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Short Communication

Exosomes from oral squamous carcinoma cell lines, SQUU-A and SQUU-B, define the tropism of lymphatic dissemination

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is often associated with lymphatic rather than hematogenous metastasis; however, a determinant factor for this process has not been elucidated. This study examined the effect of OSCC-derived exosomes on angiogenesis and lymphangiogenesis, closely related with hematogenous and lymphatic metastasis, respectively. Our data demonstrated that OSCC-derived exosomes stimulated the expression of VEGFs and their receptors, essential regulators of angiogenesis and lymphangiogenesis, in human lymphatic endothelial cells but not in human vein endothelial cells. These results suggest that specific exosomes have differential tropism toward a certain cell type, defining the modality of metastasis.

1. Introduction

In cancer pathology, the subject of metastatic organotropism has been perplexing; however, recent studies have demonstrated that exosomes could be involved in this process [1]; for example, exosomes display delimited cell tropism [2,3]. In the present study, we evaluated the effects of oral squamous cell carcinoma (OSCC)-derived exosomes on angiogenesis and lymphangiogenesis, using HUVECs (human umbilical vein endothelial cells) and HDLECs (human dermal lymphatic endothelial cells) to determine if exosomes display cell tropism toward each endothelial cell type. We recently reported that exosomes from SQUU-B cells (a highly metastatic OSCC cell line) could confer metastatic ability to SQUU-A cells (a non-metastatic OSCC cell line from the same patient) via exosomes. Herein, we used exosomes in a similar manner to that of the previous experiment [4], focusing on the expression of VEGFs and their receptors (VEGFRs), which have been shown to be essential regulators of angiogenesis and lymphangiogenesis [5].

2. Materials and methods

2.1. Cell culture

Two OSCC cell lines (SQUU-A and SQUU-B) established from local recurrent tongue cancer tumors [6] were maintained as described previously [4]. HUVECs and HDLECs, purchased from PromoCell (Heidelberg, Germany), were cultured with endothelial cell growth medium MV2 kit® (PromoCell). All cells were maintained in a 5% CO_2 humidified atmosphere at 37 °C.

2.2. Exosome purification

Exosomes were isolated with the PureExo Exosome Isolation Kit (P100) (Cosmo Bio Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions, and as previously performed [4].

2.3. Tube formation assay

Tube formation assays were performed as described previously [7,8] with minor modifications. Specifically, 96-well plates were coated with 50 µl per well of growth factor reduced Matrigel (BD Biosciences, San Jose, CA). The gel was

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Table 1
Primer sets and probe numbers for qPCR analysis.

Gene	Direction	Primer sequence	Base pairs	Probe
GAPDH	Forward Reverse	AGC CAC ATC GCT CAG ACA C GCC CAA TAC GAC CAA ATC C	66 bp	60
VEGFR1	Forward Reverse	CAG CAT ACC TCA CTG TTC AAG G CCA CAC AGG TGC ATG TTA GAG	75 bp	50
VEGFR2	Forward Reverse	GAA CAT TTG GGA AAT CTC TTG C CGG AAG AAC AAT GTA GTC TTT GC	66 pb	18
VEGFR3	Forward Reverse	CAG GCT GAC GCT GAG GAC AAA GGA CAC CCA GTT GTA ATA CCT	78 bp	64
VEGFA	Forward Reverse	CTA CCT CCA CCA TGC CAA GT CCA TGA ACT TCA CCA CTT CGT	86 bp	29
VEGFC	Forward Reverse	AAG TCC ACA GAA ATG CTT GTT AAA TCG TAC ATG GCC GTC TGT AA	77 bp	31
VEGFD	Forward Reverse	AAA GTT TTA CCA GTA TGG ACT CTC G GAG TTC TTT GCC ATT CTT CAT CTA T	114 bp	89

incubated at 37 °C for 30 min to solidify, after which 1.5×10^4 HUVEC or HDLEC cells, in 150 µl MV2 medium without VEGF, were added to each well. After treatment for 18 h, the cells were photographed at a 4 × magnification (EVOS Cell Imaging System, Life technologies Corporation, Carlsbad, CA). For quantification, branch points in each image were counted.

2.4. Real-time PCR

Total RNA extraction, reverse transcription, and real-time PCR analysis, including cycling conditions, were performed as previously described [4]. The PCR primer sequences and amplicon sizes for each gene are described in Table 1. TaqMan probes specific for each sequence were selected from the LightCycler Universal Probe Library (Roche Diagnostics, Mannheim, Germany) (Table 1).

2.5. Immunoblot analysis

SDS-PAGE and immunoblot analysis were performed as described previously [4]. Primary antibodies used in the experiments include the following: mouse monoclonal anti-GAPDH (diluted 1:40,000; Acris, San Diego, CA) and podoplanin (diluted 1:1000; Biolegend, San Diego, CA), rabbit polyclonal antibodies against VEGFR1 (diluted 1:3000; Cell Signaling Technology, Beverly, MA), VEGFR2 (diluted 1:3000; Cell Signaling Technology), VEGFR3 (diluted 1:1000; Abcam, Cambridge, UK), VEGF-A (diluted 1:3000; Abcam), VEGF-C (diluted 1:1000; Cell Signaling Technology), and VE-cadherin (diluted 1:3000; Cell Signaling Technology), and rabbit monoclonal anti-VEGF-D (diluted 1:3000; Abcam). Horseradish peroxidase-conjugated secondary antibodies targeting rabbit or mouse IgG (diluted 1:40000 for GAPDH and 1:4000 for the others; Cell Signaling Technology) were used.

2.6. Statistical analysis

The Mann–Whitney *U*-test was used to compare the relative mRNA expression, obtained by real-time PCR, and the quantification of viable cells. A *P*-value of less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Effects of SQUU-A and -B-derived exosomes on HUVECs

To examine if exosomes from SQUU-A (exoA) or SQUU-B (exoB) promote tumor angiogenesis, a process that can increase the probability of hematogenous tumor dissemination [9], we first performed *in vitro* tube formation analysis using HUVECs. For analysis, we determined the number of branch points, which is routinely considered as an index for tube-formation [10]. For this parameter (Fig. 1A,B), and for cell viability (Fig. 1C) there were no significant differences among vehicle-, exoA-, and exoB-treated HUVECs, whereas exoB resulted in the formation of numerous unpolarized aggregates at cellular junctions in HUVECs (Fig. 1A). Epithelial cellular aggregates is generally going to establish luminal structures and polarized vesicle trafficking of cell division-related molecules [11]. We therefore speculate that exoB possesses factors that confer angiogenic potential to HUVECs, in comparison to exoA, however, the molecular mechanism of this phenomenon was unclear. We next examined the expression levels of VEGFs and their receptors (VEGFRs) in exosome-treated HUVECs. Generally, VEGF-A binds to VEGFR1 and VEGFR2 and mostly induces angiogenesis, whereas VEGF-C and VEGF-D bind VEGFR3 to predominantly induce lymphangiogenesis [12]. Among the VEGF and VEGFR family members tested, only the level of VEGFR2 was increased in HUVECs after exoA and exoB treatment, and this was observed with mRNA and protein (210 kDa) levels (Fig. 1DE).

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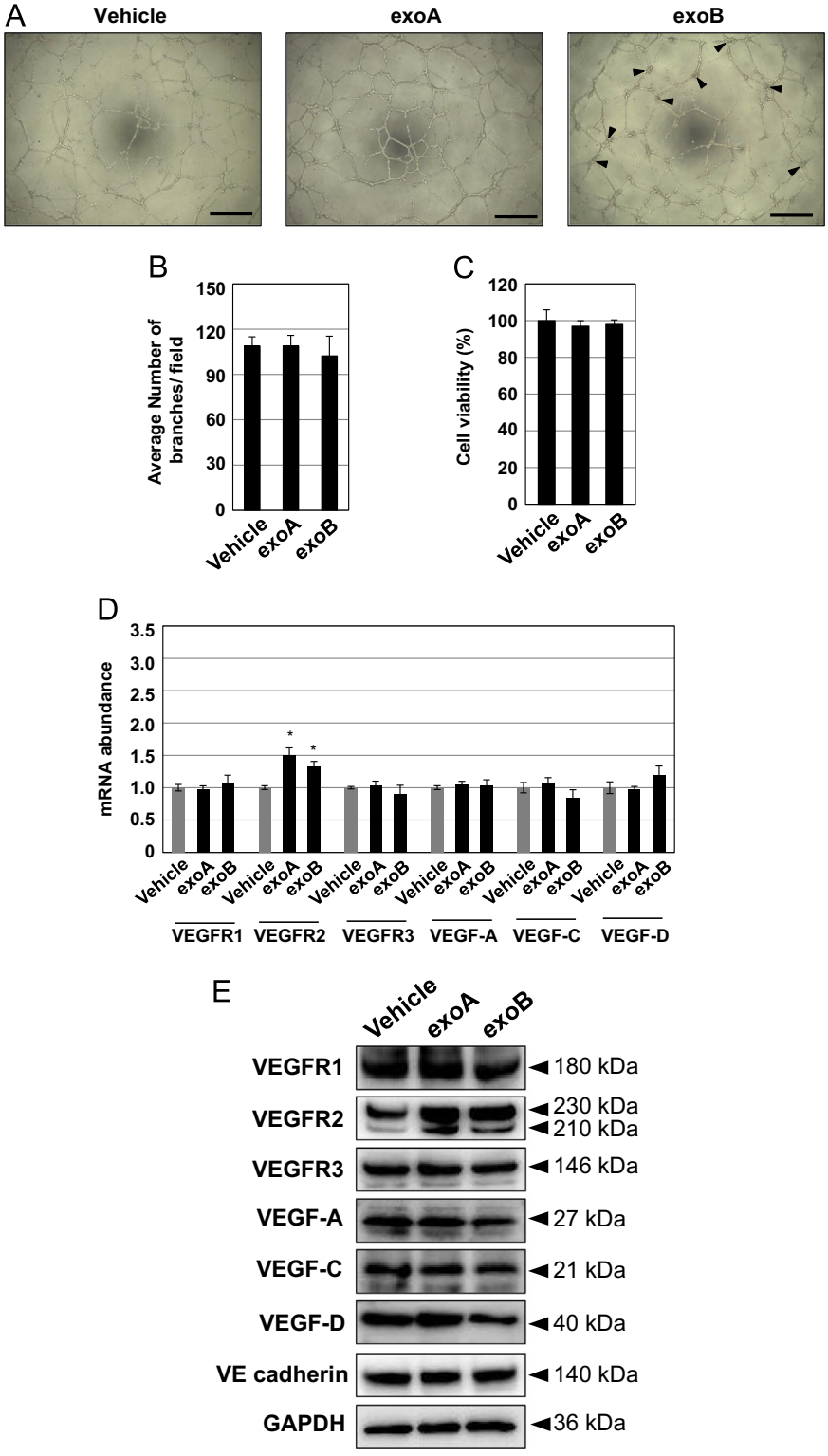

Fig. 1. Effects of the exosomes from SQUU-A or SQUU-B on HUVECs. (A) Tube formation assay of HUVECs treated with the exosomes (0.4 μg/well, equivalent amount to that previously reported [4]) in Matrigel for 18 h. The experiments were performed three times in triplicate. Arrowheads show unpolarized cell aggregates. Scale bars: 500 μm. (B) The number of branches (mean ± SD)/field formed at 18 h in the presence of vehicle, exoA, or exoB. $N=6$ separate wells. (C) Cell viability of HUVECs after treatment with exosomes. HUVECs (1.0×10^4) placed onto 96-well culture plates were treated with vehicle or exosomes (0.4 μg/well). After 18 h, cell viability was determined by a WST8 assay using Cell Count Reagent SF (Nacalai Tesque Inc., Kyoto, Japan), and presented as the relative rate compared to control conditions. (D) Relative mRNA expression levels of *VEGFR1*, *VEGFR2*, *VEGFR3*, *VEGF-A*, *VEGF-C*, and *VEGF-D* in HUVECs after vehicle or exosome-treatment for 18 h. *GAPDH* amplification was used as an internal control. The experiments were performed three times in triplicate. The error bars represent mean ± SD. $^{*}P<0.05$ and $^{**}P<0.01$ versus the control. $^{\ddagger}P<0.05$ and $^{\ddagger\ddagger}P<0.01$ versus exoA treatment. (E) Immunoblotting analysis of HUVECs after vehicle or each exosome treatment for 36 h for the above-mentioned targets. GAPDH and VE-cadherin were used as a loading control and vascular endothelial cell marker, respectively.

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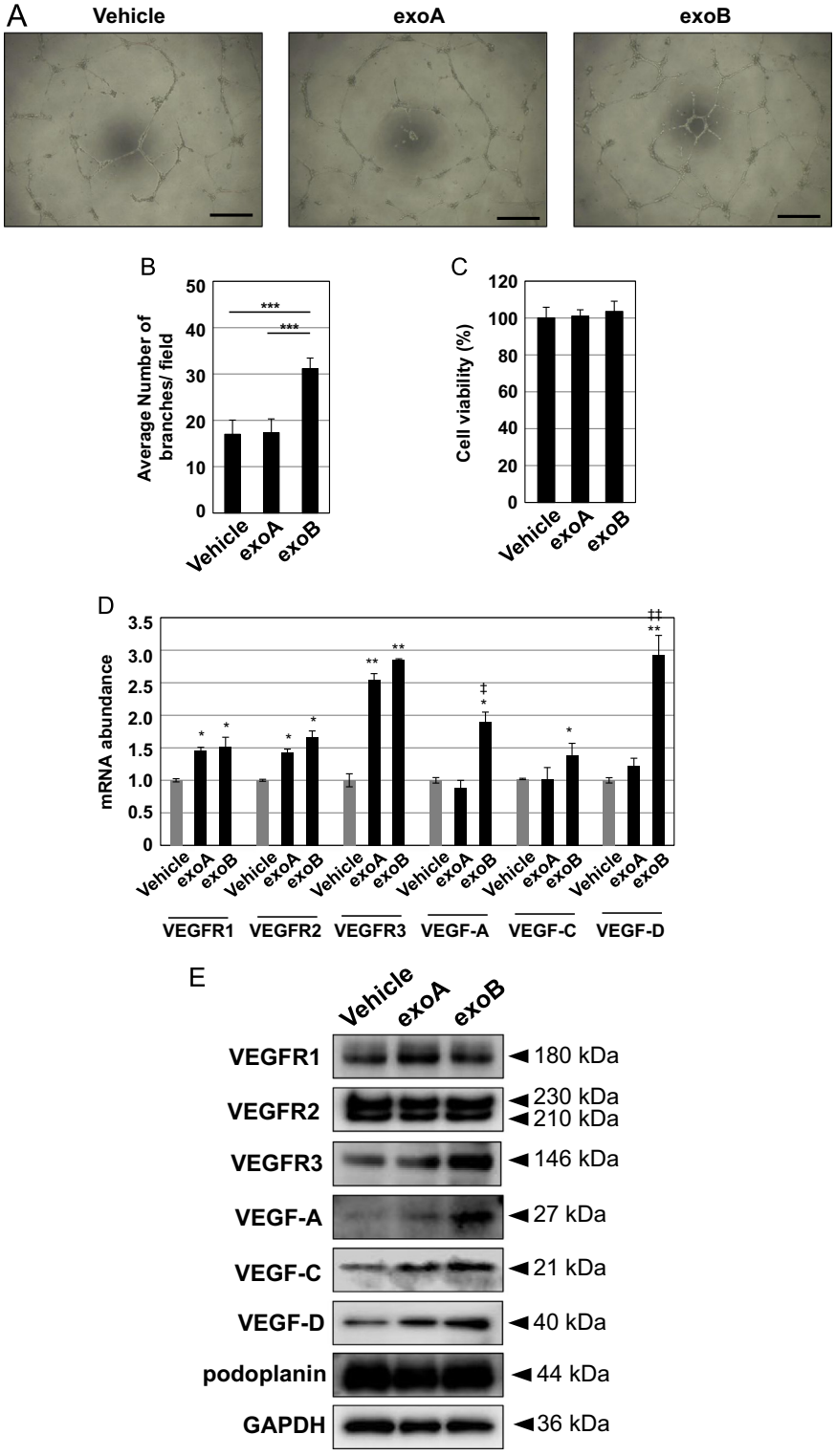

Fig. 2. *Effects of exosomes from SQUU-A or SQUU-B on HDLECs.* (A) Tube formation assay of HDLECs treated with the exosomes (0.4 μg/well, equivalent amount to that previously reported [4]) in Matrigel for 18 h. The experiments were performed three times in triplicate. Scale bars: 500 μm. (B) The number of branches (mean ± SD)/field formed at 18 h in the presence of vehicle, exoA, or exoB. $N=6$ separate wells. $^{***}P<0.001$ versus the control. (C) Cell viability of HDLECs after treatment with exosomes. HDLECs (1.0×10^4) placed onto 96-well culture plates were treated with vehicle or exosomes (0.4 μg/well). After 18 h, cell viability was determined by a WST8 assay using Cell Count Reagent SF (Nacalai Tesque Inc., Kyoto, Japan), and presented as the relative rate compared to control conditions. (D) Relative mRNA expression levels of *VEGFR1*, *VEGFR2*, *VEGFR3*, *VEGF-A*, *VEGF-C*, and *VEGF-D* in HDLECs after vehicle or exosome-treatment for 18 h. *GAPDH* amplification was used as an internal control. The experiments were performed three times in triplicate. The error bars represent mean ± SD. $^{*}P<0.05$ and $^{**}P<0.01$ versus the control. $^{‡}P<0.05$ and $^{‡‡}P<0.01$ versus exoA treatment. (E) Immunoblotting analysis of HDLECs after vehicle or each exosome treatment for 36 h for the above-mentioned targets. GAPDH and podoplanin were used as a loading control and lymphatic endothelial cell marker, respectively.

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Therefore, we suggest that both types of exosomes have the potential to sensitize vascular endothelial cells to tumor angiogenesis.

3.2. Effects of SQUU-A- and SQUU-B-derived exosomes on HDLECs

We next performed similar experiments on HDLECs. Herein, there was a tendency for exoB to facilitate the production of more tube shapes, compared to treatment with the vehicle or exoA (Fig. 2A,B). There were no differences in cell viability (Fig. 2C) among vehicle-, exoA-, and exoB-treated HDLECs. In addition, mRNA levels of VEGFRs were upregulated by exoA and exoB, whereas expression of all VEGFs was increased only by exoB, when compared to expression after control treatment (Fig. 2D). These results indicated the possibility that exoA and exoB contained miRNA(s) that regulated the expression levels of these molecules, because mRNA expression levels were altered for only 18 h. Moreover, lymphangiogenesis-inducing VEGFR3, VEGF-C, and VEGF-D proteins were produced at a higher level in exoB-treated HDLECs than in vehicle-treated cells (Fig. 2E). It is also noteworthy that VEGF-A, which was initially identified as an important promoter of angiogenesis [13] but can also induce tumor lymphangiogenesis [14], was significantly increased by exoB at the protein level. However, it is necessary to clarify if proteins were secreted. These results suggest that exoB, having metastatic potential [4,6], can potentiate lymphangiogenesis and angiogenesis, more so than exoA, which lacks metastatic potential [4,6]. Taken together, the exosomes from these two OSCC cell lines facilitated HDLEC neogenesis, and this may define the mechanism of metastasis, and hematogenous, or lymphatic spread.

4. Conclusions

The results of this study have important clinical implications. First, exosomes from OSCCs can stimulate angiogenesis and lymphangiogenesis directly through actions on vascular and lymphatic epithelial cells, and exosomes from highly metastatic cells have stronger potential for tube formation. Second, our results suggest the possibility that each type of epithelial cell has compatibility with specific exosomes, which could facilitate hematogenous or lymphatic tumor cell dissemination.

Ethical approval

The study did not require any ethical approval.

Conflict of interest

The authors have no conflict of interest to disclose.

References

[1] Peinado H, Alečkovic M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, Hergueta-Redondo M, Williams C, Garcia-Santos G, Ghajar C, Nitadori-Hoshino A, Hoffman C, Badal K, Garcia BA, Callahan MK, Yuan J, Martins VR, Skog J, Kaplan RN, Brady MS, Wolchok JD, Chapman PB, Kang Y, Bromberg J, Lyden D. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat Med 2012;18:883–91.

[2] Chaput N, Schartz NE, Andre F, Taieb J, Novault S, Bonnaventure P, Aubert N, Bernard J, Lemonnier F, Merad M, Adema G, Adams M, Ferrantini M, Carpentier AF, Escudier B, Tursz T, Angevin E, Zitvogel L. Exosomes as potent cell-free peptide-based vaccine. II. Exosomes in CpG adjuvants efficiently prime naive Tc1 lymphocytes leading to tumor rejection. J Immunol. 2004;172:2137–46.

[3] Chen W, Wang J, Shao C, Lui S, Yu Y, Wang Q, Cao X. Efficient induction of antitumor T cell immunity by exosomes derived from heat-shocked lymphoma cells. Eur J Immunol 2006;36:1598–607.

[4] Kawakubo-Yasukochi T, Morioka M, Hayashi Y, Nishinakagawa T, Hazekawa M, Kawano S, Nakamura S, Nakashima M. The SQUU-B cell line spreads its metastatic properties to nonmetastatic clone SQUU-A from the same patient through exosomes. J Oral Biosci 2016;58:33–8.

[5] Lohela M, Bry M, Tammela T, Alitalo K. VEGFs and receptors involved in angiogenesis versus lymphangiogenesis. Curr Opin Cell Biol 2009;21:154–65.

[6] Morifuji M, Taniguchi S, Sakai H, Nakabeppu Y, Ohishi M. Differential expression of cytokeratin after orthotopic implantation of newly established human tongue cancer cell lines of defined metastatic ability. Am J Pathol 2000;156:1317–26.

[7] Liang Z, Brooks J, Willard M, Liang K, Yoon Y, Kang S, Shim H. CXCR4/CXCR12 axis promotes VEGF-mediated tumor angiogenesis through Akt signaling pathway. Biochem Biophys Res Commun 2007;359:716–22.

[8] Seo M, Choi JS, Rho CR, Joo CK, Lee SK. MicroRNA miR-466 inhibits lymphangiogenesis by targeting prospero-related homeobox 1 in the alkali burn corneal injury model. J Biomed Sci 2015;22:3. http://dx.doi.org/10.1186/s12929-014-0104-0.

[9] Paku S. Current concepts of tumor-induced angiogenesis. Pathol Oncol Res 1998;4:62–75.

[10] Faulkner A, Purcell R, Hibbert A, Latham S, Thomson S, Hall WL, Wheeler-Jones C, Bishop-Bailey D. A thin layer angiogenesis assay: a modified basement matrix assay for assessment of endothelial cell differentiation. BMC Cell Biol 2014;15:41. http://dx.doi.org/10.1186/s12860-014-0041-5.

[11] Rodriguez-Fraticelli AE, Galvez-Santisteban M, Martin-Belmonte F. Divide and polarize: recent advances in the molecular mechanism regulating epithelial tubulogenesis. Curr Opin Cell Biol 2011;23:638–46.

[12] Li S, Li Q. Cancer stem cells and tumor metastasis. Int J Oncol 2014;44:1806–12.

[13] Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med 2003;9:669–76.

[14] Hirakawa S, Kodama S, Kunstfeld R, Kajiya K, Brown LF, Detmar M. VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. J Exp Med 2005;201:1089–99.

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