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The Role of VE-cadherin in Osteosarcoma Cells

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Abstract Osteosarcoma cells can generate vasculogenic-like, patterned networks to obtain nutrients and oxygen, which mimic some function of endotheial-like cells and facilitate tumor malignant progress. These cells also express vascular endothelial-cadherin (VE-cadherin), which is generally accepted as a strictly endothelial-specific transmembrane protein. However, its role is still relatively obscure in osteosarcoma cells. So we inhibit the VE-cadherin gene expression with siRNA in osteosarcoma cells (MG63), and culture those cells in three-dimensional medium, containing Type I collagen or Matrigel, to observe the role of VE-cadherin. Western blotting analysis show that sequence-specific siRNA can significantly decrease the expression of VE-cadherin in MG63 cell. After knockdown of VE-cadherin, osteosacoma cells cann't induced angiogenic sprout and form osteosarcoma-generated, endotheial-like networks. Our data indicate that VE-cadherin may be a positive and specific regulator not only in angiogenesis, but also in vasculogenic mimicry of osteosarcoma cells. And it can be considered as a new prospective option in the combining treatment of aggressive tumor with highly vascularity, including osteosarcoma.

Keywords VE-cadherin · Osteosarcoma cell · Vasculogenic mimicry · siRNA

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Introduction

Osteosarcoma, as one of the most malignant and aggressive tumors, has seriously impaired the health of patients. A large amount of blood vessels supply is one important characteristic of osteosarcoma, so osteosarcoma can be an excellent model for the study of vascular system in solid tumor. In 1999, Maniotis [1] first proposed that aggressive melanoma cells might generate vascular channels without endothelial cells or fibroblasts, where the tumor cells had the shape and function like endothelial or progenitor cells. After that, more and more researches focus on vasculogenic mimicry (VM) of aggressive melanoma cells [2–5].We had previously demonstrated the ability of the vascular lumen formation by osteosacoma cells and proved that osteosacoma cells may express many angiogenic factors and receptors, including VE-Cadherin, promoting tumor vascular formation.

VE-cadherin, originally called cadherin-5, is generally accepted as a strictly endothelial-specific transmembrane protein of the cadherin family, located at cell-to-cell adherens junctions. In addition to promoting cell adhesion and controlling vascular permeability, VE-cadherin transfers intracellular signals that contribute to endothelium integrity and vascular stabilization [6–8]. The roles of VE-cadherin in controlling endothelial cell contacts and influencing endothelial cell behavior are more and more

awared. But its expression in the osteosacoma cells is still relatively obscure. In this experiment, we inhibit the VE-cadherin gene expression with VE-Cadherin siRNA and culture osteosarcoma cells in three-dimensional medium, containing type I collagen or Matrigel, to explore its influence on vasculogenic mimicry of osteosarcoma cell.

Materials and Methods

Materials

Osteosarcoma cell MG63 (American Type Culture Collection); Dulbecco's minimal essential medium (DMEM), 0.25% Trypsin, 10%Fetal bovine serum (GIBCO Company); Matrigel-collagen, Type I collagen (American BD Company); Effectene Transfection Reagent (German Qiagen Company); DNA polymerase, Restriction endonuclease, T4 DNA ligase et al. (Japanese TaKaRa Company); Secondary peroxidase-conjugated goat antimouse antibodies (Beijing Dingguo Company); Primary anti-VE-Cadherin antibodies (American R&D Company); Acrylamide, TEMED, Ammonium persulfate (American Sigma Company); pSilencer neo (American Ambion Company); E. coliXLI-Blue (Dept of Biochemistry, Fudan University).

Methods

1. Formation of specific siRNA targeting VE-Cadherin: According to optimization principle of siRNA, we designed 19nt sequence-specific siRNA targeting VE-Cadherin. All chemically synthesized oligonucleotides were obtained from shenyou company of shanghai. DNA sequences of the double-stranded siRNAs are as follows: sense 5′-GATCCCGAACCAGAAGAAGCCTCTGATTCAAGAGATCAGAGGCTTCTTCTGGTTTTTTTTGGAAA-3′; antisense 3′-GCTTGGTCTTCTTCGGAGACTAAGTTCTCTAGTCTCCGAAGAAGACCAAAAAAAACCTTTTCGA-5′. According to directions of the manufacturer, the products were phosphated, annealed, attached by T4DNA ligase, transformed with ligated plasmid DNA. After transformation, the colonies were amplified and purified using a miniprep purification kit. At first, plasmid DNAs incised and verified on an 15% non-denaturing polyacrylamide gel. Further, the correct plasmid DNAs were sequenced by shenyou company of shanghai. After those proof-test, the plasmid DNAs with correct sequences were then amplified and purified with QIAGEN Maxi Plasmid DNA kit and used to transfect MG63 cells in the following experiment.
2. General cell culture: osteosarcoma cells(MG63) were cultured in DMEM medium, containing 10% (*v*/*v*) heat-inactivated fetal calf serum and maintained at 37°C in an incubator containing 95% air and 5% CO2. The cells were harvested at monolayer confluence with 0.25% trypsin-0.02%mM EDTA, then subcultured for experiments, such as transfection, three-dimensional culture.
3. siRNA transfection: MG63 cells (7.5×10^5) were seeded in 35 mm plates with 1,000 μL DMEM. And MG63 cells (1×10^4) were seeded in 96 well plates with 100 μL DMEM. After incubated to 40–80% confluence, according to the manufacturer's instructions, MG63 cells were transfected with siRNA. Through the G418, the surviving cell population can then be maintained and assessed for reduction of target gene expression.
4. Three-dimensional culture of osteosarcoma cells:
   - 4.1 Thin gel For all experiments, 250 ul of Matrigel or Type I collagen (Collaborative Biomedical) was 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 then seeded on top of the gels respectively and supplemented with DMEM medium, containing 10% (*v*/*v*) heat-inactivated fetal calf serum and allowed to incubated at 37°C in an incubator containing 95% air and 5% CO2. Fresh medium was added and replaced on the second day. MG63 cells cultured were observed during the incubation period in 3D cultures
   - 4.2 Thick gel 500 ul of Collagen gels were made by mixing together ice-cold gelation solution (seven volumes of type I collagen were mixed with two volumes of 5× concentrated DMEM and one volume of 0.05 N NaOH containing 2.2% NaHCO3 and 200 mmol/L HEPES). The collagen mixture was allowed to gel at 37°C. Then, cells (10^6/ml) were plated on the top of a cell-free gelled collagen layer, and the medium was added.
5. Hematoxylin and eosin (HE) stain: hematoxylin and eosin (HE) stain: After glass coverslips with sample of three-dimensional culture were take out, the sample were fixed in buffered formalin, embedded in paraffin and thin sections were cut. Sections were stained with hematoxylin and eosin in succession. Each section stained by hematoxylin and eosin (HE) were observed using light microscopy.
6. Western blot: osteosarcoma cells was harvested and placed in 100 μL buffer(Tris, NaCl, 20%Triton-X-100, and protease inhibitors)/60 mm plate. Immediately afterward, cell samples were sonicated on ice and incubated for 5 min at 95°C before centrifugation on ice. The extract Protein content was measured, and was subject to 15% PAGE, followed by transferring to polyvinylidene difluoride (PVDF) membrane electro-

phoretically. Membranes were blocked for 1 h at room temperature with 5%milk in PBST, followed by overnight incubation at 4°C in a concentration of 1:1,000 VE-Cadherin primary antibody. The appropriate secondary antibody concentration of 1:5,000 was used to incubate the membrane for 2 h at RT, after which the membrane was washed in PBST. They 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 measured by densitometry.

Results

1 pSilencer-VE-Cadherin siRNA expressing plasmid:

At first, plasmid DNAs were incised and verified on an 15% non-denaturing polyacrylamide gel (the figure was abridged). Further the correct plasmid DNAs were sequenced by shanghai shenyou company. It is confirmed that the presence of siRNA insert (GAACCAGAAGAAGCCTCTGATTCAAGAGATCAGAGGCTTCTTCTGGTT) and there is no unwanted mutation in the pSilencer plasmids.

2 Western blotting

After pSilencer-VE-Cadherin siRNA transfection, VE-Cadherin expression in MG63 cells was reduced, unlike two control(non-transfection and pSilencer siRNA transfection). Western blotting indicated that VE-Cadherin protein expression in MG63 cells transfected pSilencer-VE-Cadherin is significantly decreased in contrast to those of non-treated or pSilencer-treated cells. However, VE-Cadherin protein expression has no obviously difference between non-transfected and pSilencer-transfected cells. As shown by Western blot (Fig. 1).

3 Vasculogenic Mimicry

3.1 The structures MG63 cells formed are observed in Matrigel culture: After the transfection of pSilencer, MG63 cells form the tubular networks like endothelial cells. In contrast, no structures of the tubular networks formed by MG63 cells after transfected with pSilencer-VE-Cadherin (Fig. 2)

3.2 The structures MG63 cells formed in the thin collagen culture: the conglomeration of the cells involved in tube formation is detectable in type I collagen culture, when MG63 cells transfected with pSilencer were cultured for 2 days. Those MG63 Cells were then incubated further until the end of 1 week period, after that time the structures of the tubular networks are observed. Whereas, MG63 cells transfected with pSilencer-VE-Cadherin were observed that they cannot form structures of conglomeration of the cells or the tubular networks after 1 week (Fig. 3).

3.3 The structures MG63 cells formed in the thick collagen culture:

3.3.1 MG63 cells invading inside type I collagen is observed by phase contrast microscope about 10 h. At early stage, MG63 cells transfected with pSilencer form the loop and contact with each other inside the type I collagen. In contrast, after transfection of pSilencer-VE-Cadherin, MG63 cells cannot form the loop inside the type I collagen (Fig. 4).

3.3.2 MG63 cells invading inside type I collagen is observed by phase contrast microscope after 1 week: MG63 cells transfected with pSilencer, like endothelial cells, migrated towards a differentiation zone, where cells elongate and align in tandem, subsequently they adhere to each other to form the tubular-like networks with cell to cell contact methods inside the type I collagen. In contrast, MG63 cells transfected with pSilencer-VE-Cadherin cannot form the tubular-like networks inside the type I collagen (Fig. 5).

3.4 The cross sections of tubules formed by MG63 cells is observed in type I collagen culture with phase contrast microscope and HE stain after 1 week: MG63 cells transfected with pSilencer form the tubular networks, which can be observed in the intersecting plane of type I collagen. In contrast, it was not observed that MG63 transfected with pSilencer-VE-Cadherin cells can form the tubular networks in the cross sections of type I collagen (Fig. 6).

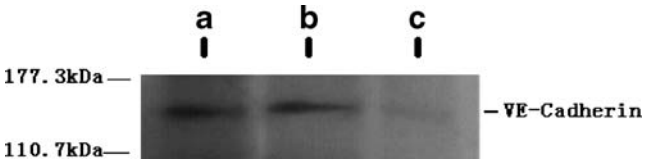

Fig. 1 The express of VE-Cadherin protein in osteosarcoma cell **a** non-transfection; **b** pSilencer transfection; **c** pSilencer-VE-Cadherin transfection

Discussion

Osteosarcoma, arising from mesenchymal bone-forming cells, is the most common highly malignant bone tumor in children and adolescents. The large amount of vascular system existing in osteosarcoma provides nutrients and oxygen necessary for tumor cell. So anti-vascular treatment has emerged as a great promising strategy. And further

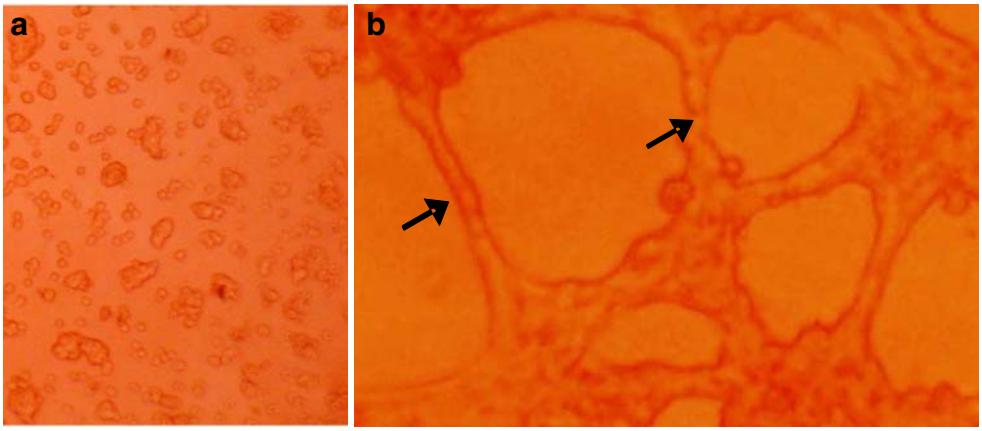

Fig. 2 The structures formed by MG63 cells are observed in Matrigel culture with phase contrast microscope about 24 h **a** no structures of the tubular networks formed by MG63 cells after transfected with pSilencer-VE-Cadherin (×400); **b** the structures of the tubular networks formed by MG63 cells transfected with pSilencer (×400)

research on the manchanism of vascular formation is necessary. Recently, all the theory discussed extensively, such as vasculogenesis, angiogenesis, vasculogenic mimicry, to explain vascularization of tumor reveal that the angiogenically activated phenotype plays a key role in the vascular formation [9–11]. However, the intrinsic property of tumor cell is the major determinant for angiogenic phenotype transition and may be the critical reason for

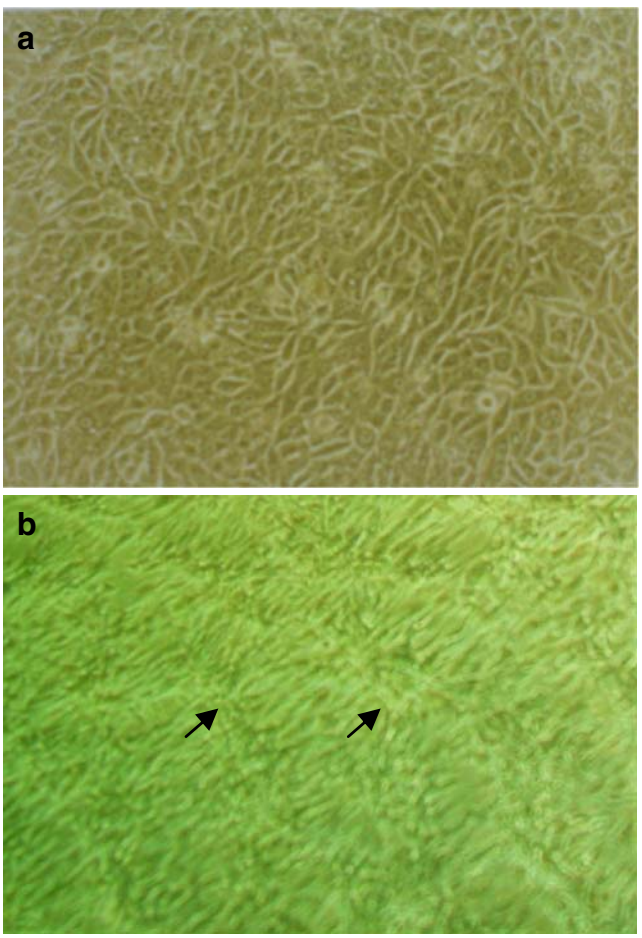

Fig. 3 The structures formed by MG63 cells are observed in type I collagen culture with phase contrast microscope **a** no structures of the tubular networks formed by MG63 cells transfected with pSilencer-VE-Cadherin after 1 week, (×400); **b** the representative structures of the tubular networks formed by MG63 cells transfected with pSilencer after 1 week (×400)

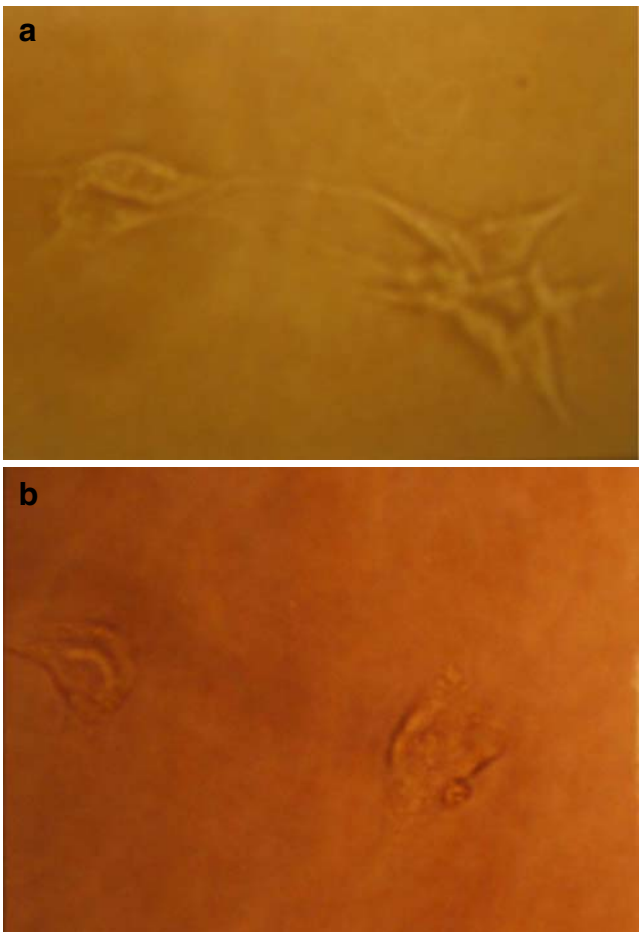

Fig. 4 MG63 cells was observed in type I collagen with phase contrast microscope for 10 h **a** MG63 cells transfected with pSilencer form the loop-like structures about 10 h (×600) **b** no loop-like structures formed by MG63 cells transfected with pSilencer-VE-Cadherin (×600)

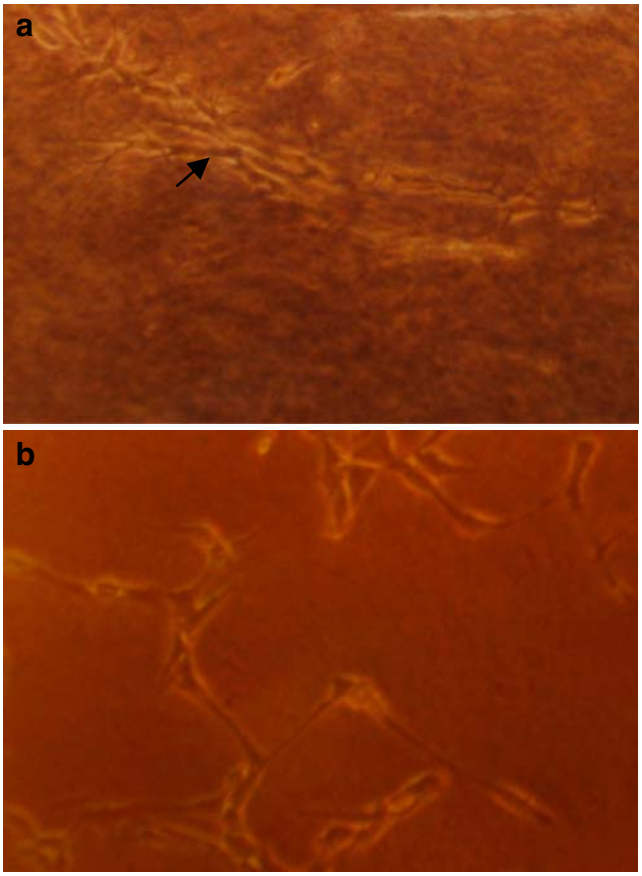

Fig. 5 The structures formed by MG63 cells are observed inside type I collagen with phase contrast microscope after 1 week **a** cells transfected with pSilencer form the tubular-like networks with cell to cell contact methods (×400) **b** MG63 cells transfected with pSilencer-VE-Cadherin do not form the structures of tubular-like networks (×400)

initiating and maintaining the distinctive blood vessel of the whole tumor, which differents from normal physiological and other pathological conditions. Further study on the vasculogenic mimicry of osteosarcoma cells and the angiogenic marker's function will provide the basis for the osteosarcoma therapy.

VE-cadherin, the Ca(2+)-dependent cell–cell adhesion molecules, can express in the endothelial precursor (EPC) and mature endothelial cells. And it provides an excellent gene marker of identifying genes which are expressed during endothelial differentiation and distinct from hematopoiesis [12]. So more experiments about it focus on the endothelial cell. It is proposed that VE-cadherin may promote cell–cell adhesion through its cytoplasmic domain, linking intracellular partners such as b-catenin, p120 and plakoglobin [13]. Because the differential activities of VE-cadherin reflect the versatile behavior of endothelial cells switching from a vascular quiescence to an angiogenic state [14]. In addition to being an essential protein of adherens junctions of endothelial cells, VE-cadherin plays a pivotal role in vascular homeostasis. On the other hand, VE-cadherin at adherens junctions can upregulate the gene encoding the tight junctions adhesive protein claudin-5 in endothelial cell [15]. Moreover annexin 2, an actin-binding protein, can connect the VE-cadherin-catenin complex to the actin cytoskeleton. Those novel link promotes endothelial cell–cell-adhesion-based motility and is labile for the advancement of vascular sprouts within a moving tissue environment [16].

Besides the specificial role of VE-cadherin on vascular endothelial cells, the constitutive presence of VE-cadherin in some non-endothelial cells has caused more attention, notably those of metastatic tumors [17]. In melanoma, VE-cadherin and EphA2 are found to be colocalized in cell–cell adhesion junctions, and they can coordinate as a key regulator in the process of melanoma VM. Moreover, VE-cadherin can regulate the expression of EphA2 at the cell

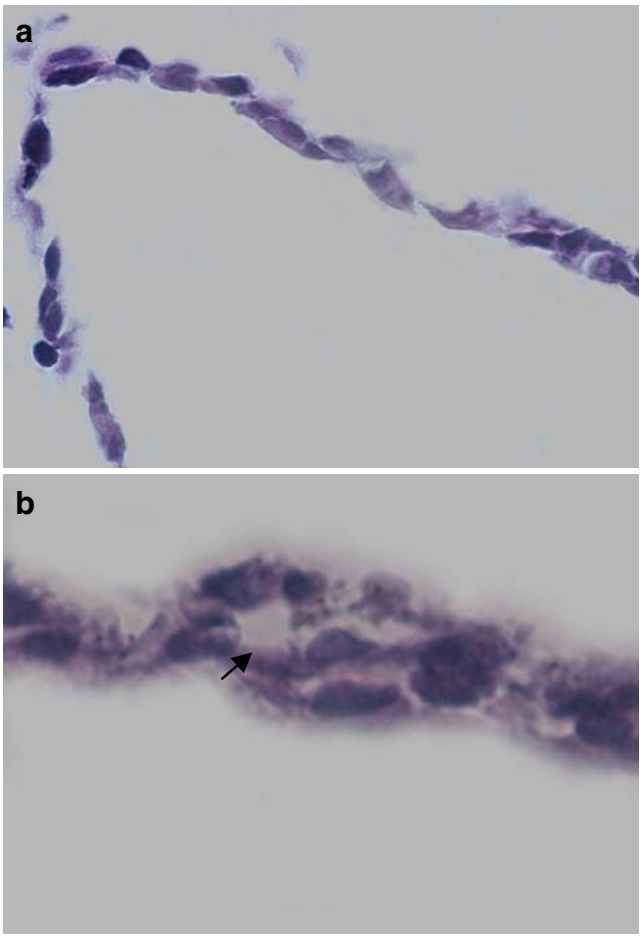

Fig. 6 The intersecting plane formed by MG63 cells is observed in type I collagen culture with phase contrast microscope and HE stain after 1 week **a** no tubular structure is formed by MG63 cells transfected with pSilencer-VE-Cadherin (×400); **b** one representative tubular structure is formed by MG63 cells transfected with pSilencer (×600)

membrane by mediating its phosphorylation, through interacting with its membrane bound ligand, ephrin-A1 [5, 18]. Previously, we have demonstrated that EPHA2 gene may be necessary for osteosarcoma cells in vasculogenic mimicry. Additional papers have shown that the factors, such as Twist, Slug, Snail, Serum response factor (SRF), Id2, PTEN, (protein tyrosine phosphatase 1B) PTP1B, ADAM10, appear to be involved in VE-cadherin regulatory process. Moreover, Gal-3 and Id2 plays an essential role in the process of vasculogenic mimicry and angiogenic properties associated with melanoma progression.

In this experiment, three dimensional culture medium contains Matrigel or type I collagen. Type I collagen is found in most tissues and organs, and is most plentiful in bones tendon and dermis. Whereas, Matrigel Basement Membrane Matrix extracted from the Engelbreth-Holm-Swarm(EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins. And Matrigel contains many growth factors, such as TGF, FGF, TPA et al. So in contrast to Matrigel, type I collagen may give cells a simpler influence. After cultivated in type I collagen medium, some osteosarcoma cells invading inside the collagen altered significantly in morphology with different cell to cell contact methods. Elongated endothelial-like cells are observed, then some elongated cells aligned in tandem and form the tubular-like networks.

Specific siRNA targeting VE-cadherin can degrade VE-cadherin expression in MG63 cells. Thus the biological function of VE-cadherin is checked completely. Moreover, the osteosarcoma cells growth, in three dimensional culture medium, are further observed under the comparison between the knockdown and unknockdown cells. Lumen formation is blocked in three dimensional culture medium of tumor cells VE-cadherin knockdown. Furthermore, siRNA-mediated knockdown of VE-cadherin can block angiogenic migration. VE-Cadherin may play an essential role in senseing the presence of other adjoining cells, and react by limiting their signalling and cell–cell-adhesion-based motility. VE-cadherin may be required for the advancement of vascular sprouts, where cells migrate along towards its tip, in lumen formation of osteosarcoma cells. So it can reveal that VE-cadherin, an endothelial specific cell–cell adhesion molecule, expressed in osteosarcoma cells may have the role that is re-markably similar to lumen formation in endotheial cells. In this sense, vasculogensic mimicry of osteosarcoma cells is the angiogenesis process involved by endothelial cells origined from tumors [19].

Another should be mentioned is that when endothelial cells are cultured in type I collagen medium, it is generally accepted that growth factors are added. However, any additional growth factors are not added in medium in this experiment. Many proangiogenic factor(we have confirmed previously) excreted by osteosarcoma cells may be partly responsible for the complete lumen formation. So compared with endothelial cells, osteosarcoma cells seem to have more potentia than the endothelial cells in lumen formation. And it is no surprising to hypothesize that they may have the ability of interplay between themselves and the host's endothelial cells, or endothelial progenitor cells based on the paracrine or autocrine method. Thus blood vessels are organized, which makes it possible to provide nutrients and oxygen necessary for tumor cells and the malignancy of tumor. However, the mechanistic details of vasculogenic mimicry need further research. VE-cadherin may be involved in the transdifferentiation of tumor cells into endothelial-like cells and mimic endothelial function in osteosarcoma.

From above mentioned, three-dimensional culture models may not only provide an important tool for the study on angiogenic phenotype and vasculogenic mimicry, but also one optional methods for the observation of tumor cells early invasion, angiogenesis process and efficiency of anti-vascular formation therapy. On the other hand, VE-cadherin involving the vasculogenesis and angiogenesis has been described extensively by other previous research. Our data also support that it may be a positive and specific regulator of vasculogenic mimicry of osteosarcoma cells. So it can be considered as a promising target in the combining treatment of aggressive tumor with highly vascularity, including osteosarcoma.

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