SCIENTIFIC ARTICLE

Inhibition of Growth and Metastasis of Tumor in Nude Mice after Intraperitoneal Injection of Bevacizumab

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Objective: To explore the inhibitory effect of bevacizumab, a vascular endothelial growth factor antibody, on angiogenesis in human osteosarcoma of nude mice.

Methods: Twenty-one nude mice were inoculated with red fluorescent protein (RFP)-labeled human osteosarcoma cell line 143B-RFP, that is, clones that expressed RFP in the cytoplasm, and randomly assigned to one of three groups: G1 (Control group, injected with saline solution); G2 (intraperitoneal bevacizumab 2 mg/kg twice per week) and G3 (intraperitoneal bevacizumab 5 mg/kg, twice per week). The tumor-bearing mice were examined in a fluorescence light box that was illuminated periodically. The primary tumors were measured by fluorescence imaging weekly and their volumes calculated.

Results: The mean tumor volumes were significantly smaller in the G3 (186.4 ± 100.8 mm^3) than the control group (587.0 ± 406.8 mm^3) ($P < 0.05$) on Day 31, and again significantly smaller in the G3 (677.3 ± 461.9 mm^3) than the control group (3162.6 ± 1529.2 mm^3) on Day 38 ($P < 0.01$). The average tumor volume in the G2 group was 493.5 ± 425.4 mm^3 on Day 31 and 1870.1 ± 1524.8 mm^3 on Day 38. The effect on tumor volume was greater in the G3 than the G2 group. Three mice in the G2 group, four in the G3 group and four in the control group developed lung metastases that were confirmed by pathological examination; these differences were not statistically significant ($P < 0.05$).

Conclusions: Bevacizumab exhibits strong antiangiogenesis activity in experimental osteosarcoma in a nude mouse model but does not influence the incidence of lung metastasis. Our findings may have considerable potential for the treatment of osteosarcoma.

Key words: Bevacizumab; Green fluorescent protein; Mice; Osteosarcoma model; Tumor cells, cultured

Introduction

Osteosarcoma, the commonest malignant bone tumor, mainly affects children and adolescents[1]. Despite advances in chemotherapy and surgery, the 5-year survival rate is 50%–60% for localized disease and only 20% for metastatic disease. Failure of therapy is mainly attributable to metastatic dissemination to bone and lung[2]. Clinical outcomes and therapeutic strategies, including surgical excision and combination chemotherapy, have remained essentially unchanged in the past 20 years and current strategies have limited efficacy for treatment of metastatic disease. It is therefore very important to develop more effective and less toxic novel agents for the treatment of primary metastatic osteosarcoma.

Angiogenesis is necessary for growth and invasion of primary tumors and plays an important role in the

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establishment of metastases. Several studies have focused on the role of angiogenesis in osteosarcoma[3]. Vascular endothelial growth factor (VEGF), a specific stimulator of vascular endothelial cell proliferation and tumor angiogenesis, is manufactured in response to various environmental and cellular stimuli. VEGF is overexpressed in many human neoplasms, including osteosarcoma, and this expression is associated with tumor angiogenesis. Strong expression of VEGF in human osteosarcoma has been shown to correlate with a significantly worse prognosis and development of lung metastases[4]. New blood vessels that grow within tumors as a result of VEGF expression are structurally and functionally irregular, which leads to further tumor hypoxia and subsequent increases in VEGF production[5]. VEGF has significant effects on the growth and progression of neoplasia, stimulating the growth of endothelial cells and apparently being central to angiogenesis. Increasingly more studies have shown that a murine antihuman monoclonal antibody against VEGF can inhibit the growth of human tumors. Accordingly, such antibodies are being evaluated in clinical trials as treatment for various cancers. Given its key role, it is not surprising that much effort has been put into developing new drugs that block the effects of VEGF and consequently inhibit the carcinogenic process.

Bevacizumab (BV; Avastin; Genentech, South San Francisco, CA, USA), a humanized monoclonal antibody that inhibits VEGF, is the first antiangiogenic therapy to be approved for use in humans with cancer. In combination with chemotherapy or biologics, bevacizumab has been shown to improve progression-free survival and/or overall survival in patients with metastatic breast, colorectal, non-small-cell lung and renal cell cancers[6]. Bevacizumab may also improve the delivery of chemotherapy by altering tumor vasculature and decreasing the high interstitial pressure within them[7].

However, bevacizumab therapy has not been shown to have a role in osteosarcoma. Because the vascularity of osteosarcomas correlates with the incidence of metastatic spread, this tumor is a potential target for antiangiogenic therapy. Fukaya *et al.* have reported that different osteosarcoma cell lines have different metastatic capabilities[7].

While parental Dunn is poorly metastatic to lung, LM8, which was derived from Dunn by *in vivo* selection through pulmonary metastasis, displays a clear capability for pulmonary metastasis. Fukaya *et al.* have shown that expression of an active form of Akt in Dunn substantially activates its matrix metalloproteinase secretion and that Akt signaling plays an important role in pulmonary metastasis from osteosarcoma[7]. It is therefore necessary to test bevacizumab on different osteosarcoma cell lines to clarify its ability to inhibiting metastases to the lung.

Fossey *et al.* showed that oncostatin stimulation of human and canine osteosarcoma cell lines induces STAT3 activation, thereby enhancing the expression/activation of metalloproteinase-2 and VEGF, ultimately promoting invasive behavior and tumor angiogenesis[8]. This research indicates that more agents are needed to inhibit lung metastasis. These authors did not observe tumor necrosis in their experiments. Thus, it is necessary to identify other chemotherapy drugs for controlling lung metastasis. Yang *et al.* showed that baseline p53, apoptosis and human epidermal growth factor receptor 2 are each significantly associated with outcome in patients with breast cancer who receive bevacizumab plus chemotherapy[9]. All of these data suggest that bevacizumab plus chemotherapy can cause very rapid, irreversible damage to osteosarcoma cells and lead to rapid cell death that is distinct from apoptosis.

The purpose of this study was to determine whether anti-VEGF therapy inhibits the growth of primary osteosarcoma and its micrometastases. In this study, we established an implantation model of osteosarcoma in nude mice by transplanting osteosarcoma cell line 143B-RFP into the right hind tibia. The 143B human osteosarcoma cells were labeled with red fluorescent protein (RFP). The tumor-bearing mice were examined in a fluorescence light box that was illuminated periodically. The length and width of the primary tumors were measured by fluorescence imaging system and the tumor volume (length × width^2/2) thereby calculated.

Materials and Methods

Experimental Animals

This study was performed in accordance with the guidelines of the China Laboratory Animal Management Committee and the animal experiments were approved by the ethics committee under the animal experiment scheme of Nanjing Origin Biosciences (China). Male BALB/c mice aged 4–6 weeks and weighing 15–20 g were obtained from the Model Animal Research Center of the Nanjing University (Nanjing, China). They were housed in a particulate air-filtered environment in which the room temperature was 24–25 °C and the humidity 50%–60% and fed a special laboratory diet.

Cell Culture and Reagents

The human osteosarcoma cell line 143-B-RFP was obtained from the American Type Culture Collection (Manassas, VA, USA) and these 143B human osteosarcoma cells (Fig. 1) labeled with RFP, thus establishing clones expressing RFP in the cytoplasm. The cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, and maintained in a 37 °C, 10% CO_2, humidified incubator. Bevacizumab (Avastin) was obtained from Genentech (South San Francisco, CA, USA) in the form of a colorless solution with a concentration of 25 mg/mL.

Fluorescence Optical Tumor Imaging

A fluorescence stereo microscope model LZ12 (Jiang Nan Yu Chen Optical Instruments, Nanjing, China) equipped with a mercury 50 W lamp power supply was used. Selective excitation of GFP was produced through a D425/60 band-pass filter and 470DCXR dichotic mirror (LG-150-model, Chaoteng

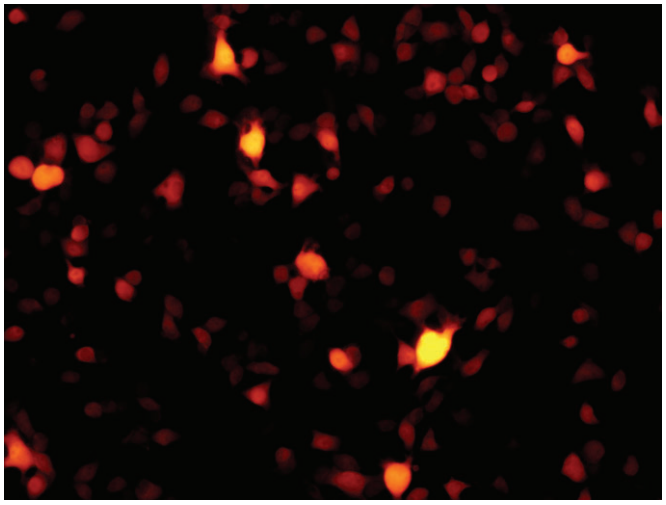

Fig. 1 143B-RFP human osteosarcoma cells have been labeled with RFP. × 400.

Technology Development. Nanjing, China). Emitted fluorescence was collected by a color CCD camera (Retiga Exi cooled Digital Color model, Qimaging, Surrey, BC, Canada). The tumor-bearing mice were also periodically examined in a fluorescence light box illuminated by fiberoptic lighting at 440/20 nm, images being collected with the camera described above. High resolution images of 1392 × 1040 pixels were captured directly on an IBM PC, processed for contrast and brightness, and analyzed with the use of Image Pro Plus 4.0 software (Media Cybernetics, Silver Springs, MD, USA). During the study, the animals were monitored at each time point of external fluorescent optical tumor imaging. At the end of the study, all mice were killed and open fluorescent imaging performed. Primary tumors and all metastases to distant organs, including the mesentery lymph nodes, lung and liver, were carefully imaged.

Experimental Protocol

On experiment Day 0, the mice were anesthetized, their right hind limbs sterilized with 0.2% iodine tincture, a suitable incision made and about 2×10^6 143-B-RFP cells in 5 μL saline injected into the right hind tibia. The skin was then closed with sterile 6-0 surgical sutures. When the primary tumor was measurable and the tumor volume (length × width^2/2) calculated in 80% of the mice, they were randomly allocated to one of the following three groups (seven mice per group), and bevacizumab was injected: control group G1 (injected with saline solution), bevacizumab group G2 (2 mg/kg intraperitoneally twice per week), and bevacizumab group G3 (5 mg/kg intraperitoneally, twice per week).

The weights of the mice were monitored and recorded twice weekly. The lengths and widths of the primary tumors were measured by the fluorescence imaging system weekly and the tumor volumes (length × width^2/2) was calculated. The mice were killed on Day 42 to assess lung metastases. Tumor volumes were calculated according to the formula: volume = (the shortest diameter)^2 × (the longest diameter) × 0.5[10].

Statistical Analysis

Student's *t*-test was used to evaluate differences in tumor growth between groups and Fisher's exact test to compare differences in tumor metastasis. $P < 0.05$ was considered statistically significant. All statistical analysis was performed using SPSS 16.0.

Results

Efficacy of Bevacizumab in Osteosarcoma Cell Lines 143-B-RFP Transplantation Model

Representative photographs of osteosarcomas are shown in Figures 2 (room light) and 3 (fluorescent light). The mean tumor volume was significantly smaller in both bevacizumab groups than the control group ($P < 0.05$). Additionally, the mean tumor volume was significantly smaller in the G2 (bevacizumab 5 mg/kg) than in the G3 (bevacizumab 2 mg/kg) group ($P < 0.05$). On Day 31, the mean tumor volume was significantly smaller in the animals treated with bevacizumab 5 mg/kg (G2 group, 186.4 ± 100.8 mm^3) than in the saline solution-treated group (control, 587.0 ± 406.8 mm^3) ($P < 0.05$, Table 1, Fig. 4). On Day 38, the mean tumor volume was significantly smaller in the G2 group (677.3 ± 461.9 mm^3) than in the control group (3162.6 ± 1529.2 mm^3) ($P < 0.01$). Additionally, on Day 38 the tumor volume was significantly smaller in the G2 group (677.3 ± 461.9 mm^3) than in the G3 group (1870.1 ± 1524.8 mm^3) ($P < 0.05$). Thus, a dose of 5 mg/kg was more effective than one of 2 mg/kg. In the G3 group, the mean tumor volume was 493.5 ± 425.4 mm^3 on Day

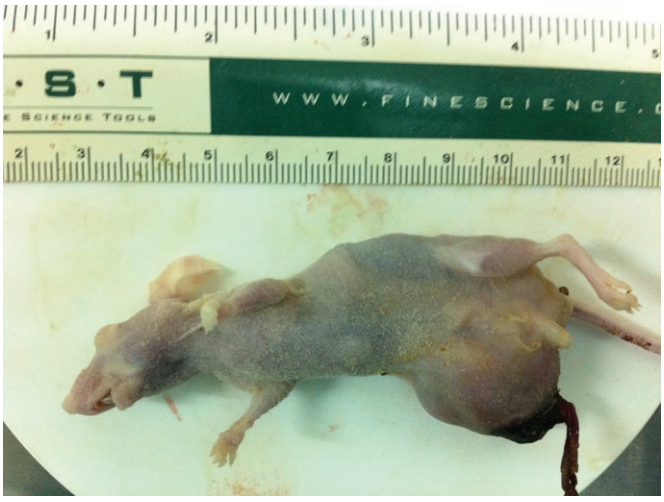

Fig. 2 The proximal tibias of athymic nude mice were injected with 143B-RFP cells. A representative photograph on Day 43 showing a large primary tumor.

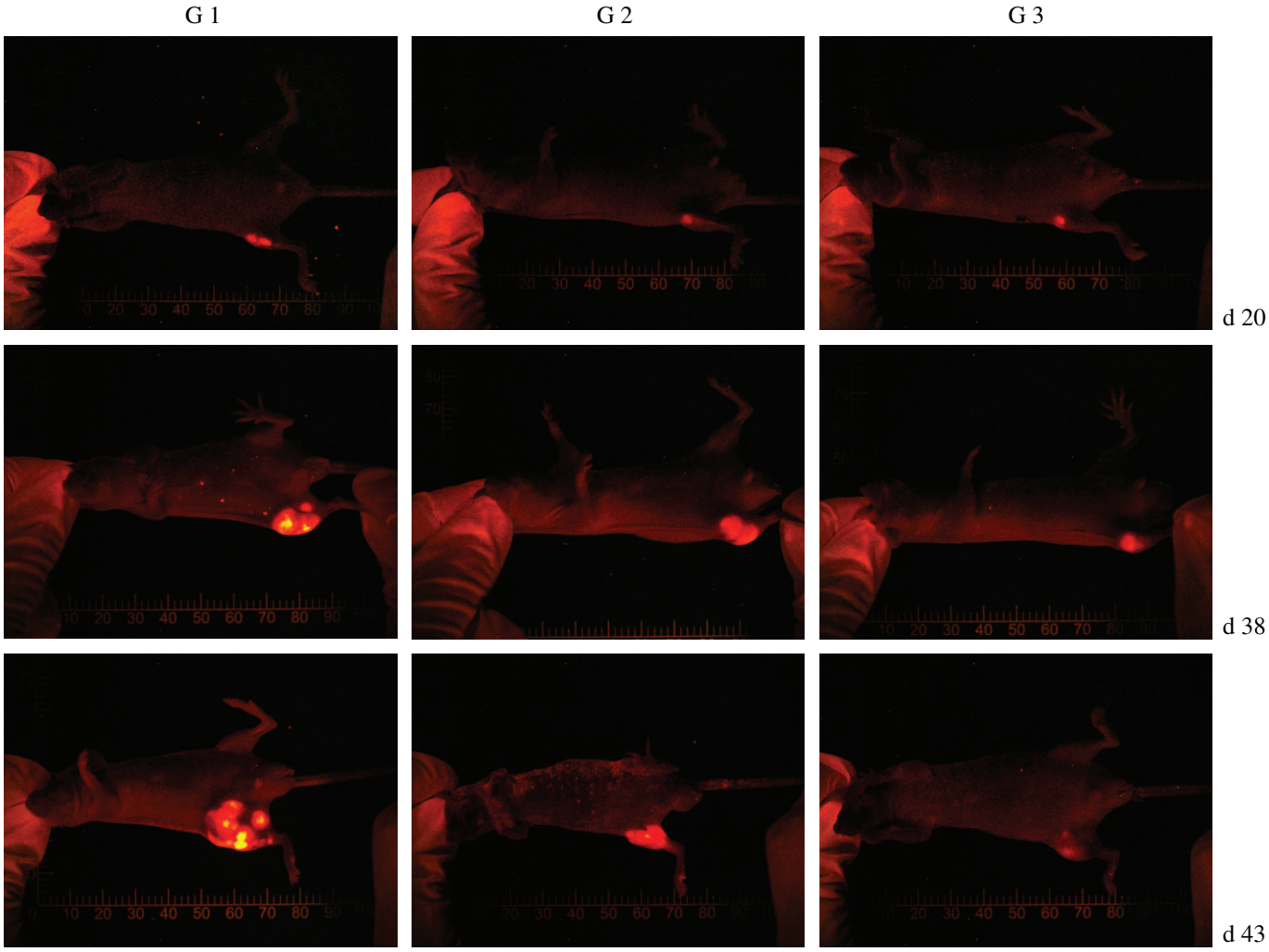

Fig. 3 Whole-body fluorescent photographs of mice on Days 20, 38 and 43. The tumor volumes are smaller in treated than control group mice ($P < 0.05$) and significantly smaller in the G2 than G3 group, indicating that inhibition by bevacizumab of primary tumor growth is dose-dependent.

TABLE 1 Mean tumor volumes (mm^3) according to treatment group on Days 20, 31 and 38 (mean ± SD)

Groups	Day 20	Day 31	Day 38
G1	27.7 ± 7.6	587.0 ± 406.8	3162.6 ± 1529.2
G2	26.5 ± 9.3	493.5 ± 425.4	1870.1 ± 1524.8
G3	27.9 ± 12.8	186.4 ± 100.8	677.3 ± 461.9
P value	G1 *vs.* G2: 0.808 G1 *vs.* G3: 0.97	G1 *vs.* G2: 0.695 G1 *vs.* G3: 0.02	G1 *vs.* G2: 0.156 G1 *vs.* G3: 0.00

G1, control group (injected with saline solution); G2, bevacizumab group (2 mg/kg, twice per week); G3, bevacizumab group (5 mg/kg, twice per week).

31 and 1870.1 ± 1524.8 mm^3 on Day 38; these values are significantly lower than on the same days in the control group ($P < 0.05$).

Fluorescent light photographs of mice in all groups are shown in Fig. 3. The tumors were easily observed repeatedly under fluorescent light and their volumes were observed to decrease after bevacizumab treatment. Tumor growth rates were slower in treated than in untreated mice and the tumor volumes were significantly smaller in the treated than control groups ($P < 0.05$). These data suggest that the antitumor effects of the antibody against VEGF are attributable to inhibition of angiogenesis. The present findings support the contention that the inhibition of primary tumor growth by bevacizumab is dose-dependent. No tumor necrosis was

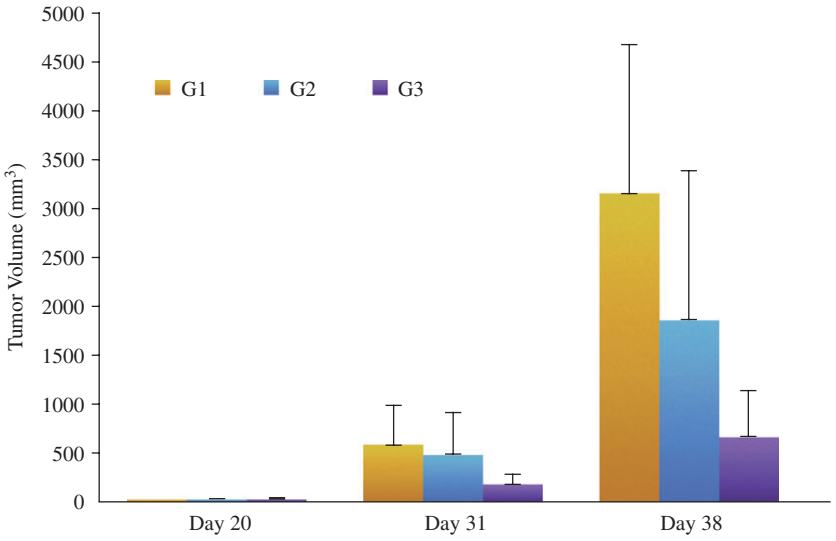

Fig. 4 Mean tumor volumes according to group on Days 20, 31 and 38.

observed (results not shown). Additionally, the higher dose of bevacizumab did not incur greater toxicity.

Effect of Bevacizumab on Lung Metastasis by Osteosarcoma Cells

The relevant results are shown in Table 2 and Figure 5. In the current study, there was no evidence that bevacizumab reduced the incidence of spontaneous metastasis of 143B-RFP cells to the lung. Four mice in the untreated group, three mice in the G3 group and four in the G2 group developed lung metastases (as confirmed by microscopic examination) (Table 2, Fig. 5); these differences are not significant ($P > 0.05$). Thus, according to our data, bevacizumab does not inhibit metastasis of 143B-RFP cells to the lung.

Discussion

Use of Osteosarcoma Cell Lines 143-B-RFP Transplantation Model

Osteosarcoma, the most common primary malignant bone tumor in children and young adults, is characterized by an aggressive clinical course, most patients dying of pulmonary metastases[11–17]. In this study, we established an osteosarcoma mouse model with a high tumor take rate, easy real-time visualization and easy repeated sampling, these characteristics making it an ideal model for evaluating osteosarcoma[18–24].

TABLE 2 Effect of bevacizumab on incidence of osteosarcoma metastasis (cases)

Group	*n*	Lung metastasis
G1	6	4
G2	5	3
G3	6	4

Fisher exact test: $P > 0.05$; G1, control group (injected with saline solution); G2, bevacizumab group (2 mg/kg, twice per week); G3, bevacizumab group (5 mg/kg, twice per week).

Bevacizumab and Metastasis of Osteosarcoma to the Lung

The most well-developed anti-osteosarcoma agent is bevacizumab (Avastin; Genetech), which is a humanized murine monoclonal antibody against VEGF. Recent studies have indicated that bevacizumab treatment results in a 25%–95% dose-dependent inhibition of tumor growth in mouse and rat models[18,19,24–27]. In the present study, we found that bevacizumab causes a dose-dependent reduction in the size of murine osteosarcomas and that this is attributable to reduced intratumoral endothelial cell proliferation and angiogenesis, leading to a shrinkage of tumors. Bevacizumab did not

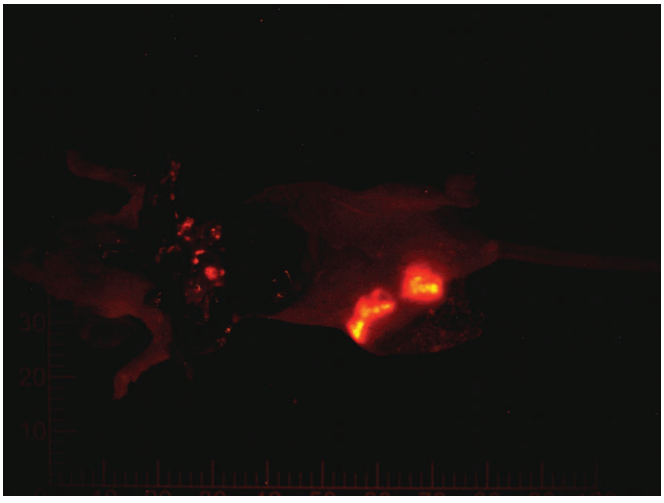

Fig. 5 Whole-body fluorescent imaging of representative nude mouse with lung metastases.

induce apoptosis in the osteosarcoma cells. Some of the findings in this study of treatment of mice with 143B-RFP cell-derived primary tumors and spontaneous metastases in the lung with bevacizumab were unexpected findings. Our data indicate that bevacizumab treatment as administered in the current study does not affect circulating cells metastasizing to the lung early during primary tumor development. The expression of VEGF is regulated by different factors.

Although VEGFA over expression and the regulation of its expression have been studied, the main focus has been on its regulation by transcriptional factors such as hypoxia inducible factor-1A[19–23]. Yang *et al.* reported for the first time that VEGF pathway genes, including the VEGFA gene, are amplified in osteosarcoma[11]. Amplification of the gene is not only an important mechanism for VEGFA protein expression, but is also a poor prognostic factor for tumor-free survival[1,24,25]. Furthermore, rapamycin reportedly inhibits metastatic tumor growth and angiogenesis in *in vivo* mouse models by reducing translational production of VEGFA[20–26]. These data indicate that bevacizumab alone does not completely inhibit expression of VEGF; more agents with different mechanisms of action (e.g., at the molecular level) are needed to achieve this. Warren *et al.* showed that administration of an anti-VEGF antibody in a colorectal cancer xenograft model caused a 90% reduction in the mass of the primary tumor and a 10- to 18-fold reduction in the number of liver metastases compared with the control[20]. As is well known, pulmonary metastases from osteosarcoma result in fatal outcomes in the majority of patients[27–31].

Our findings show that bevacizumab does not inhibit metastasizing cells in the circulation and in the lung. Bevacizumab binds to VEGF, inhibiting angiogenesis and thereby preventing tumor growth and metastasis[25,26]. This agent has been shown to have unprecedented survival benefit in patients with metastatic colorectal cancer[18,28–30]. These data directly led to its approval by the US Food and Drug Administration and the European Medicines Agency for the treatment of patients with metastatic cancer of the colon. However, there are insufficient data about the ability of bevacizumab to inhibit metastasis to the lung. In our study, the tumors had already metastasized to the lung when bevacizumab treatment started. We therefore speculate that bevacizumab cannot inhibit lung metastases once the tumor has reached a critical size. Thus, more research is needed to investigate the ability of bevacizumab to inhibit metastasis to the lung in an osteosarcoma mouse model[31–37].

Limitations of this Study

In conclusion, with the advantages of easy real-time visualization and easy repeated sampling of tumors, our implantation model is valuable for the study of osteosarcoma and evaluation of new anticancer therapy[38–40]. Our findings show that VEGF inhibition reduces osteosarcoma proliferation and angiogenesis, resulting in suppression of the primary tumor. Anti-VEGF therapy is a potential strategy for the treatment of osteosarcoma[41–43]. Although several studies have investigated the dose of bevacizumab required to prevent neovascularization[28–30], few studies have addressed bevacizumab dosage. Its efficacy may be dose-dependent. Further studies *in vitro* and *in vivo* are needed to investigate how bevacizumab in combination with chemotherapy drugs affects primary tumor growth and metastasis of osteosarcoma in an orthotopic osteosarcoma mouse model. This trial was not designed to assess the safety of bevacizumab in an osteosarcoma mouse model[44–47]. Considering that bevacizumab is reportedly moderately safe in animals and its predominant antiangiogenesis activity demonstrated in the current study, it appears to be a promising novel chemotherapeutic agent for the treatment of osteosarcoma, particularly in patients with metastases at diagnosis and a correspondingly poor prognosis[48–51].

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