ORIGINAL ARTICLE

VEGF-C/VEGFR-3/iNOS Signaling in Osteosarcoma MG63 Cells Mediates Stimulatory Effects on Human Umbilical Vein Endothelial Cell Proliferation

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Objectives To assess the effects of vascular endothelial growth factor-C (VEGF-C)/vascular endothelial growth factor receptor-3 (VEGFR-3) signaling on nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression in human osteosarcoma MG63 cells and the subsequent impact on the proliferation of human umbilical vein endothelial cells (HUVECs).

Methods MG63 cells were treated with VEGF-C alone (VEGF-C group), VEGF-C + iNOS inhibitor aminoguanidine (AG; AG group), and VEGF-C + VEGFR-3 inhibitor MAZ51 (MAZ51 group); untreated MG63 cells were used as controls. NO production was evaluated by a colorimetric method involving nitrate reductase. Meanwhile, mRNA and protein levels of iNOS were examined by reverse transcription polymerase chain reaction (RT-PCR) and Western blot. To explore the effect of VEGF-C/VEGFR-3/iNOS signaling of MG63 cells on proliferation of HUVECs, we set up six groups: HUVECs, HUVECs+MG63, HUVECs+VEGF-C, HUVECs+MG63+VEGF-C, HUVECs+MG63+VEGF-C+AG, and HUVECs+MG63+VEGF-C+MAZ51 groups. The proliferation of HUVEC cells was assessed by Cell Counting Kit-8 (CCK-8), 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay, and proliferating cell nuclear antigen (PCNA) expression quantitation.

Results VEGF-C treatment enhanced iNOS expression at both gene and protein levels (mRNA: LSD- $t=4.152$, $P<0.01$; protein: LSD- $t=3.486$, $P<0.01$) and increased NO release of MG63 cells (LSD- $t=3.774$, $P<0.01$); treatment with either AG or MAZ51 decreased these effects (mRNA: LSD- $t=9.183$, $P<0.001$; LSD- $t=8.639$, $P<0.001$; protein: LSD- $t=5.170$, $P<0.001$; LSD- $t=7.255$, $P<0.001$; NO production: LSD- $t=10.326$, $P<0.001$; LSD- $t=10.540$, $P<0.001$). Interestingly, co-incubation of HUVECs with MG63 cells and/or VEGF-C significantly promoted HUVEC proliferation (EdU: LSD- $t=5.374$, $P<0.001$; LSD- $t=2.984$, $P<0.05$; LSD- $t=8.526$, $P<0.001$; PCNA: LSD- $t=9.267$, $P<0.001$; LSD- $t=5.515$, $P<0.001$; LSD- $t=14.873$, $P<0.001$). The proliferation effects of

HUVEC induced by MG63 cells and VEGF-C attenuated by the treatment of AG (EdU: LSD- $t=10.770$, $P<0.001$; PCNA: LSD- $t=19.940$, $P<0.001$) or MAZ51 (EdU: LSD- $t=6.950$, $P<0.001$; PCNA: LSD- $t=14.001$, $P<0.001$).

Conclusion In human osteosarcoma MG63 cells, activation of VEGFR-3 by VEGF-C promotes iNOS expression and NO production, which subsequently induces HUVEC proliferation.

OSTEOSARCOMA (OS) is the leading primary bone cancer and prevalent in children and young adults. It accounts for 20% of primary bone cancer and the 5-year survival rate is ranged of 60% -78%.^[1-3] Although 90% of OS patients can benefit from limb-salvage surgery and achieve an improved survival with combination treatments, 25-50% of patients develop metastasis, the major cause of deaths.^[4-6] For instance, local recurrence of OS with pulmonary metastasis has a poor 5-year survival rate of 6%.^[7] Elucidating the precise mechanism underlying the relationship between survival and metastasis in OS patients could provide a basis for developing effective treatments.

Studies suggest that angiogenesis plays an important role in cancer growth, development, invasion and metastasis.^[8] Specifically, tumor progression and metastatic process in osteosarcoma can be promoted through angiogenesis induction.^[9] Multiple reports have proposed molecules modulating angiogenesis as targets for the treatment of metastatic OS.^[10-12] Angiogenesis is tightly controlled by the balance between angiogenesis-stimulating factors and angiogenesis inhibitors, and maintained by endothelial cells,^[13] it involves vascular endothelial cell activation, proliferation, migration and sprouting.^[14] Ultimately, the capillaries are stabilized through pericyte recruitment to complete vasculature remodeling.^[15]

Vascular endothelial growth factor (VEGF) silencing was shown to inhibit angiogenesis and promote apoptosis in human osteosarcoma,^[16] which suggested that VEGF/VEGF receptor (VEGFR) signaling plays a central role in vasculogenesis and angiogenesis. In agreement, Abdeen A *et al.*^[17] demonstrated that VEGFR-3 was overexpressed in OS patients, and its level was negatively correlated with overall survival. As a ligand of VEGFR-3, VEGF-C is also expressed in advanced OS.^[18,19] Although the exact role of VEGF-C and VEGFR-3 overexpression in OS remains unclear, the VEGF-C/VEGFR-3 signaling pathway was regarded to contribute to tumor progression and angiogenesis by enhancing production of nitric oxide (NO).^[20] Lahdenranta *et al.*^[21] reported that VEGF-C/VEGFR-3 activated

endothelial nitric oxide synthase (eNOS) and enhanced NO production via PI3K/Akt signaling. In turn, NO promotes the proliferation of vascular and lymphatic endothelial cells,^[22] subsequently induces tumor angiogenesis.

Based on the above findings, we hypothesize that overexpression of VEGF-C and VEGFR-3 in OS may induce nitric oxide synthase, which produces excess NO to promote vascular endothelial cell proliferation. Therefore, this study aimed to assess the role of VEGF-C/VEGFR-3 signaling in NO production and inducible nitric oxide synthase (iNOS) expression in human osteosarcoma MG63 cells, as well as downstream effects on the proliferation of human umbilical vein endothelial cells (HUVECs). Our results demonstrated that activation of VEGFR-3 in MG63 cells by VEGF-C enhanced iNOS expression and NO production, which subsequently promoted HUVEC proliferation.

MATERIALS AND METHODS

Materials

We purchased MG63 cells from the Cell Bank of Shanghai Institutes for Biological Sciences, and HUVECs from the Biology Laboratory, Southern Medical University. Both cell lines were cultured in DMEM (Hyclone, Utah, USA) supplemented with 10% fetal bovine serum (FBS; Zhejiang Tianhang Biotechnology Co., Ltd, China), 100 IU/mL penicillin, and 100 μ g/mL streptomycin at 37°C in humidified atmosphere with 5% CO₂.

MG63 cell treatments

To assess the role of VEGF-C/VEGFR-3 signaling in iNOS expression and NO production in MG63 cells, the latter were treated for 48h with 100 ng/mL VEGF-C alone (VEGF-C group), or 100 ng/mL VEGF-C in combination with the iNOS inhibitor aminoguanidine (AG, Sigma, Missouri, USA; dissolved in double distilled water to obtain a 100 μ mol/L stock solution; the AG group), or in combination with the VEGFR-3 inhibitor MAZ51 (Sigma, Missouri, USA; dissolved in DMSO to obtain a stock solution of 500 μ M; the MAZ51 group); MG63 cells treated with DMSO were used as controls.

Cells were cultured according to the requirements of various assays, including iNOS mRNA and protein levels assessment, as well as NO release quantitation.

Human umbilical vein endothelial cell culture

To explore the effect of VEGF-C/VEGFR-3/iNOS signaling in MG63 cells on the proliferation of HUVECs, MG63 and HUVEC cells were co-cultured in Transwell plates at a density of 1×10^5 /mL. Six groups were set up: HUVECs, HUVECs+MG63, HUVECs+VEGF-C, HUVECs+MG63+VEGF-C, HUVECs+MG63+VEGF-C+AG, and HUVECs+MG63+VEGF-C+MAZ51. MG63 cells (200 μ L) and HUVEC cells (500 μ L) were seeded in upper and lower compartments, respectively. After overnight incubation, the culture medium in both chambers was replaced. Then, MG63 cells were pretreated with MAZ-51 (5 mM) or AG (100 μ M) for 2h, and VEGF-C (100 ng/mL) was added to the transwell insert. After incubation for various times, HUVEC cell proliferation was assessed by Cell Counting Kit-8 (CCK-8, Beyotime, China), 5-ethynyl-2'-deoxyuridine (EdU, Guangzhou RiboBio, China) incorporation assay, and proliferating cell nuclear antigen (PCNA, Beckman Coulter Inc, USA) expression quantitation.

Reverse transcription polymerase chain reaction

Total RNA from MG63 cells of different groups was extracted using TRIzol reagent (Takara Bio, Dalian, China) following the manufacturer's instructions. Two micrograms of total RNA were reverse-transcribed using the PrimeScript RT reagent kit (Takara Bio, Dalian, China) according to the manufacturer's protocols. PCR reactions were performed using SYBR Premix Ex Taq II (Takara Bio, Dalian, China) on IQ5 real-time PCR detection system (Bio-Rad, California, USA) using the following primers: iNOS, Forward 5'-CATGCTCCTCTAG-TAACTG-3', Reverse 5'-TTTCCTGAAGAAGGGGAC-3'; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Forward 5'-ACAGTTGCCATGTAGACC-3', Reverse 5'-TTTTTGGTTGAGCACAGG-3'. Reactions were performed at 95°C for 20s, followed by 40 cycles at 95°C for 3s and at 60°C for 30s. Relative amounts of mRNA were derived using 2- $\Delta\Delta$ Ct method, with GAPDH as the internal control. All experiments were carried out in triplicate.

Western blot

After treatment, MG63 cells were harvested and lysed. Total protein (40 μ g/sample) components were

separated by electrophoresis on SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane. Subsequently, the membrane was blocked with BSA for 1 hour at room temperature, and incubated with anti-iNOS antibodies (1:500 dilution, Santa Cruz Technology, Santa Cruz, CA) overnight at 4°C. After washing with phosphate buffer saline containing Tween-20, the membranes were probed with horseradish peroxidase-conjugated secondary antibodies (rabbit anti-Goat IgG IRDye680, Odyssey). β -actin was used as the internal control. Data were quantified by analyzing gray scale intensity using Gel-Pro Analyzer software (Media Cybernetics Inc., Rockville, MD, USA).

Nitric oxide (NO) release assessment

NO in MG63 cell culture medium of different groups was measured by colorimetric method using the Nitric Oxide Assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instruction.

CCK-8 assay

After co-incubation for 48h, HUVEC culture supernatants were removed, and CCK-8 test solution (10% v/v CCK-8 in culture medium) was added to each well. After 4h of incubation at 37°C, 100 μ L supernatant was moved to 96-well plates, and optical density (OD) was measured at 450 nm on a Multiscan MK3 microplate reader (Thermo Fisher Scientific, Waltham, USA).

5-Ethynyl-2'-deoxyuridine (EdU) incorporation assay

After co-incubation for 48h, HUVECs were treated with 50 μ M EdU (Guangzhou RiboBio, China) for an additional 12h at 37°C. Then, discarded the culture medium and washed cells twice with PBS. After fixation with 4% formaldehyde (800 μ L) for 30 min, add 800 μ L glycine (2 mg/mL) to each well for 5 min, washed cells twice with PBS and incubated cells with 0.5% Triton X-100 for 10 min at room temperature. After washing with PBS for 5 min, 1 $\times$ Apollo reaction reagent was added (1600 mL/well), and plates were incubated at room temperature in dark for 30 min. Then, cells were stained with 1600 μ L 1 $\times$ Hoechst 33342 for 30 min in dark. After two washes with PBS, micrographs were obtained by confocal microscopy. A total of 300 cells were counted by flow cytometry and fluorescence intensity was determined.

Flow cytometry for PCNA expression quantitation

HUVECs were washed with PBS twice. Trypsin-EDTA (300 μ L/well) was added to cells for 1 min; digestion was terminated by adding 2 mL culture medium. Cells were then collected, counted and washed with PBS. After membrane rupture and incubation with fixation solution, cells were incubated in presence of PCNA antibody (Santa Cruz Technology, Santa Cruz, CA) for 15 min. PCNA expression was determined by flow cytometry (FC500, Beckman Coulter Inc, California, USA).

Statistical analysis

Data are described as mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare differences among the groups. If the ANOVA was significant, then LSD-*t* test was employed for multiple comparisons between two groups. All statistical analyses were carried out with SPSS statistics (version 19.0, IBM, USA). All experiments were repeated three times and the results were averaged. $P < 0.05$ was considered statistically significant.

RESULTS

VEGF-C/VEGFR-3 promoted NO production of MG63 cells

To assess the effects of VEGF-C on NO production in MG63 cells, the cells were co-incubated with VEGF-C for 48h. As shown in **Fig. 1A**, treatment with VEGF-C significantly induced NO release in MG63 cells (LSD-*t*=3.774, $P < 0.01$). AG group and MAZ-51 group showed significantly decreased NO production compared with MG63 group (LSD-*t*=6.552, $P < 0.001$; LSD-*t*=6.765, $P < 0.001$) or VEGF-C group (LSD-*t*=10.326, $P < 0.001$; LSD-*t*=10.540, $P < 0.001$). The iNOS inhibitor AG or VEGFR-3 inhibitor MAZ51 not only attenuated the VEGF-C induced NO release, but also greatly decreased the basal production of NO in MG63 cells. These results indicated that iNOS was involved in increasing NO amounts induced by VEGF-C/VEGFR-3 interaction.

VEGF-C/VEGFR-3 enhanced iNOS expression in MG63 cells

Treatment with VEGF-C significantly enhanced iNOS mRNA expression in MG63 cells (LSD-*t*=4.152, $P < 0.01$) (**Fig. 1B**). Nevertheless, the mRNA expression values in both AG group and MAZ51 group were significantly lower compared with MG63 group (LSD-*t*=5.030,

$P < 0.01$; LSD-*t*=4.487, $P < 0.01$) or VEGF-C group (LSD-*t*=9.183, $P < 0.001$; LSD-*t*=8.639, $P < 0.001$), suggesting that addition of AG or MAZ-51 decreased iNOS mRNA amounts (**Fig. 1C**), which was correlated with their effects on NO production. Similarly, VEGF-C treatment increased iNOS protein levels in MG63 cells (LSD-*t*=3.486, $P < 0.01$), this effect was attenuated with the treatment of AG (LSD-*t*=5.170, $P < 0.001$) or MAZ51 (LSD-*t*=7.255, $P < 0.001$) (**Fig. 1C, 1D**). These results indicated that VEGF-C enhanced NO production by up-regulating iNOS in MG63 cells.

Co-incubation of HUVECs with MG63 cells and VEGF-C promoted HUVEC proliferation

The iNOS overexpression and NO production increase in MG63 cells induced by VEGF-C improved HUVEC proliferation, as shown by CCK-8 (**Fig. 2A**), EdU incorporation (**Fig. 2B, 2C**) and PCNA expression (**Fig. 2D, 2E**) assays. HUVECs treated with VEGF-C showed increased cell proliferation compared to untreated HUVECs (EdU: LSD-*t*=2.984, $P < 0.05$; PCNA: LSD-*t*=5.515, $P < 0.001$). However, the HUVECs+MG63+VEGF-C group showed stronger proliferation effects on HUVECs than HUVECs + VEGF-C group (EdU: LSD-*t*=5.124, $P < 0.001$; PCNA: LSD-*t*=10.573, $P < 0.001$). Interestingly, the promoting effects of MG63 cells plus VEGF-C on HUVEC proliferation were markedly attenuated by addition of AG (EdU: LSD-*t*=10.770, $P < 0.001$; PCNA: LSD-*t*=19.940, $P < 0.001$) or MAZ51 (EdU, LSD-*t*=6.950, $P < 0.001$; PCNA: LSD-*t*=14.001, $P < 0.001$). These results indicated that the VEGF-C/VEGFR-3 pathway and iNOS played an important role in MG63 induced HUVEC proliferation.

DISCUSSION

The current study demonstrated that VEGF-C treatment enhanced iNOS expression, and increased NO release in MG63 cells. In addition, HUVECs co-incubated with VEGF-C and/or MG63 cells significantly promoted HUVEC proliferation. The VEGF-C dependent effects were significantly attenuated after treatment with MAZ51 or AG.

In the current study, activation of the VEGF-C/VEGFR-3 pathway significantly enhanced NO production in MG63 cells, and its blockage by the VEGFR-3 inhibitor MAZ51 resulted in remarkable decrease of NO level. Our hypothesis that the VEGF-C/VEGFR-3

Figure 1. Nitric oxide (NO) production and iNOS expression (mRNA and protein levels) in MG63 cells with different treatments.

(A) NO levels in MG63 cell culture medium were assessed by a colorimetric method. (B) iNOS expression assessed by qRT-PCR. (C, D) iNOS expression levels determined by Western blot. VC (VEGF-C) binding with VEGFR-3 promoted NO release by up-regulating iNOS. $n = 3$ for each group.

** $P < 0.01$, *** $P < 0.001$ compared to the MG63 group; ### $P < 0.001$ compared to the MG63+VC group.

induced NO production being mediated by iNOS was confirmed by the results of qRT-PCR and Western blot analyses. Indeed, VEGF-C/VEGFR-3 activation resulted in increased expression of iNOS while it was downregulated by VEGFR-3 inhibition. Collectively, these findings indicate that the VEGF-C/VEGFR-3 signaling pathway may induce NO production, which is accordant with previous reports.^[20,21]

We assessed the role of the VEGF-C/VEGFR-3/iNOS pathway in MG63 cells on HUVEC proliferation subsequently in this study. Although VEGF-C itself could promote HUVEC proliferation, HUVECs co-incubated with MG63 cells resulted in higher proliferative rate compared with those treated with VEGF-C alone. It is reported that VEGF-C was highly expressed in MG63 cells;^[18] however, increased NO release by exogenous VEGF-C may also contribute to stimulation of HUVECs growth induced by MG63 cell co-incuba-

tion. These findings indicate that MG63 cells probably synergize with VEGF-C to enhance the proliferation of HUVECs. The iNOS inhibition by AG attenuated the pronounced promoting effects of VEGF-C and MG63 cells, suggesting that the iNOS-NO axis may greatly contribute to HUVEC growth.

Previous studies reported that VEGF-C and VEGFR-3 were up-regulated in OS cells.^[18] Meanwhile, iNOS was also overexpressed in OS of jaw.^[23] Our study filled the gap between VEGF-C/VEGFR-3 pathway and the iNOS-NO axis: activation of the VEGF-C/VEGFR-3 pathway promotes NO production by up-regulating iNOS in MG63 cells; then, NO production by MG63 cells facilitates the proliferation of HUVECs and promotes angiogenesis in OS, which is in agreement with findings by Chen *et al.* that overexpression of iNOS in OS was correlated with tumor microvessel density.^[23] Taken together, these findings demonstrate that the

Figure 2. The effects of MG63 cells with treatment of VEGF-C on HUVEC proliferation.

Proliferation of HUVECs was assessed by CCK-8 (A), EdU (B and C) and PCNA expression (D and E) assays in co-incubation with MG63 cells and/or VC (VEGF-C), with MG63+VC+AG, and MG63+VC+MAZ51. $n = 3$ for each group. Bars in (B): 200 μm .

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the HUVEC group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared with the HUVEC+MG63+VC group.

VEGF-C/VEGFR-3/iNOS axis in MG63 cells could induce HUVEC proliferation and promote tumor angiogenesis. Overexpression of iNOS has been demonstrated in several malignancies, including colon carcinoma, brain tumor, breast cancer, and melanoma, [24-27] suggesting the importance of VEGF-C/VEGFR-3/iNOS signaling in cancers.

A few limitations of this study should be mentioned. Because both MG63 cells and HUVECs express VEGF-C and VEGFR-3, [28, 29] the extent of iNOS-NO effect on HUVEC proliferation is not easy to evaluate. Further comprehensive experiments should be designed to determine the contribution of the iNOS-NO axis. In addition, the effect of VEGF-C/VEGFR-3 signaling on other

forms of nitric oxide synthase was not elucidated, and should be addressed in future investigations.

In conclusion, this study demonstrated that stimulation of VEGFR-3 by VEGF-C in MG63 cells promotes iNOS expression and NO production, inducing the proliferation of HUVECs.

Conflict of interests

None.

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论著

骨肉瘤 MG63 细胞中 VEGF-C/VEGFR-3/iNOS 信号转导对人脐静脉内皮细胞增殖的促进作用

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摘要

目的 探讨血管内皮生长因子 -C (vascular endothelial growth factor-C, VEGF-C) / 血管内皮生长因子受体 -3 (vascular endothelial growth factor receptor-3, VEGFR-3) 信号转导对人骨肉瘤 MG63 细胞中一氧化氮 (nitric oxide, NO) 的生成和诱导型一氧化氮合酶 (inducible nitric oxide synthase, iNOS) 的表达以及对人脐静脉内皮细胞 (human umbilical vein endothelial cells, HUVECs) 增殖的影响。

方法 MG63 细胞分别以 VEGF-C 单独处理 (VEGF-C 组), VEGF-C 联合 iNOS 抑制剂氨基胍 AG (AG 组) 以及 VEGF-C 联合 VEGFR-3 抑制剂 MAZ-51 (MAZ-51 组) 处理, 将未处理的 MG63 细胞作为对照。采用硝酸还原酶比色法测定 NO 生成量, 逆转录 - 聚合酶链反应 (reverse transcription polymerase chain reaction, RT-PCR) 和 Western blot 分别检测 iNOS 的 mRNA 和蛋白水平。为研究 MG63 细胞中 VEGF-C/VEGFR-3/iNOS 信号通路对 HUVECs 增殖的影响, 实验设立如下 6 组: HUVECs 组、HUVECs+MG63 组、HUVECs+VEGF-C 组、HUVECs+MG63+VEGF-C 组、HUVECs+MG63+VEGF-C+AG 组及 HUVECs+MG63+VEGF-C+MAZ51 组。用细胞计数试剂盒 (Cell Counting Kit-8, CCK-8)、5- 乙炔基 -2'- 脱氧尿嘧啶核苷 (5 ethynyl 2' deoxyuridine, EdU) 掺入法和增殖细胞核抗原 (proliferating cell nuclear antigen, PCNA) 定量表达评估各组 HUVECs 的增殖情况。

结果 VEGF-C 促进了 MG63 细胞中 iNOS 在基因 (LSD- $t=4.152$, $P<0.01$) 和蛋白水平的表达 (LSD- $t=3.486$, $P<0.01$), 增加了 NO 释放 (LSD- $t=3.774$, $P<0.01$)。经 AG 或 MAZ51 处理均可降低上述作用 (mRNA: LSD- $t=9.183$, $P<0.001$; LSD- $t=8.639$, $P<0.001$; protein: LSD- $t=5.170$, $P<0.001$; LSD- $t=7.255$, $P<0.001$; NO: LSD- $t=10.326$, $P<0.001$; LSD- $t=10.540$, $P<0.001$)。HUVECs 与 MG63 细胞和 / 或 VEGF-C 共同培养可显著促进 HUVECs 的增殖 (EdU: LSD- $t=5.374$, $P<0.001$; LSD- $t=2.984$, $P<0.05$; LSD- $t=8.526$, $P<0.001$; PCNA: LSD- $t=9.267$, $P<0.001$; LSD- $t=5.515$, $P<0.001$; LSD- $t=14.873$, $P<0.001$)。经 AG (EdU: LSD- $t=10.770$, $P<0.001$; PCNA: LSD- $t=19.940$, $P<0.001$) 或 MAZ51 (EdU: LSD- $t=6.950$, $P<0.001$; PCNA: LSD- $t=14.001$, $P<0.001$) 处理后, MG63 细胞和 VEGF-C 对 HUVECs 增殖的诱导作用减弱。

结论 VEGF-C 激活 VEGFR-3 可促进在人骨肉瘤 MG63 细胞 iNOS 的表达和 NO 的产生, 进而诱导 HUVECs 的增殖。

关键词: 骨肉瘤; 血管内皮生长因子 -C; 血管内皮生长因子受体 -3; 诱导型一氧化氮合酶; 肿瘤血管生成

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