were analyzed by flow cytometry.

Statistical Analysis. Data analysis was performed with medical SAS6.12. Statistical significance was assessed with Students t-test. Data is presented as mean \pm standard deviation (SD) and $p < 0.05$ was considered to be statistically significant.

RESULTS

pSilencer-VEGF siRNA expressing plasmid. Initially, plasmid DNAs were incised and verified in a 15% non-denaturing polyacrylamide gel (data not shown). Further, the correct plasmid DNAs were sequenced. The presence of siRNA inserted sequence was confirmed, and there were no mutations in the insert to pSilencer plasmid. Additionally, we could see unmodified H1-RNA promoter, BamH I and Hind III restriction sites within the cloned insert as shown on Fig. 1.

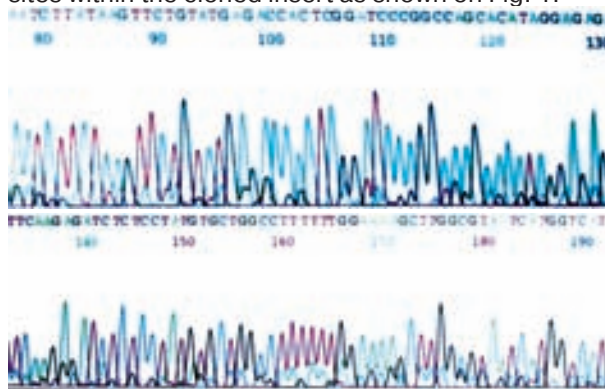


Fig. 1. The results of pSilencer-VEGF DNA sequencing

Western blotting. VEGF expressed in at least six isoforms with distinct biologic activities and clinical implications. Western blotting results showed that four isoforms of VEGF expressed in osteosarcoma

cells, and VEGF₁₆₅ was the predominant isoform in the medium. VEGF expression in MG63 cells was reduced on the 2nd and 3rd day after pSilencer-VEGF siRNA transfection compared with control group. In medium, VEGF protein expression in MG63 cells transfected by pSilencer-VEGF was significantly decreased in comparison with non-treated cells or treated by pSilencer. However, VEGF protein expression level did not differ between non-transfected and transfected by pSilencer cells as shown by Western blotting (Fig. 2).

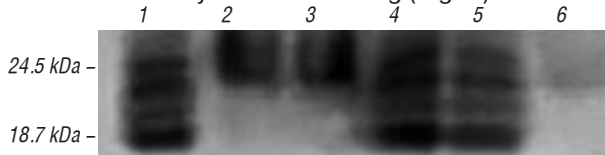


Fig. 2. The expression of VEGF protein after transfecting pSilencer-VEGF to osteosarcoma cells, Western blotting. (Lanes 1–6 are normal cells and media, media and cells transfected by pSilencer, cells and media transfected by pSilencer- VEGF siRNA, respectively)

MTT. The absorbance value of MG63 cells treated with pSilencer-VEGF showed decreased proliferation as measured by MTT compared with MG63 cells treated with pSilencer, and there was significant difference between the absorbance value of two groups at 48 h, 72 h, and 96 h.

Inhibitory effect of pSilencer-VEGF on proliferation of osteosarcoma cells with MTT were 41.67%, 43.92%, 35.32% at 48 h, 72 h, 96 h respectively (Fig. 3).

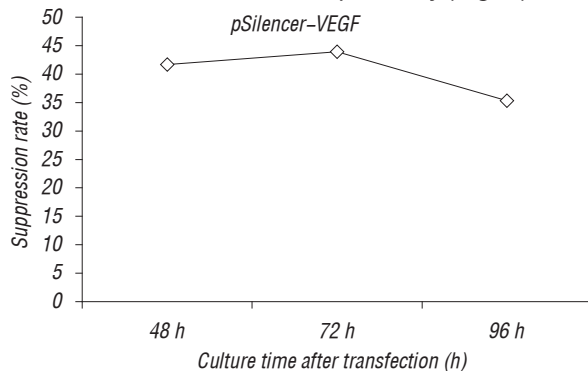


Fig. 3. Inhibitory effect of pSilencer-VEGF on proliferation of osteosarcoma cells

Apoptosis and proliferation of osteosarcoma cells examined by light microscopy. As shown on Fig. 4, transfection of pSilencer did not induce any obvious changes in the shape of MG63 cells. The cells outspreaded themselves, had an asteroidal or polygonal appearance, contacted tightness and had the ability for overlapping growth like fibroblasts in morphology. However, the morphology dramatically changed to an elongated shape with filamentous protrusions after 48 h upon transfection by pSilencer-VEGF. Some cells were morphologically characterized by severe alterations in shape like cell shrinkage and disintegration of cell-cell contacts. Shuttle-shaped appearance, line sparseness and slow growth were also obviously observed.

Apoptosis assays using Annexin-V FITC/Propidium Iodide Staining. Apoptosis was induced in osteosarcoma cells after transfection of pSilencer-

VEGF as shown on Fig. 5. Apoptosis assays revealed that pSilencer-VEGF induced apoptosis in 28.27% osteosarcoma cells upon culturing for 48–72 h.

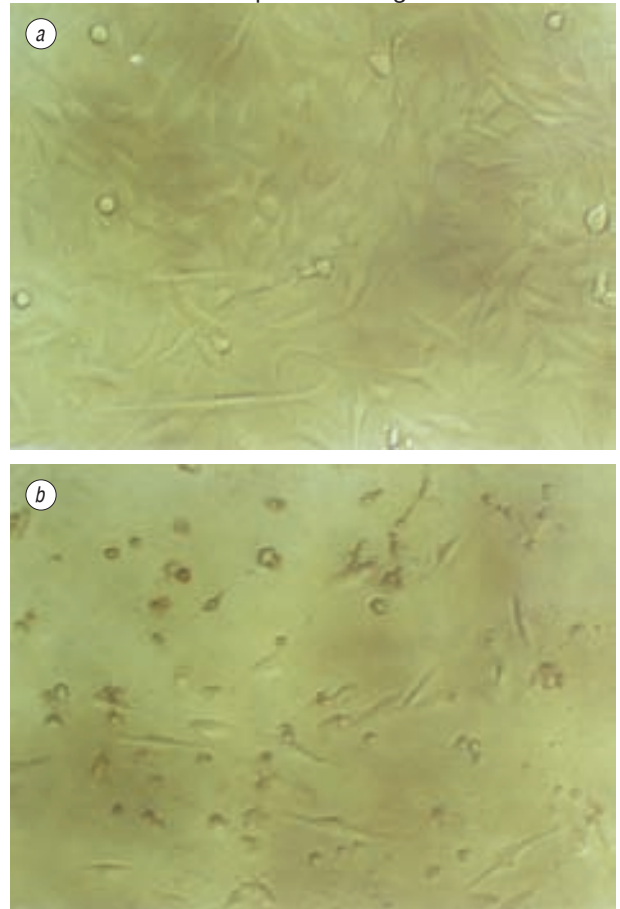


Fig. 4. Effect of pSilencer-VEGF on apoptosis and proliferation of osteosarcoma cells with light microscopy: (a) MG63 cells transfected by pSilencer ($\times 400$); (b) MG63 cells transfected by pSilencer-VEGF ($\times 400$)

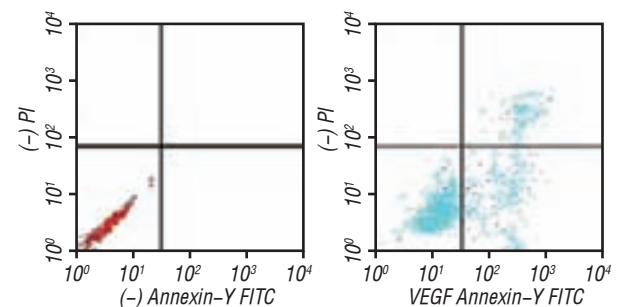


Fig. 5. Effect of pSilencer-VEGF on apoptosis of osteosarcoma cells

Vasculogenic mimicry and the structures MG63 cells formed were observed in Matrigel culture. MG63 cells formed the tubular networks as well as endothelial cells did after transfection by pSilencer. In contrast, MG63 cells did not form structures of the tubular networks after transfection by pSilencer-VEGF (Fig. 6).

Vasculogenic mimicry and the tube formation MG63 cells formed in type I collagen culture. The conglomeration of MG63 cells involved in tube formation was detectable in type I collagen culture after transfection by pSilencer for two days. Then, MG63 cells were incubated further up to one week period,

when the structures of the tubular networks were observed. At the same time, MG63 cells could not form structures of the tubular networks in one week after transfection by pSilencer-VEGF (Fig. 7).

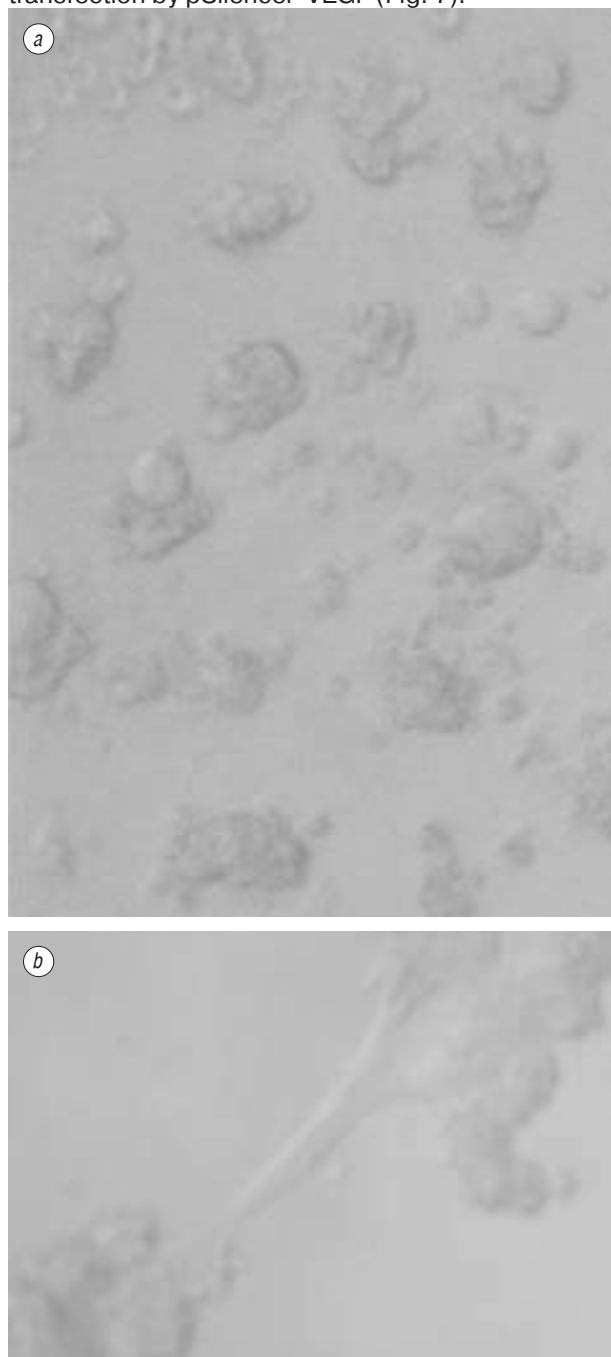


Fig. 6. The structures formed by MG63 cells were studied in Matrigel culture with phase contrast microscopy 24 h after transfection: (a) The structures of the tubular networks were not formed by MG63 cells after transfection by pSilencer-VEGF ($\times 400$); (b) The structures of the tubular networks, which MG63 cells formed after transfection by pSilencer ($\times 400$)

The cross sections of tubules formed by MG63 cells were observed in type I collagen culture with phase contrast microscope and HE staining. MG63 cells transfected by pSilencer formed the tubular networks in the intersecting plane of type I collagen. Adversely, MG63 cells could not form the tubular networks in the sections of type I collagen one week after transfection of pSilencer-VEGF (Fig. 8).

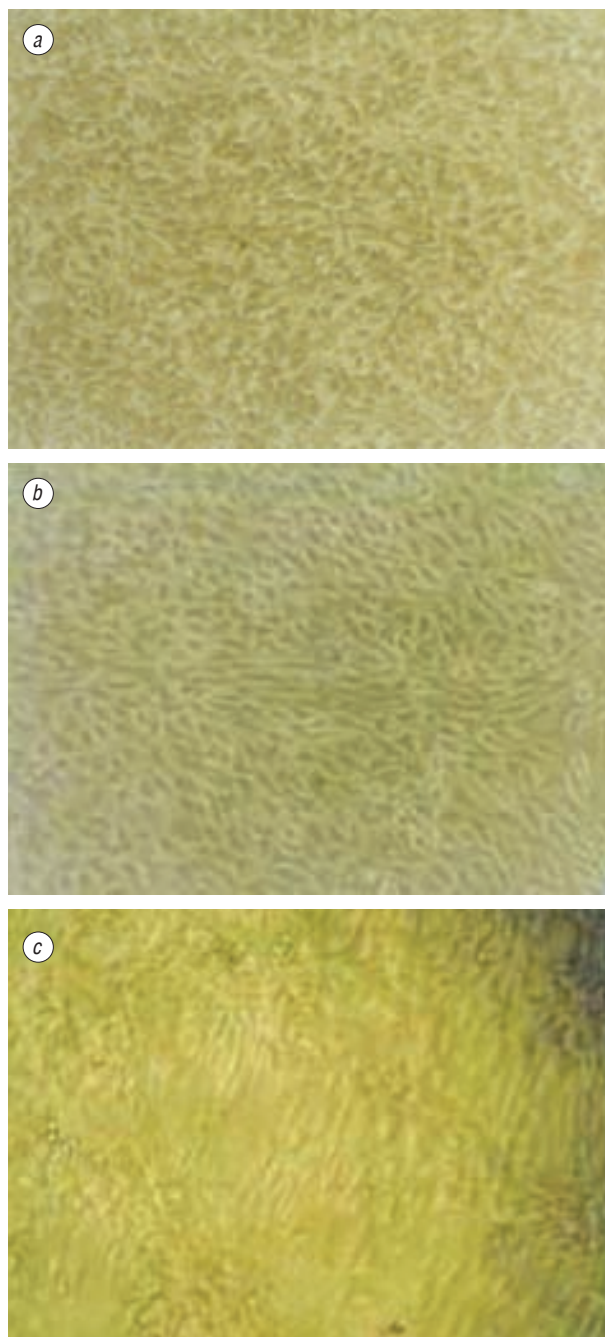


Fig. 7. The structures that MG63 cells formed were analyzed in type I collagen culture with phase contrast microscopy: (a) Structures of the tubular networks were not formed by cells transfected with pSilencer-VEGF in one week ($\times 400$); (b) The conglomeration of the MG63 cells in two days after transfection by pSilencer ($\times 400$); (c) The structures of the tubular networks that MG63 cells formed after transfection by pSilencer in one week ($\times 600$)

DISCUSSION

Osteosarcoma is the most common highly malignant bone tumor in children and adolescents. Limb-salvage resection and neoadjuvant chemotherapy or amputation is the usual treatment of choice with the 5-year survival rate being between 40% to 70%. Several studies demonstrated that vascular genesis might be involved in growth and development of osteosarcoma. When new vascular networks are formed or expanded, tumor spreads almost exclusively via the bloodstream. VEGF is an important regulator of angiogenesis and vasculogenesis [8, 9],

which exhibits endothelial cell-specific mitogenic properties. There is considerable experimental evidence that VEGF isoforms are strongly involved in neoangiogenesis of neoplastic cells, extracellular matrix remodeling, inflammatory cytokine regeneration, formation of an invasive and metastatic immunophenotype. VEGF is both a vascular growth and vascular permeability promoter. Its expression can upregulate several proangiogenic and prometastatic molecules. VEGF expression in untreated osteosarcoma is predictive marker for pulmonary metastasis and poor prognosis.

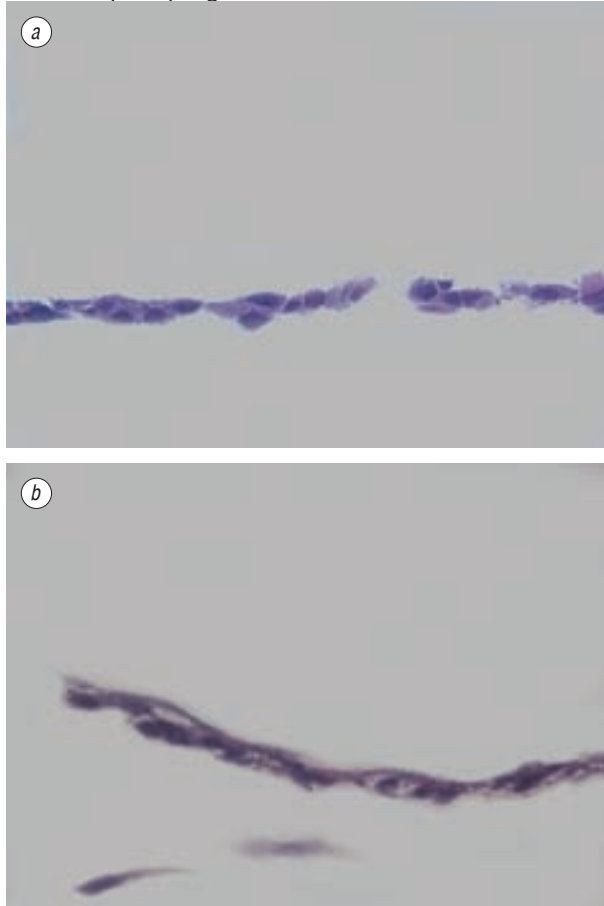


Fig. 8. Tubular networks formed by MG63 cells in type I collagen culture and analyzed with HE staining followed by phase contrast microscopy: (a) There was no tubular networks formation in a week after transfection by pSilencer-VEGF ($\times 400$); (b) The structures of the tubular networks, which MG63 cells formed in a week after transfection by pSilencer ($\times 400$)

Anti-angiogenesis therapy targeting VEGF is inferior to RNAi in silencing the target gene or establishing the functions of gene efficiently and differentially. RNA interference methods provide several major advantages over standard methods (antisense DNA or antibody-based techniques) for suppressing gene expression [10, 12]. Our current studies, confirmed by reverse-transcriptase polymerase chain reaction (RT-PCR) analyses and Western blotting, showed that VEGF isoforms and their receptors were expressed in osteosarcoma cells, with VEGF₁₆₅ being the predominant form in medium.

It has been discussed previously that expanding of the tumor endothelial mass results in the *de novo* formation of endothelial cell-lined vessels. In response, tumor-derived angiogenic factors, such as VEGF, support tumor

growth and angiogenesis in a paracrine fashion. Induction of apoptosis of tumor cells is caused by suppressive vascular formation in anti-angiogenesis treatment. Our previous data [13] demonstrated that osteosarcoma cells not only produced VEGF, but also acquired the capacity to express functional receptor (such as VEGFRs), which resulted in the generation of an autocrine loop, independent of endothelial cells that supported osteosarcoma cells proliferation, inhibited apoptosis and promoted vasculogenic mimicry. Recently, researches have shown that interaction of VEGF with particular subtypes of receptors, which all possess an internal tyrosin kinase domain, activates a circuit of signaling pathways, e.g. PI3K/Akt, Ras/Raf-MEK/Erk, eNOS/NO, and IP₃/Ca²⁺ [14]. In addition, VEGF decreased the expression of gene products involved in anti-apoptosis (xIAP, Bcl-2, HIF-1, and survivin) and proliferation (cyclin D1 and c-Myc) [15, 16]. These proteins participate in the generation of specific biological responses connected with cellular proliferation, cell cycle progression, viability, motility, neovascularization or increasing vascular permeability. Furthermore, as one of the immune suppressive factors, VEGFs induced immature myeloid cells and regulatory T-cells in accordance with tumor progression, resulting in the inhibition of dendritic cell maturation and T-cell activation in a tumor-specific immune response [17].

Previously, we had also demonstrated the presence of osteosarcoma cells expressing the gene markers associated with tumor vasculogenic mimicry *in vitro* [13]. We had also described that the formation of the vascular lumen of osteosarcoma cells was remarkably similar to lumen formation in epithelial cells. Highly aggressive cancer cells line vascular channels and form a perfused network in the absence of endothelial cells which are independent of tumor angiogenesis. As a result, vasculogenic mimicry secures that the newly regenerated portion of tissue develops in parallel with the appropriate vascular supply. Proteinases secreted by tumor cells activation causes local degradation of extracellular matrix components of the vascular channels, so that tumor cells contacted the rapid blood flow of tumor. It is conceivable that vasculogenic mimicry is in favor of the growth invasion and metastasis.

Our findings suggest that down-regulation of VEGF expression has two functions: not only inhibits proliferation and induces apoptosis, but also markedly inhibits vascular cord and tube formation of osteosarcoma cells. It may be implemented from the obstruction of tyrosine kinase receptor signal. Oda et al. [16] found that VEGF expression in the primary site had predictive value for the osteosarcoma patients who would develop lung metastasis. Although RNAi gene therapy has less side effects, it should not be ignored that the mutant osteosarcoma cells might secret other factors instead of VEGF, leading to elusive suppression.

Taken together, signal transduction via VEGF may play multiple roles in the growth and development of osteosarcoma: VEGF not only activates endothelial cells, participates in the angiogenesis, vasculogenesis, but also activates signal transduction involved in vas-

culogenic mimicry, proliferation and apoptosis of tumor cells themselves. Our data indicate that the inhibition of VEGF expression by siRNA silencing has the potential to inhibit tumor growth and progression of osteosarcoma. A VEGF-based siRNA silencing approach may become a promising treatment for osteosarcoma in the future.

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ИНДУКЦИЯ АПОПТОЗА, ИНГИБИРОВАНИЕ ПРОЛИФЕРАЦИИ И ВАСКУЛЯРНОЙ МИМИКРИИ КЛЕТОК ОСТЕОСАРКОМЫ *IN VITRO* ПРИ ПРИМЕНЕНИИ siRNA-VEGF

Цель: ингибировать экспрессию фактора роста эндотелия сосудов (VEGF) с помощью метода РНК интерференции (RNAi) и изучить влияние введения сайленсинга siRNA-VEGF на апоптоз, пролиферацию, васкулярную мимикрию клеток остеосаркомы *in vitro*. **Методы:** плазмида pSilencer-VEGF, рекомбинированная за счет клонирования специфической siRNA последовательности, была трансфицирована в клеточную линию остеосаркомы MG63. Экспрессию VEGF после трансфекции проверяли с помощью Вестерн-блот анализа. Биологические особенности трансфицированных клеток MG63, а именно пролиферацию, апоптоз и васкулярную мимикрию, изучали с помощью МТТ-анализа, проточной цитофлуориметрии, аннексина V-FITC/пропидиум йодида, световой микроскопии и окрашивания гематоксилином и эозином (HE). **Результаты:** последовательность, клонированная в плазмиду pSilencer-VEGF, проверена при секвенировании ДНК. Специфические к VEGF последовательности siRNA значительно снижали его экспрессию клетками MG63. В сравнении с клетками, трансфицированными плазмидой без siRNA, рост клеток с pSilencer-VEGF был ингибирован, а также в них был индуцирован ранний апоптоз. Ингибирование экспрессии VEGF было ассоциировано со снижением васкулярной мимикрии клеток MG63. **Выводы:** ген VEGF может быть тесно связан с регуляцией апоптоза и пролиферации клеток остеосаркомы и может быть ключевым регулятором васкулярной мимикрии. Ингибирование экспрессии VEGF с помощью специфических siRNA можно рассматривать как новый подход к лечению опухолей, характеризующихся повышенной экспрессией VEGF, таких как остеосаркома.

Ключевые слова: фактор роста эндотелия сосудов, остеосаркома, васкулярная мимикрия, РНК интерференция.