Anoikis-resistant human osteosarcoma cells display significant angiogenesis by activating the Src kinase-mediated MAPK pathway

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Abstract. Tumor cells must resist anoikis to metastasize. There is a key role of angiogenesis in the growth and metastasis of tumors. However, the relationship between anoikis resistance and angiogenesis has not been well explored in human osteosarcoma. In the present study, we reported the higher expression of vascular endothelial growth factor-A (VEGF-A) in osteosarcoma cells that were resistant to anoikis than in parental osteosarcoma cells, promoting the proliferation, tube formation, and migration of human umbilical vein endothelial cells (HUVECs). Src, JNK (Jun amino-terminal kinase) and ERK (extracellular signal-regulated kinase) signaling pathway phosphorylation was activated in anoikis-resistant cells; Src inhibitor reduced the expression of VEGF-A and angiogenesis and inhibited JNK and ERK pathway activity. Overexpression of phosphorylated (p)-Src and VEGF-A was positively correlated to the metastatic potential in human osteosarcoma tissues, as quantified by immunohistochemistry. In addition, p-Src expression was directly correlated with VEGF-A expression and microvessel density *in vivo*. Our findings revealed that anoikis resistance in osteosarcoma cells increased the expression of VEGF-A and angiogenesis through the Src/JNK/ERK signaling pathways. Thus, Src may be a potential therapeutic alternative in osteosarcoma angiogenesis and metastasis.

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Abbreviations: AI, anchorage-independent; ECM, extracellular matrix; ERK, extracellularsignal-regulated kinase; HUVECs, human umbilical vein endothelial cells; IL-8, interleukin 8; JNK, Jun amino-terminal kinase; MAPK, mitogen-activated protein kinase; MVD, microvessel density; VEGF-A, vascular endothelial growth factor-A

Key words: angiogenesis, anoikis resistance, osteosarcoma, VEGF-A, Src kinase

Introduction

The most common primary malignant bone tumor is osteosarcoma, and it is derived from primitive bone-forming mesenchymal cells (1). There is an association between lung metastasis and poor prognosis in osteosarcoma, and it occurs in the initial stages of the disease (2). Although recent innovations in multi-method treatment, i.e., chemotherapy, surgery and radiotherapy have improved prognosis, metastasis and recurrence persist due to high rates of multi-drug resistance (3,4). Therefore, novel, targeted treatment of metastasis in osteosarcoma is urgently needed.

Cancer cells exhibit anoikis resistance when they avoid anoikis, or programmed death in a foreign microenvironment without extracellular matrix (ECM) adhesion (5). Similar to most tumor cells, osteosarcoma cell metastasis is complex and includes the absence of ECM attachment, cell migration, anoikis resistance, angiogenesis and metastatic colonization (6). By regulating cell homeostasis in tissues, anoikis plays a critical role in cellular physiology; it is a type of apoptosis induced by separating cells from their surrounding matrix and neighboring cells (7). A crucial hallmark of malignant tumor metastasis and progression is anchorage-independent (AI) growth; the potential for metastasis and progression in prostate and breast cancer, as well as melanoma, and lung cancer, is associated with AI growth (8,9). Tumor cells can survive in the circulatory or lymphatic system due to resistance to anoikis, and it enables the formation of metastasis in distant organs (10).

In recent years, the function of angiogenesis in the invasion, growth and metastasis of tumors has attracted much attention from the scientific community (11). Angiogenesis is a fundamental process required for physiological and pathological processes, referring to new blood vessel formation from the vasculature already present (12). An important angiogenic factor, vascular endothelial growth factor A (VEGF-A) plays a critical role in tumor progression and prognosis (13,14). There is an

association between VEGF-A expression and the development of lung metastases in osteosarcoma (15,16). Neovascularization is necessary for anoikis-resistant cells adherence to distant sites and formation of metastases. Therefore, elucidating the molecular mechanism of angiogenesis via VEGF-A in anoikis-resistant human osteosarcoma is vital.

Tyrosine kinase Src is an important tumor biological factor (17). Yes, Fyn, Lck, Lyn, Hck, Blk, Yrk, Fgr and c-Src are among the Src family kinases; it is one of the most important cellular pathways responsible for proliferation, apoptosis, migration, and metastasis in osteosarcoma, chondrosarcoma and Ewing sarcoma (18). Several studies have reported that Src kinase activation is frequently detected in a variety of anoikis-resistant tumor cells, and there is an association between its activation and angiogenesis through increased VEGF-A and interleukin 8 (IL-8) expression, which are proangiogenic factors (19,20). Previously, we found that Src kinase plays important roles in apoptosis, metastasis, anoikis resistance, autophagy and stem cell self-renewal of osteosarcoma (21-23). However, it remains unclear whether Src kinase activation promotes the production of VEGF-A to enhance angiogenesis and metastasis of tumors in anoikis-resistant human osteosarcoma cells.

The mitogen-activated protein kinase (MAPK) pathway is a downstream molecule of Src kinase that plays a key regulatory role in VEGF-A expression (24,25). The MAPK [JNK (Jun amino-terminal kinase)/ERK (extracellular-signal-regulated kinase)/p38] pathway is another pivotal signaling pathway that contributes to tumor cell anoikis resistance. Nevertheless, whether the MAPK pathway is involved in the anoikis-resistant human osteosarcoma angiogenesis cascade requires further clarification.

In the present study, we investigated the role of human osteosarcoma cell resistance to anoikis in angiogenesis and aimed to uncover the underlying mechanisms. We revealed that angiogenesis was enhanced in such cells through control of the expression of VEGF-A by anoikis via the Src/JNK/ERK signaling pathways. The findings revealed that Src kinase is a potential target in anti-angiogenesis therapy of osteosarcoma.

Materials and methods

Cell culture. The U2OS human osteosarcoma cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The malignant transformed hFOB1.19 cell line (MTH) was established by treatment with an initiating agent, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG, 1.0 μg/ml) and a promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA, 200 ng/ml), as previously reported (26). Human umbilical vein endothelial cells (HUVECs) were obtained from Southwest Hospital, Third Military Medical University (TMMU) (Chongqing, China). The MTH and U2OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA); HUVECs were cultured in RPMI-1640 medium (HyClone; GE Healthcare Life Sciences) in 5% CO_2 and 95% humidified air at 37˚C. Fetal bovine serum (FBS, 10%; Biological Industries, Beit Haemek Ltd., Beit HaEmek, Israel) and 1% penicillin-streptomycin (HyClone; GE Healthcare Life Sciences) were used to supplement the cultures.

AI culture. To establish the anoikis-resistant model, we cultured the MTH and U2OS cells in ultra-low attachment 6-well plates (Corning Inc., Corning, NY, USA) for 3 days, transferred to normal plates with attachment culture until they formed monolayers, cultured again in ultra-low attachment 6-well plates for 14 days, and finally transferred to normal plates; the re-adherent cells were considered anoikis-resistant cells, and were designated MTHar and U2OSar, respectively (21). An inverted light microscope (Olympus Corp., Tokyo, Japan) was used to observe the cell morphology.

Flow cytometry for apoptosis. We cultured the MTH, U2OS, MTHar and U2OSar cells in ultra-low attachment 6-well plates for 3 days before detection. Then, $1x10^6$ cells were harvested and an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA) was used to detect the cells. We analyzed the apoptotic cell percentage using a flow cytometer (BD Biosciences) after staining and repeated the experiment independently 3 times.

Preparation of conditioned medium (CM). Complete medium (DMEM, 10% FBS, 1% penicillin-streptomycin) was used to culture the MTH, U2OS, MTHar and U2OSar cells, or the cells were pretreated with 10 μmol/l Src inhibitor PP2 (Selleckchem, Houston, TX, USA) for 24 h. Phosphate-buffered saline (PBS) was used to wash the cells three times and the cells were transferred to serum-free medium when the cell density was ~70-80%. Then, the CM was collected 24 h after we had changed the medium; we stored the CM at -80˚C until use.

Wound healing assay. We seeded the HUVECs in 6-well plates and cultured in complete medium until they reached approximately 90% confluence. A 10-μl pipette tip was used to create wounds, and the HUVECs were treated with CM. We monitored cell migration to the wound at 0 and 20 h using an inverted light microscope, and quantified it by measuring the distance between the start point of the scratch wound to the migrated point, and the experiment was repeated thrice.

Cell proliferation assay. We examined the cell proliferative potential using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). We seeded $1x10^3$ HUVECs in 96-well plates; the cells were treated with CM. Following 1 to 5 days of culture, we added 10 μl/well CCK-8 reagent to each plate and cultured the cells for 1 h. A microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) was used to measure the absorbance at 450 nm.

Tube formation assay. We dissolved Matrigel (BD Biosciences) at 4˚C overnight. We coated a pre-chilled 96-well plate with thawed Matrigel (50 μl), and incubated it at 37˚C for 30 min. After the gel had solidified, $1x10^4$ HUVECs/well were suspended in culture medium comprised of 50% complete medium and 50% CM and seeded in the wells. After 4-12 h of incubation at 37˚C, we photographed tube formation at 6 h using an inverted microscope. A capillary-like tube formation assay is often used to determine angiogenesis *in vitro*. Tube branches and total tube length were measured with ImageJ software (http://rsb.info.nih.gov/ij/). Their number or total

length represents the capacity of angiogenesis (27). In this study, the number of tube branches was used for statistical analysis.

Enzyme-linked immunosorbent assay (ELISA). We cultured the MTH, U2OS, MTHar, and U2OSar cells in 6-well plates. We changed confluent cells to serum-free medium or pretreated them with PP2 (10 μmol/l). After 24 h, we detected supernatant VEGF-A levels using a human VEGF-A ELISA kit (Neobioscience, Shenzhen, China).

Quantitative real-time PCR. We extracted total RNA from the osteosarcoma cells with TRIzol (Takara Bio Inc., Shiga, Japan). We reverse-transcribed RNA into complementary DNA (cDNA) using a QuantScript kit for reverse transcription (RT) (Takara Bio Inc.). We performed RT-PCR with SYBR Green using primers based on human VEGF-A and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) sequences. The primer sequences were: VEGF-A forward, 5'-CTTCGCTTA CTCTCACCTGCTTCTG-3' and reverse, 5'-GCTGCTTCT TCCAACAATGTGTCTC-3'; GAPDH forward, 5'-CTTTGG TATCGTGGAAGGACTC-3' and reverse, 5'-GTAGAGGCA GGGATGATGTTCT-3'. The cycling conditions were polymerase activation at 95˚C for 30 sec, and then 40 cycles at 95˚C for 15 sec and 60˚C for 60 sec. We performed triplicate quantitative PCR (qPCR) with StepOnePlus (Applied Biosystems, Foster City, CA, USA). The levels of the experiment group mRNA were calculated with the $2^{-\Delta\Delta Cq}$ method (28).

Western blotting. We performed western blotting as previously described (21). Proteins were extracted using RIPA buffer (cat. no. 89900; Thermo Fisher Scientific, Inc.) and their concentrations were measured by BCA Protein Assay kit (cat. no. P0010S; Beyotime Institute of Biotechnology, Haimen, China). Proteins (40 μg/lane) were separated by 10% SDS-PAGE (cat. no. P0012A; Beyotime Institute of Biotechnology) and transferred to polyvinylidene difluoride (PVDF) membranes (cat. no. 3010040001; Roche, Shanghai, China). Skim milk (5%) was applied to block the membranes for 1 h at room temperature. Then, the membranes were incubated with primary antibodies overnight at 4˚C. After 3 washes with PBST (0.05% Tween-20 in PBS), the blots were incubated with the corresponding secondary antibodies for 1 h at room temperature. We used the following primary antibodies: Rabbit anti-phosphorylated (p)-Src (dilution 1:1,000; cat. no. 2105) and Src (dilution 1:1,000; cat. no. 2109; both from Cell Signaling Technology, Inc., Danvers, MA, USA); mouse anti-VEGF (dilution 1:200; cat. no. sc-7269; Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit anti-JNK (dilution 1:1,000; cat. no. AJ518), rabbit anti-ERK (dilution 1:1,000; cat. no. AM076), rabbit anti-p-ERK (dilution 1:1,000; cat. no. AF1891), rabbit anti-p-JNK (dilution 1:1,000; cat. no. AF1762), mouse anti-p38 (dilution 1:1,000; cat. no. AM065) and mouse anti-p-p38 (dilution 1:1,000; cat. no. AM063; all from Beyotime Institute of Biotechnology). The secondary antibodies were horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (IgG) (dilution 1:5,000; cat. no. BA1056) and HRP-conjugated goat anti-mouse IgG (dilution 1:5,000; cat. no. BA1050; both from Biodragon Immunotech, Beijing, China). GAPDH (dilution 1:1,000; cat. no. 5174; Cell Signaling Technology, Inc.) was used as a loading control. Finally, the blots were visualized using an ECL kit (cat. no. P0018FFT; BeyoECL Moon; Beyotime Institute of Biotechnology) and quantitative data were obtained using ImageJ software (http://rsb.info.nih.gov/ij/).

Human osteosarcoma specimen preparation. We collected specimens from 26 patients (13 males and 13 females, aged from 8 to 49 years old, average age, 19.31±9.30 years) who had been diagnosed with osteosarcoma before radiation therapy or chemotherapy from the Southwest Hospital, TMMU from January 2011 to April 2014 (Table I). We had obtained informed consent previously from the patients or their guardians according to the standards set by the Declaration of Helsinki. The TMMU Institutional Ethical Committee approved the present study.

Immunohistochemistry (IHC) analysis. p-Src, VEGF, CD34 and CD31 expression were determined with a kit (ZSGB-BIO, Beijing, China) as previously described (21). Two pathologists blinded to the pathological and clinical data examined the specimens under a light microscope. We used primary rabbit anti-p-Src (dilution 1:200; cat. no. 2105; Cell Signaling Technology, Inc.), mouse anti-VEGF (dilution 1:50; cat. no. sc-7269; Santa Cruz Biotechnology, Inc.) and mouse anti-CD31 (dilution 1:100; cat. no. ZM-0044; ZSGB-BIO, Beijing, China) for human osteosarcoma specimens. For mouse xenograft specimens, primary rabbit anti-p-Src (dilution 1:200; cat. no. 2105; Cell Signaling Technology, Inc.), mouse anti-VEGF (dilution 1:50; cat. no. sc-7269; Santa Cruz Biotechnology, Inc.) and rabbit anti-CD34 (dilution 1:100; cat. no. Ab81289; Abcam, Cambridge, MA, USA) were used. Semi-quantitative estimation was calculated to interpret the IHC results based on the stained cells per 100 cells (percentage) in >5 randomly selected fields under high-power microscopy (magnification, x400). We calculated the IHC scores by multiplying the intensity of staining (0, no staining; 1, stained yellow; 2, stained brown; and 3, stained dark brown) and staining extent scores, i.e., percentage of positive tumor cells (0, 0-5%; 1, 6-25%; 2, 26-50%; 3, 51-75%; 4, >75%). Protein expression was categorized using the final IHC scores as follows: 0 (-); 1-3 (+); 4-7 (++); and 8-12 (+++); positive expression was denoted by scores ≥4. We assessed microvessel density (MVD) using a CD31 antibody. We scanned the immunostained sections at a low magnification (x40) to detect hotspots, i.e., areas with the greatest vascular intensity. Then, the number of microvessels were counted in 5 randomly-selected fields per hotspot under a high-power field (magnification, x200). The average number of microvessels in each sample was determined and was defined as the MVD.

Mouse xenograft assay. We performed the animal experiments adhering to the Institutional Animal Care and Use Committee-approved protocols at Xinqiao Hospital, TMMU, according to the Directive 2010/63/EU. Eight female 4-week-old nude mice were obtained from the Laboratory Animal Centre of Xinqiao Hospital, TMMU (Chongqing, China).We randomly divided mice into two groups (n=4 mice in each group) and the average weight was ~15 g. Housing conditions included temperature (22±2˚C), humidity (40-60%), 12-h light/dark cycle and fed *ad libitum*. We resuspended

Table I. Correlations of the clinicopathological features with p-Src and VEGF-A expression in patients with osteosarcoma.

			p-Src			VEGF-A		
Clinical characteristics	Group	n	Positive	Negative	P-value[a]	Positive	Negative	P-value[a]
Sex	Male	13	6	7	1	7	6	0.688
	Female	13	7	6		9	4	
Age (years)	<20	19	8	11	0.378	10	9	0.19
	≥20	7	5	2		6	1	
Tumor location	Femur	14	5	9	0.215	7	7	0.397
	Tibia	10	6	4		7	3	
	Other	2	2	0		2	0	
Histological type	Osteoblastic	12	7	5	0.876	10	2	0.185
	Chondroblastic	5	2	3		2	3	
	Fibroblastic	2	1	1		1	1	
	Other	7	3	4		3	4	
Enneking stage	IIA	3	0	3	0.029	1	2	0.05
	IIB	13	5	8		6	7	
	III	10	8	2		9	1	
Tumor size (cm)	<8	15	9	6	0.428	11	4	0.228
	≥8	11	4	7		5	6	
Recurrence	Yes	8	5	3	0.673	6	2	0.42
	No	18	8	10		10	8	
Metastasis	Yes	10	8	2	0.041	9	1	0.037
	No	16	5	11		7	9	

[a]Fisher's exact test.

MTH/MTHar cells ($1x10^6$ for each mouse) in serum-free medium, and the cells were subcutaneously injected into each mouse. The mice were sacrificed by cervical dislocation after 3 weeks and the harvested tumors were fixed with formalin. The maximal volumes of xenografts were 1,058 mm^3 (MTH) and 1,573 mm^3 (MTHar). p-Src, VEGF-A and CD34 expression were determined by IHC.

Statistical analysis. We performed all statistical analyses using SPSS 19.0 (version 19.0; IBM Corp., Armonk, NY, USA). We reported the data as the mean ± standard deviation (SD). We analyzed between-group differences with Student's t-test of variance, Spearman's rank correlation coefficients or Pearson's χ^2 test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Establishment of anoikis-resistant model. The anoikis-resistant human osteosarcoma cell model was established by cell suspension culture according to a previous study (21). The osteosarcoma cells were round and gathered into clusters gradually in the suspension culture. The clusters grew bigger and tighter over time (Fig. 1A). The regular polygonal osteosarcoma cells became irregular, elongated and spindle-shaped when they were transferred into normal plates and these cells (the re-adherent cells) were considered anoikis-resistant cells (Fig. 1B). Flow cytometry demonstrated significantly less apoptosis in the anoikis-resistant osteosarcoma cells under suspension conditions compared with the parental osteosarcoma cells (Fig. 1C).

Anoikis-resistant human osteosarcoma cells promote angiogenesis by controlling the expression of VEGF-A. Tumor metastasis requires angiogenesis, a critical factor in controlling cancer progression (27,29). Anoikis-resistant cells play a key role in metastasis; however, the effects of resistance to anoikis in human osteosarcoma cells on angiogenesis remain largely unknown. Angiogenesis includes HUVEC migration, proliferation, and tube formation for the formation of new blood vessels (30). Therefore, we investigated the effects of resistance to anoikis in osteosarcoma cells from humans on HUVEC angiogenesis using *in vitro* migration, tube formation and proliferation assays. CM from anoikis-resistant osteosarcoma cells promoted HUVEC migration, tube formation and proliferation (Fig. 2A-C). RT-PCR, western blotting and ELISA revealed increased mRNA and protein expression of VEGF-A in the anoikis-resistant osteosarcoma cells (Fig. 2D-F). These data demonstrated that osteosarcoma cells that were resistant to anoikis had increased expression of VEGF-A and angiogenesis.

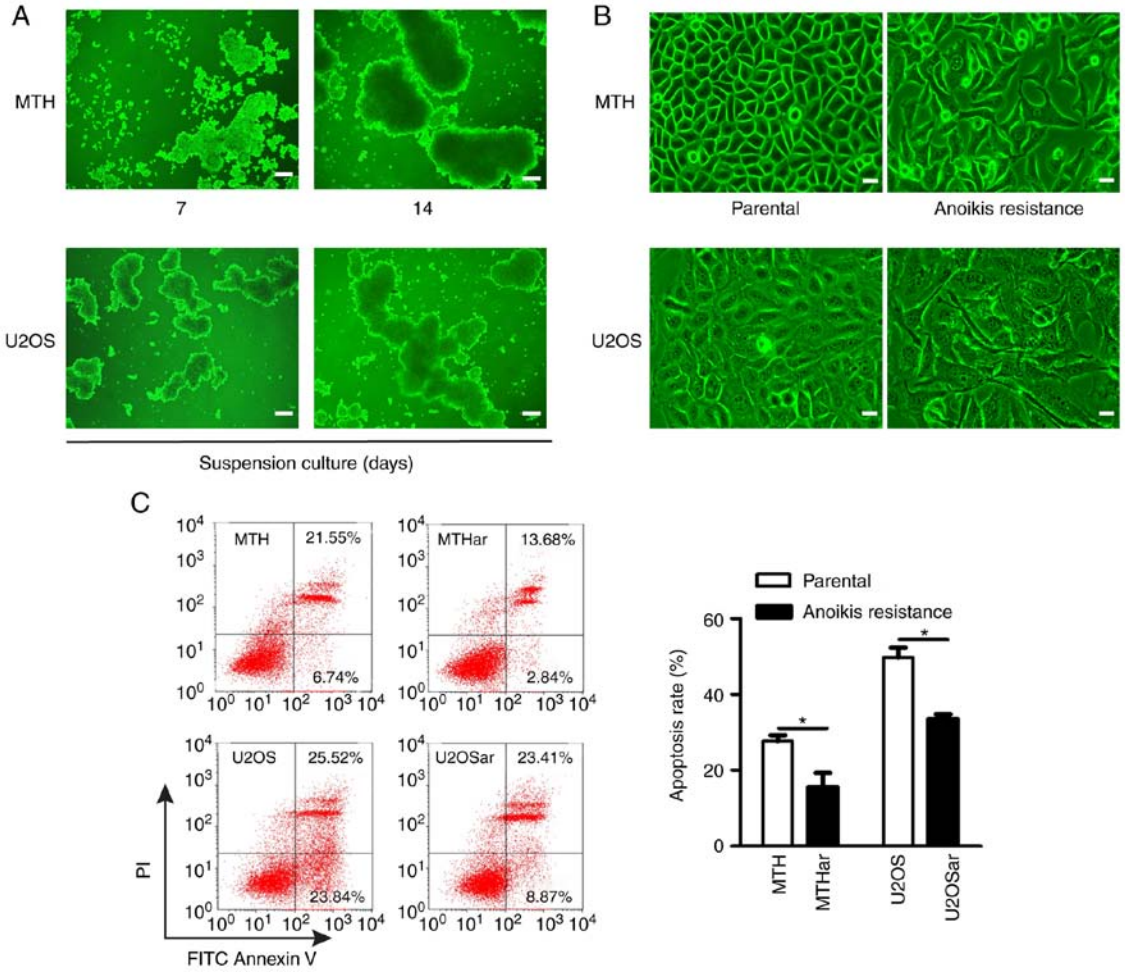

Figure 1. Characteristics of parental and anoikis-resistant osteosarcoma cells (MTH, MTHar, U2OS and U2OSar). (A) Osteosarcoma cells were round and grew in clusters gradually in suspension culture. Scale bar, 100 μm. (B) The morphology of parental and anoikis-resistant osteosarcoma cells. Scale bar, 25 μm. (C) Flow cytometry demonstrating that anoikis-resistant osteosarcoma cells had significantly decreased apoptosis compared with parental cells under suspension conditions. Results are expressed as the mean ± SD. *P<0.05.

Src inhibitor reduces the expression of VEGF-A and angiogenesis and inhibits JNK and ERK pathway activity. Src kinase activationis frequently detected in a variety of anoikis-resistant tumor cells as demonstrated in Fig. 3G. Recent research has focused on kinases directly modulating the apoptosis machinery in anoikis resistance. However, the relationship between p-Src and VEGF-A has been poorly explored in anoikis-resistant osteosarcoma cells in humans. To verify whether the expression of VEGF-A involved Src kinase activation in anoikis-resistant osteosarcoma cells, we pretreated anoikis-resistant cells with an Src inhibitor (PP2) for 24 h. PP2 inhibited the expression of p-Src and VEGF-A in the osteosarcoma cells resistant to anoikis (Fig. 3D-G). Compared with the control group, PP2 also reduced HUVEC proliferation, migration and tube formation (Fig. 3A-C). In various cancers, the MAPK (JNK/ERK/p38) signaling pathway is a downstream molecule of Src kinase (24). Accordingly, we investigated whether MAPK (JNK/ERK/P38) signaling pathway molecules were involved in the expression of VEGF-A induced by p-Src in anoikis-resistant osteosarcoma cells. We investigated JNK and ERK phosphorylation in anoikis-resistant cells and p-p38 was not altered; PP2 decreased JNK and ERK phosphorylation in anoikis-resistant cells (Fig. 3G). These results revealed that JNK/ERK may be an Src target downstream in controlling VEGF-A expression. Altogether, our findings indicated that Src kinase activation induced the expression of VEGF-A and angiogenesis via the JNK/ERK pathway activation in anoikis-resistant osteosarcoma cells.

VEGF-A, Src and MVD clinical importance in osteosarcoma. We quantified the expression of P-Src and VEGF-A in osteosarcoma tissues from humans using IHC staining, and the areas of positive expression were mainly localized in the (Fig. 4). To elucidate their clinical significance, we assessed the correlation between P-Src and VEGF-A expression and the patients' available clinicopathological parameters (Table I). Both P-Src and VEGF-A expression was significantly associated with lung metastasis, and there was an association between P-Src expression and Enneking stage, but not with age, sex, tumor size, pathological type and recurrence. The positive rate of P-Src and VEGF-A expression was 50% (13/26) and 61.5% (16/26), respectively, in the osteosarcoma samples (Table I). The relationship between P-Src and VEGF-A expression was calculated and showed that high P-Src expression correlated with expression of VEGF-A (r=0.474; P=0.014; Table II). To assess the P-Src-angiogenesis association, we detected the MVD via

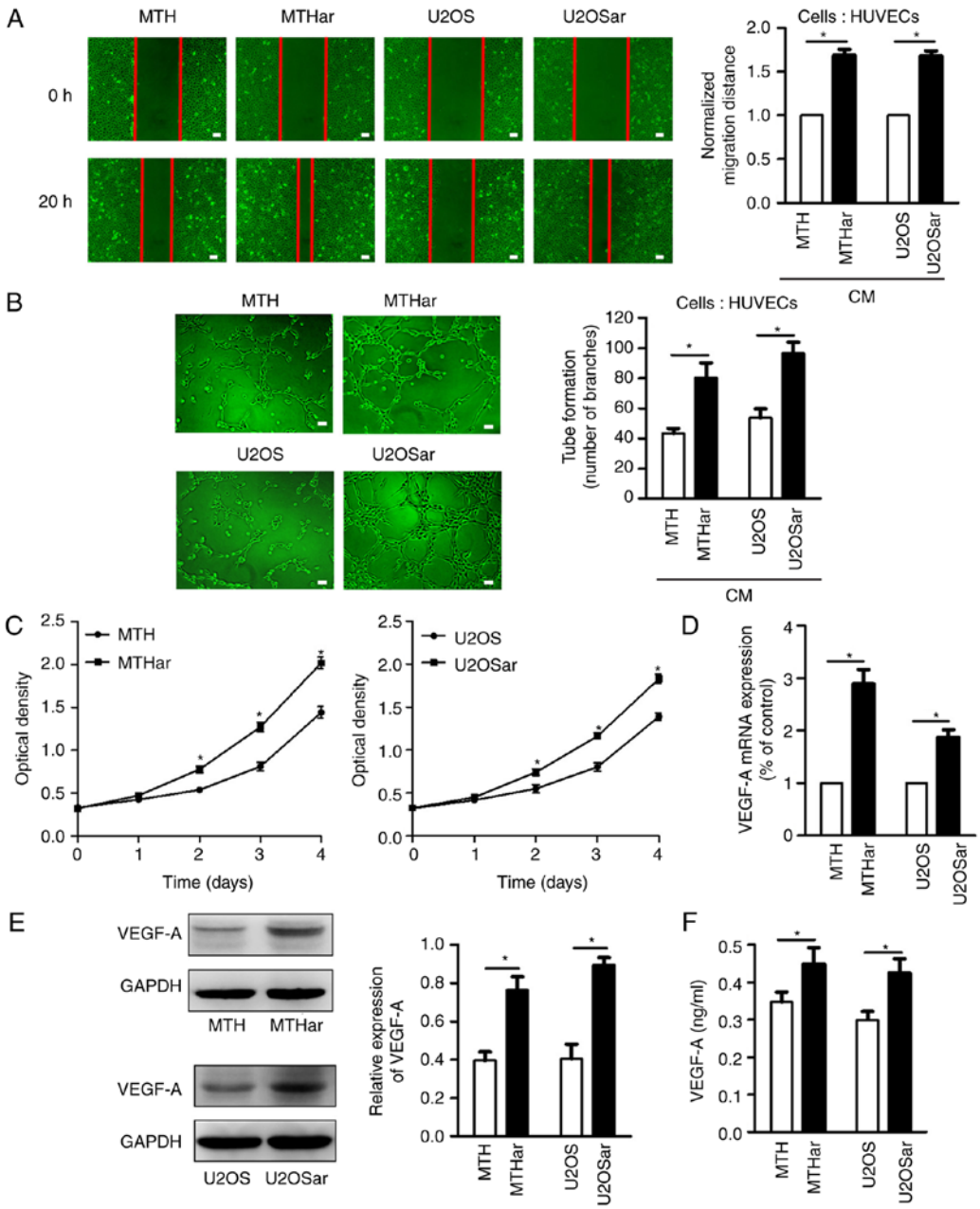

Figure 2. Anoikis-resistant osteosarcoma cells enhances angiogenesis by increasing VEGF-A expression. (A-C) Cultured medium was collected as CM (MTH, MTHar, U2OS and U2OSar cells) and applied to HUVECs. HUVEC capillary-like cell migr ation, structure formation, and proliferation were examined by (A) wound healing, (B) tubeformation and (C) cell proliferation assays, respectively. Scale bar, 100 μm. (D-F) VEGF-A mRNA and protein expression in parental and anoikis-resistant osteosarcoma cells was detected by (D) RT-qPCR, (E) western blotting and (F) ELISA. Each experiment was performed in triplicate. Results are expressed as the mean ± SD. *P<0.05.

CD31 IHC staining in osteosarcoma tissues (Fig. 4). MVD was significantly higher in tumors with positive expression of P-Src (r=0.545; P=0.004; Table II). Taken together, these data demonstrate that P-Src and VEGF-A may be important clinical markers in human osteosarcoma with lung metastasis, and Src kinase may promote angiogenesis via VEGF-A expression in osteosarcoma development.

Resistance to anoikis in osteosarcoma cells from humans promotes angiogenesis in a mouse xenograft model. We found that VEGF-A expression and HUVEC proliferation, migration, and tube formation were enhanced in anoikis-resistant osteosarcoma cells via Src kinase activation *in vitro*. To verify

Table II. Associations between p-Src expression and VEGF-A expression and MVD in patients with osteosarcoma.

		VEGF-A		MVD	
p-Src	n	Positive	Negative	High	Low
Positive	13	11	2	11	2
Negative	13	5	8	4	9
r		0.474		0.545	
P-value		0.014[a]		0.004[a]	

[a]Spearman's correlation analysis.

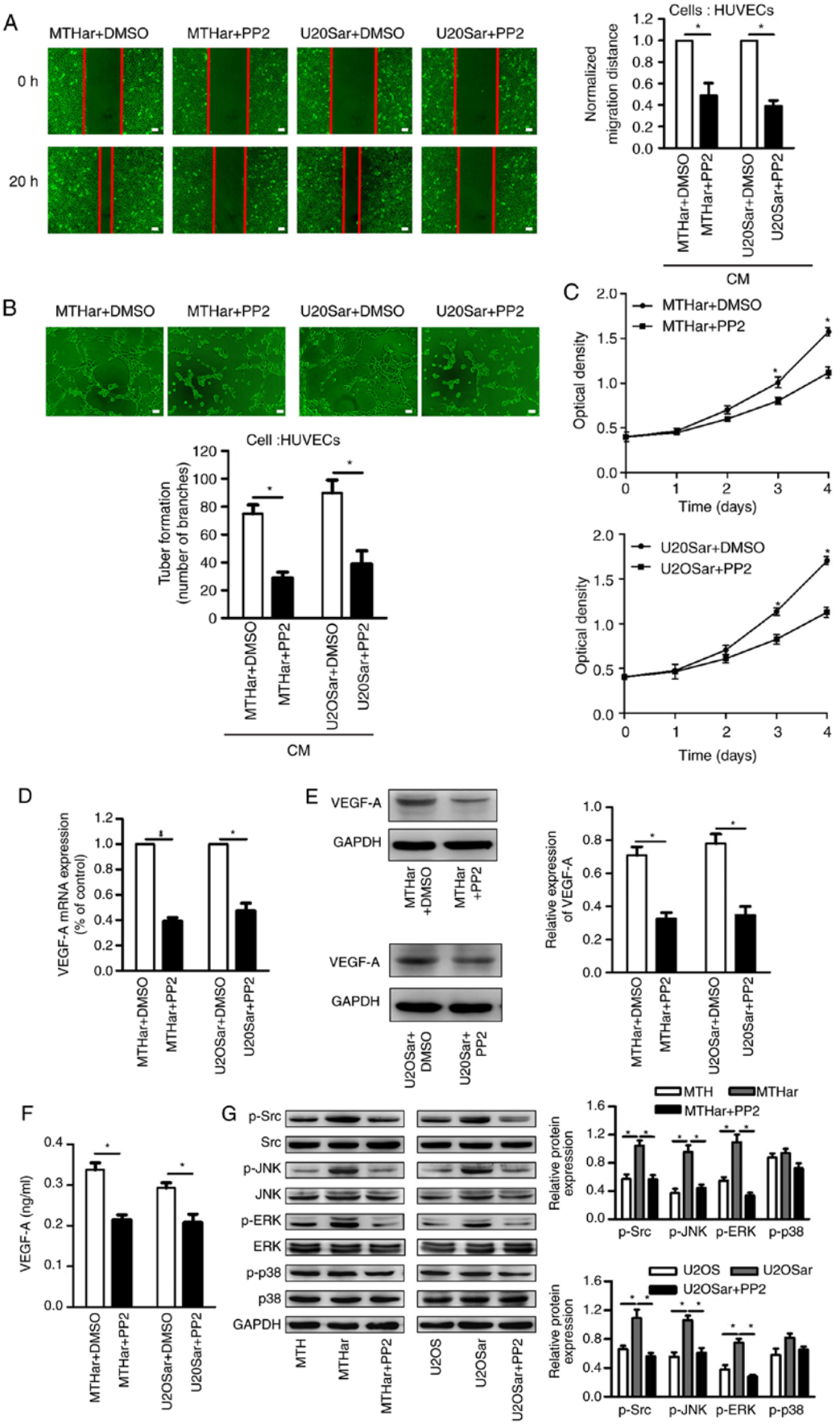

Figure 3. Src inhibitor reduces VEGF-A expression and inhibits angiogenesis in anoikis-resistant osteosarcoma cells. Anoikis-resistant osteosarcoma cells were pre-treated with Src inhibitor (PP2) and dimethyl sulfoxide (DMSO) for 24 h. (A-C) CM was applied to HUVECs. HUVEC capillary-like cell migration, structure formation, and proliferation were examined by (A) wound healing, (B) tube formation and (C) cell proliferation assays, respectively. Scale bar, 100 μm. (D-F) VEGF-A mRNA and protein expression were detected by (D) RT-qPCR, (E) western blot analysis and (F) ELISA. (G) Western blot detection of Src, JNK, ERK and p38 phosphorylation. Each experiment was performed in triplicate. Results are expressed as the mean ± SD. *P<0.05.

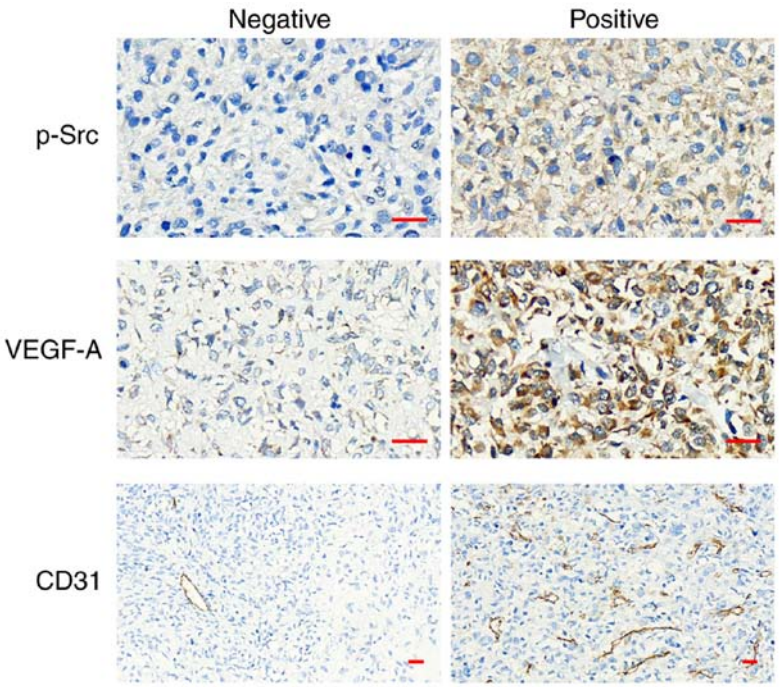

Figure 4. Representative IHC staining of p-Src, VEGF-A and CD31 in osteosarcoma specimens. Images for p-Src and VEGF-A were obtained under an x400 magnification and that for CD31 was obtained under an x200 magnification. Scale bar, 30 μm.

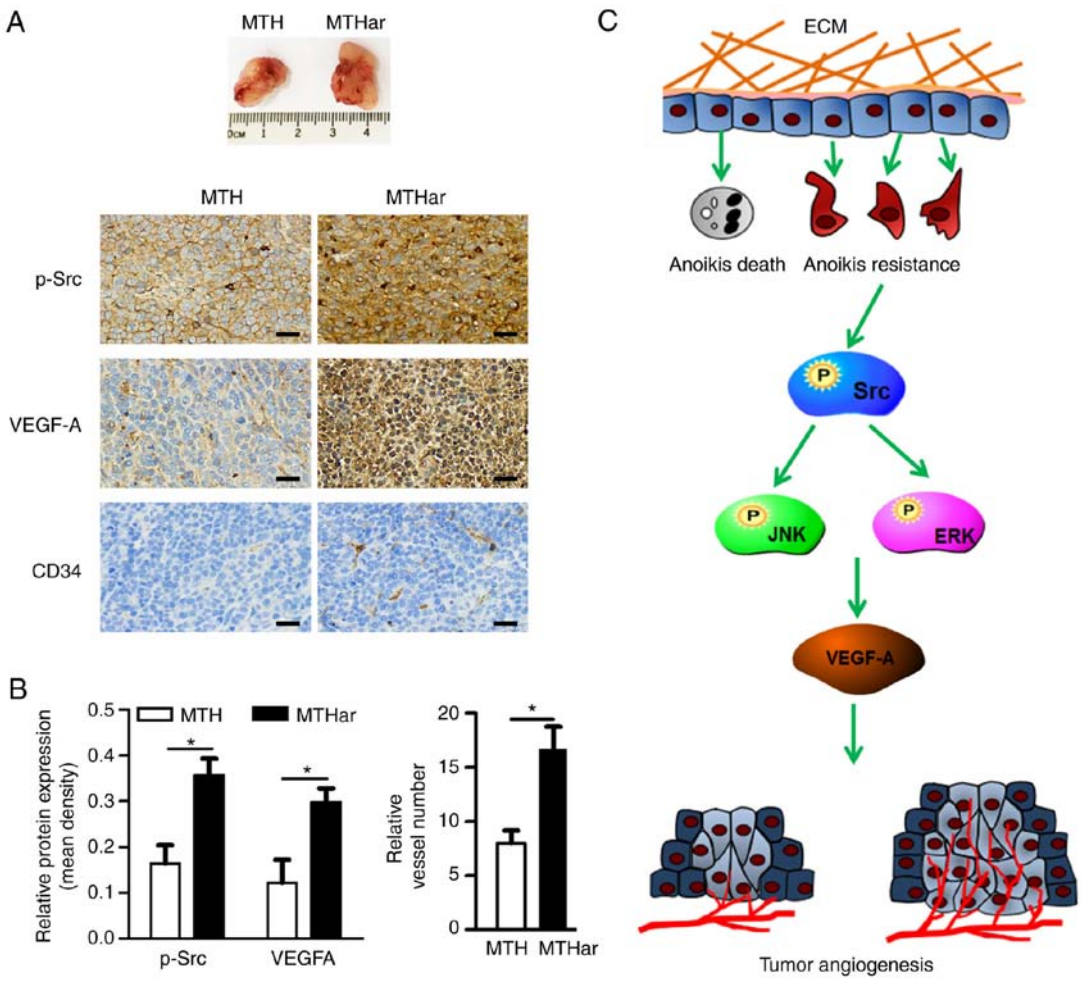

Figure 5. Anoikis resistance in human osteosarcoma cells promotes angiogenesis in a nude mouse xenograft model. (A) Representative immunohistochemical staining of p-Src, VEGF-A and CD34 inxenograft tumors. Scale bar, 30 μm. (B) p-Src and VEGF-A expression was higher in anoikis-resistant cells, as was MVD. Results are expressed as the mean ± SD. *P<0.05. (C) Proposed model for illustrating the mechanism of angiogenesis:anoikis-resistant human osteosarcoma cell enhanced VEGF-A expression andangiogenesis via the Src/JNK/ERK signaling pathways.

our experimental results, a xenograft model in mice was used to assess the association between anoikis resistance and angiogenesis. p-Src, VEGF-A and CD34 expression in the xenograft tumors formed in the nude mice were detected using IHC (Fig. 5A). p-Src and VEGF-A expression was higher in anoikis-resistant cells, as was MVD (Fig. 5B). Collectively, these data indicated that anoikis-resistant osteosarcoma cells enhanced osteosarcoma angiogenesis via p-Src and VEGF-A overexpression.

Discussion

Osteosarcoma is a highly malignant bone cancer whose leading cause of death is metastasis, and treatment options are unsatisfactory (31). Resistance to anoikis is a vital aspect of metastasis in several human cancers, including osteosarcoma, in which an indispensable role is played by angiogenesis in its metastasis and growth (31,32). Anti-angiogenesis enables the normalization of tumor blood vessels to improve oxygen transport and enhance chemotherapy efficiency, thereby prolonging survival (33). Anti-angiogenesis is a potential osteosarcoma treatment target. In the present study, we determined the effect of anoikis resistance on the expression of VEGF-A in human osteosarcoma, i.e., it subsequently increased angiogenesis. Anoikis-resistant subpopulations of human osteosarcoma cells had upregulated VEGF-A and increased angiogenesis via the Src/JNK/ERK signaling pathways (Fig. 5C).

Due to the high pulmonary metastasis in osteosarcoma, it is vital to elucidate the metastasis-controlling mechanisms in order to improve poor prognosis. Metastasis involves cancer cells migrating from primary sites, invading through the basement membrane, surviving in the vascular system, and proliferating in a distant target organ (7,10). A barrier is presented when tumor cells typically detach from the primary site before entering the bloodstream; apoptosis resulting from the loss of cell connection to the ECM, or cell adhesion in an inappropriate location, is termed anoikis (Greek for homelessness) (34). The intrinsic or extrinsic apoptosis pathway mediates anoikis initiation and execution, and it likely prevents ectopic growth of cells at unsuitable sites. Anoikis is simply a special type of apoptosis, there is no special marker. Some studies have reported that the overexpression of cell adhesion molecules CEACAM6 and CD44V6 were associated with cancer cell mobility, poor overall survival and lung metastasis in osteosarcoma (35,36). However, no studies have reported that these molecules were regarded as the specific markers to predict anoikis in osteosarcoma tissues. Anoikis suppression following cancer cells disassociating from the ECM is an important step in tumor malignancy and metastatic progression. Therefore, acquiring the ability to resist anoikis is a critical step in these metastatic cell subpopulations. Many investigations have indicated the involvement of multiple molecular pathways in conferring anoikis resistance in cancer cells, including activation of the pathways of phosphatidylinositol 3-kinase (PI3K)/AKT, Src and Wnt/β-catenin signaling, caveolin-1 downregulation, c-Met activation, interaction of ezrin/β4 integrin and the Bcl family (37,38). Previously, we determined that the Src/AKT pathway played vital roles in osteosarcoma anoikis resistance and metastasis (21).

Osteosarcoma is a highly malignant, blood vessel-rich bone tumor, and the maintenance of tumor growth and metastasis rely on angiogenesis (6,39). Without the formation of a new vascular network, tumor cells would not acquire the necessary oxygen and nutrients for tumor invasion, metastasis and growth (6). The pro-angiogenic and anti-angiogenic factor imbalance results in tumor angiogenesis (40). In particular, there is an important function of VEGF-A in tumor angiogenesis, including in osteosarcoma.

Commonly, osteosarcoma has a tendency to develop distant metastasis, particularly to the lungs, by blood circulation, resulting in death. Angiogenesis is essential for anoikis-resistant cells adherence to distant sites and establishment of metastatic lesions. VEGF-A was revealed to be upregulated in anoikis-resistant sublines in intestinal epithelial cells and prostate cancer cells (32,41). Research has reported VEGF-A upregulation in osteosarcoma and positive correlation with poor prognosis and lung metastases (15,16). In the present study, VEGF-A expression was significantly higher in anoikis-resistant human osteosarcoma cells in contrast to that of parental cells (Fig. 2D-F), subsequently promoting HUVEC migration, tube formation and proliferation *in vitro* (Fig. 2A-C). In addition, we determined that VEGF-A expression was significantly associated with lung metastasis in osteosarcoma specimens (Table I). Our findings indicated that human osteosarcoma cells resistant to anoikis had significant angiogenesis by increasing VEGF-A expression, and VEGF-A expression may be an important clinical predictive marker of pulmonary metastasis in osteosarcoma.

Src kinase activation is considered a prometastatic pathway in several types of tumors, since vital oncogenic mechanisms such as cell proliferation, resistance to apoptosis, invasion and adhesion are involved in it (42). p-Src expression was revealed to be high in osteosarcoma tissue (64.7%) and was significantly associated with metastasis and clinical stage (43). Notably, Src kinase activation was frequently detected in anoikis-resistant osteosarcoma cells, and its activation was associated with angiogenesis through increased proangiogenic factor expression, e.g., VEGF-A and IL-8 (19,21). Using IHC, we determined a significant association between the expression of p-Src and lung metastasis and Enneking stage in osteosarcoma specimens (Table I). Furthermore, the expression of p-Src and VEGF-A was positively correlated (r=0.474; P=0.014; Table II), and there was an association between positive expression of p-Src and significantly higher MVD *in vivo* (r=0.545; P=0.004; Table II). In the present study, Src activation was detected in anoikis-resistant human osteosarcoma cells, and the Src inhibitor reduced the expression of VEGF-A and angiogenesis (Fig. 3A-F). We determined that Src kinase activation played a key part in stimulating the expression of VEGF-A and angiogenesis in anoikis-resistant osteosarcoma cells in humans, indicating that, in osteosarcoma, Src kinase may be a novel treatment target against angiogenesis and metastasis.

As a downstream molecule of Src kinase, the MAPK cascade pathway is a key regulator of oncogenic characteristics such as invasion, proliferation, apoptosis, angiogenesis and metastasis in osteosarcoma, Ewing sarcoma and chondrosarcomas (44). The MAPK pathway is comprised of 3 families: JNK, ERK and p38/SAPK (stress-activated protein kinase) (45). ERK and p38 signaling pathway activation was detected in ovarian cancer

under AI growth conditions (46). In this study, JNK and ERK phosphorylation in anoikis-resistant osteosarcoma cells was investigated; p38 was not activated in this cancer type, and Src inhibitor inhibited JNK and ERK pathway activity (Fig. 3G). p38 activation is believed to regulate apoptosis (anoikis) and/or inflammation (47). However, a number of studies have shown that p38 plays an important role in the developmental processes and progression of manysolid tumors (48,49). These seemingly contradictory biological properties may beexplained by the fact that genetic and epigenetic alterations involve cancer onset and progression. The data indicated that the MAPK pathway may be important in resistance to anoikis in osteosarcoma and that it has an important function in the expression of VEGF-A.

In conclusion, VEGF-A expression and angiogenesis were enhanced in anoikis-resistant subpopulations of osteosarcoma cells from humans via the Src/JNK/ERK signaling pathways. Therefore, Src kinase may be a new treatment target against metastasis and angiogenesis in osteosarcoma. Our findings demonstrated the underlying mechanisms by which resistance to anoikis in osteosarcoma cells from humans promoted angiogenesis.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

The conceptualization and the formal analysis was conducted by QNG. ZG was responsible for the conceptualization, formal analysis, data analysis and interpretation, the figure preparation and was also involved in the writing. GSZ was involved in the conceptualization, data analysis and interpretation, and performed the mouse xenograft model experiments. YL was involved in the conceptualization and data analysis. DP, HS and XT performed the clinical sample collection, the IHC assay and the data analysis. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experiments were approved by the Ethical Committee of theThird Military Medical University (Army Medical University, Chongqing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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