ORIGINAL RESEARCH

Yan Du MD, PhD
Yiwen Liu MD
Yingzhi Wang MD
Yiqing He MD
Cuixia Yang MD
Feng Gao M.D, PhD

Department of Molecular Biology, Shanghai
Sixth People's Hospital, Shanghai Jiaotong
University School of Medicine, Shanghai,
China

LYVE-1 enhances the adhesion of HS-578T cells to COS-7 cells via hyaluronan

Abstract

Purpose: Lymphatic vessel endothelial hyaluronan receptor (LYVE-1), a specific molecular marker for lymph systems, has only one known ligand, hyaluronan (HA). Many studies have reported that HA, on the surface of tumor cells, is associated with the metastatic behavior of cancer cells. The interaction of LYVE-1 with HA may facilitate tumor cell attachment and enhance dissemination of tumor cells to lymph nodes. The aim of this study was to explore the biological function of LYVE-1 and to determine whether the interaction between LYVE-1 and HA was directly involved in the adhesion of tumor cells to lymphatic vessels.

Methods: COS-7 cells were transfected with cDNA encoding LYVE-1 and expressed LYVE-1 assembled exogenously added HA. A high HA-expressing breast cancer cell line, HS-578T, was chosen to be the upper layer of cells that adhered to a lower layer of COS-7^{LYVE-1(+)}, COS-7^{pEGFP-N1}, or COS-7 cells for the adhesion analyses. The mechanism of adhesion was investigated by an experiment in which the HA on the surface of HS-578T cells was digested by *Streptomyces* hyaluronidase before the HS-578T cells were allowed to adhere to COS-7^{LYVE-1(+)} cells.

Results: Results showed that more adhesion was observed between HS-578T and COS-7^{LYVE-1(+)} cells, while less adhesion was observed between HS-578T cells and either COS-7^{pEGFP-N1} or COS-7 cells ($p < 0.01$). Decreased HA on the HS-578T cell surface could reduce the adhesion of HS-578T cells to COS-7^{LYVE-1(+)} cells, suggesting that this adhesion might be mediated through HA.

Conclusion: Our results suggest that LYVE-1 allows the adhesion of tumor cells through the interaction of HA on the tumor cell membrane with LYVE-1.

Manuscript submitted 8th September, 2010
Manuscript accepted 28th December, 2010

Clin Invest Med 2011; 34 (1): E45-E54.

Correspondence to:

Dr. Feng Gao
Department of Molecular Biology
Shanghai Sixth Peoples' Hospital
Shanghai Jiaotong University School of Medicine
600 Yi-shan Road
Shanghai 200233, P. R. China
E-mail address: gaofengc@online.sh.cn

The common pathways of cancer cell dissemination are via the lymphatic system and the bloodstream. Previous studies, using various inhibitors and other approaches, have indicated that angiogenesis is important for tumor growth and hematogenous metastasis [1-6]; however, little is known about the role of the lymphatic system in metastasis because of a lack of specific markers that discriminate between the lymphatics and blood vessels. Recently, the identification of lymphatic endothelial molecules, such as LYVE-1 [7], Prox-1 [8], podoplanin [9], β -chemokine receptor [10], and macrophage mannose receptor [11], has provided novel tools for examining tumor lymphatic vessels.

LYVE-1, a homologue of CD44 [7], is a cell surface receptor for hyaluronan (HA) in the lymphatic endothelium. Previous studies in breast cancer cell lines suggested that a high density of lymph vessels expressing LYVE-1 correlated with a high frequency of regional lymph node metastases [12, 13]. LYVE-1 is one of the most specific lymphatic markers and much attention has been paid to its role in tumor lymphangiogenesis and metastasis; however, its biological function has not been extensively studied. Over-expression of HA has been associated with cancer metastasis [14, 15], and LYVE-1, which is expressed only in the lymphatic endothelium, may be involved in cancer cell metastasis through binding to HA on the cell surface. Nevertheless, there has been no evidence that LYVE-1 is involved in the transport of tumor cells by lymph vessels.

In this study, the biological role of LYVE-1 in cancer metastasis was investigated. The LYVE-1 gene was cloned and co-expressed with the green fluorescent protein (GFP) in COS-7 cells. To observe the adhesion reaction of LYVE-1 with HA on the tumor surface, three measurements were made: 1) LYVE-1 protein expression on COS-7 cells, 2) HA expression on the surface of HS-578T cells, and 3) binding of HS-578T cells, before and after treatment with *Streptomyces* hyaluronidase, to COS-7^{LYVE-1(+)}, COS-7^{pEGFP-N1} and COS-7 cells. The results showed that LYVE-1, which was expressed in COS-7 cells, could bind exogenously added hyaluronan and could enhance the adhesion capability of COS-7 cells to HS-578T cells by binding the HA on the surface of the HS-578T cells.

Materials and Methods

Reagents and Supplies

PrimeSTAR HS DNA Polymerase, T4 DNA Ligase, HindIII and EcoRI restriction enzymes were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). A pair of PCR primers specific for LYVE-1 was designed according to the published human LYVE-1 cDNA sequence (GenBank AF118108)

and synthesized by Invitrogen LifeTechnologies (CA, USA). Plasmids containing full-length human LYVE-1 cDNA and pEGFP-N1 were constructed in our laboratory. A polyclonal antibody against human LYVE-1 (goat anti-human) and a Cy3-donkey anti-goat antibody were purchased from Santa Cruz Biotechnology, Inc. (CA, USA). DMEM was purchased from Gibco (CA, USA). Fetal bovine serum (FBS) was from Hyclone (Lanzhou, China). *Streptomyces* hyaluronidase, chemicals, and other reagents were from Sigma (MO, USA).

Cell Lines and Culture Conditions

Mammalian cells (simian virus 40-transformed African Green monkey kidney cell line COS-7) and breast cancer cells (HS-578T) were purchased from the cell bank at Shanghai Institute of Cell Biology. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin, and they were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Preparation of Full-Length LYVE-1 cDNA in pEGFP-N1 and Transient Transfection of COS-7 Cells

For cell surface expression of full-length LYVE-1, the entire LYVE-1 coding sequence was reamplified (94°C for 1 min, 55°C for 1 min and 72°C for 1 min; 30 cycles) from the original clone with the primers LYVE-1 F HindIII (GTA-CAAGCTTGCCGCCACCATGGCCAGGTGCTT CAGCC) and LYVE-1 R EcoRI (GTCAGAATTCCAAC TTCAGCTTCCAGGC) using PrimeSTAR HS DNA Polymerase. After digestion with HindIII and EcoRI, the PCR product was ligated into a HindIII/ EcoRI digested pEGFP-N1 vector. The pEGFP-N1 construct was transfected into COS-7 cells using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Western Blotting

For detection of LYVE-1 protein expression in transfected COS-7 cells and HS-578T cells, cell lysates were boiled in SDS-PAGE sample buffer, electrophoresed on 10% polyacrylamide SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes. Blots were incubated with a LYVE-1 polyclonal antibody (1:1000 dilution in TBS, pH 7.5 with 5% dried milk powder and 0.1% Tween 20) and developed with donkey anti-goat IgG-HRP using chemiluminescent detection (Pierce ECL kit; Thermo Scientific, IL, USA).

Confocal Microscopy

For detection of LYVE-1 protein expression on the COS-7 cell membrane, COS-7^{LYVE-1(+)}, COS-7^{pEGFP-N1} and COS-7 cells, at 60% confluence, were plated on sterile glass coverslips in 35 mm tissue culture plates with DMEM supplemented with 10% FCS. The cells were washed twice with PBS, fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (PBS) for 15 min, blocked with 1% BSA, and then washed with PBS. Cells were incubated with or without LYVE-1 polyclonal antibody (1:100) overnight at 4°C, and incubated with Cy3-donkey anti-goat IgG (H+L) (1:50) for 30 minutes at room temperature. Slides were viewed on a confocal microscope (Nikon A1, Tokyo, Japan) for immunofluorescence detection.

HA Binding Assay

Texas red-labeled HA (TR-HA) was prepared according to published methods [16]. To detect the binding of COS-7 membrane-expressed LYVE-1 to HA, COS-7^{LYVE-1(+)}, COS-7^{pEGFP-N1}, and COS-7 cells were plated in 24-well plates in DMEM supplemented with 10% FCS. The cells were then exposed to 200 µg TR-HA conjugate in 1 ml of medium for varying durations (30 min to 12 h), rinsed twice in cold PBS, and fixed in a solution of 2% paraformaldehyde in PBS for 10 min at room temperature. Cells were then washed three times for 5 min each in PBS, and viewed using an OLYMPUS IX70 inverted phase-contrast microscope (Japan).

Visualization of the HS-578T Pericellular Matrix

Visualization and morphometric analysis of HA matrices were done using a particle-exclusion assay as previously described [17, 18]. Briefly, 1×10^5 HS-578T cells and COS-7 cells were separately plated in 35 mm tissue culture dishes in DMEM containing 10% FBS. After 24 hours, 750 µl of a suspension of fixed and washed human erythrocytes ($\approx 10^8$ /ml) were added to the cells and allowed to settle for 15 minutes. The HA-specific *Streptomyces* hyaluronidase was then added at a concentration of 1 U/ml of medium. HA matrices were visualized before and after digestion of HA using an OLYMPUS IX70 inverted phase-contrast microscope.

Adhesion assay

Adhesion of HS-578T cells to COS-7 cells was measured as previously described [19]. Briefly, COS-7^{LYVE-1(+)}, COS-7^{pEGFP-N1}, and COS-7 cells were isotopically grown to confluent monolayers on glass coverslips in 24-well plates and

maintained in DMEM supplemented with 10% FBS. HS-578T cells pre-incubated with DAPI (Roche, Switzerland) (50 µg/ml, 37°C, 5 min) were harvested with 0.02% EDTA, and 300 µl of cell suspension (5×10^4 cells/well) treated with control medium or with 30 U/ml of *Streptomyces* hyaluronidase were added to the monolayer of COS-7 cells and incubated at 37°C for 15 min under normal conditions. Co-cultured cells were then gently washed three times with adhesion buffer containing DMEM supplemented with 0.1% BSA and fixed in 4% glutaraldehyde in PBS for counting the number of bound tumor cells.

Results

The LYVE-1 Is Expressed on the Cell Surface

A full-length human LYVE-1 cDNA was successfully amplified. The product was cloned into a pEGFP-N1 vector. The LYVE-1 cDNA construct was sequenced and proven to be the same as human LYVE-1 (GenBank AF118108). Colony selection was carried out using plasmid and LYVE-1 primers. The extracted plasmid (Fig. 1A, lane 2) was subsequently digested using EcoRI and HindIII enzymes, which demonstrated that the plasmid insert was the correct size (Fig. 1A, lane 3).

The expression level of LYVE-1 protein in the transfected cells was different from that in HS-578T cells (Fig. 1B, C).

Consistent with the previous characterization of the COS-7 cell line, COS-7 cells themselves and GFP control transfectants did not yield detectable amounts of LYVE-1 product (lanes 1 and 2 respectively). COS-7 cells were considered naked cells, since few HA binding molecules were detected on their surface. Using a polyclonal antibody, Western blotting of the COS-7^{LYVE-1(+)} cells revealed a major band that migrated with an apparent molecular mass of 70 KD. LYVE-1 expression was not detected in HS-578T cells (Fig. 1C).

To further confirm the identification of the LYVE-1 molecule as an integral membrane glycoprotein, COS-7 cells were transiently transfected with vector alone (pEGFP-N1) or with vector containing full-length human LYVE-1 cDNA and stained with polyclonal LYVE-1 antibodies. As shown in Fig. 1D, COS-7^{LYVE-1(+)} cells displayed intense surface staining that is absent from both the COS-7^{pEGFP-N1} cells and the nontransfected COS-7 cells.

TR-labeled HA Is Taken Up by COS-7^{LYVE-1(+)} Cells

To assess the functionality of LYVE-1, binding of TR-HA to the COS-7^{LYVE-1(+)}, COS-7^{pEGFP-N1} and COS-7 cells was measured. As shown in Fig. 2, the majority of LYVE-1 trans-

FIGURE 1. Gel electrophoresis of LYVE-1 cloning and LYVE-1 expression in COS-7 cells and HS-578T cells. A: 1: Marker, 2-3: Purified pEGFP-N1 plasmid containing the LYVE-1 insert, 2: Plasmid before digestion, 3: Plasmid digested with HindIII and EcoRI. B: The parental nontransfected COS-7 cells, COS-7^{pEGFP-N1} cells and COS-7^{LYVE-1(+)} cells were electrophoresed on a 10% polyacrylamide SDS-PAGE gel, transferred to polyvinylidene fluoride membranes, and Western blotted with LYVE-1 antibody and peroxidase-conjugated donkey anti-goat IgG (see Materials and Methods). The samples were COS-7 cells (lane 1), COS-7^{pEGFP-N1} cells (lane 2) and COS-7^{LYVE-1(+)} cells (lane 3). C: The expressions of LYVE-1 in HS-578T cells were evaluated as mentioned previously. The samples were COS-7^{LYVE-1(+)} (lane 1) and HS-578T cells (lane 2). D: The three types of COS-7 cells mentioned above were transiently transfected with either full-length LYVE-1 cDNA in the expression vector pEGFP-N1 (a, b and c) or with a control empty pEGFP-N1 vector (d, e and f). Untransfected COS-7 cells (g, h and i) were also used as a control. The transfection was analyzed by surface immunofluorescent staining with goat polyclonal LYVE-1 antibody (1:100 dilution) and Cy3-donkey anti-goat IgG. Bar = 20 μ m.

fectants bound TR-HA, while the control, mock-transfected cells did not. These results provided clear evidence that the LYVE-1 in COS-7 cells interacted with HA.

Pericellular Matrix Formation by Human HS-578T

A particle-exclusion assay [20] was used to determine if human HS-578T cells or COS-7 cells were capable of producing and organizing HA-rich pericellular matrices. In this assay, HS-

578T cells displayed a wide pericellular space that excluded fixed erythrocytes (Fig. 3A). *Streptomyces* hyaluronidase gradually removed the HA layer on the plasma membrane, and erythrocytes approached the cell body, leading to the disappearance of the exclusion space (Fig. 3B): these data are consistent with previous work [17]. Our results showed that COS-7 cells, having low capacity for HA production, could not exclude the fixed erythrocytes (Fig. 3C), and almost no change was observed after adding hyaluronidase.

FIGURE 2. *LYVE-1 mediates HA-binding.* COS-7 cells were transiently transfected with pEGFP-N1-LYVE-1 cDNA to assess the capacity of the receptor to bind Texas-Red-labeled HA. GFP-LYVE-1 in cells is shown in A, TR-HA around or in the same cell is visualized in B, and a composite image is shown in C. Control cells transfected with an empty pEGFP-N1 vector are presented in D, E and F. Bar = 50 μ m.

LYVE-1 Enhances the Adhesion of HS-578T Cells to COS-7 Cells

LYVE-1 may play a role in the adhesion of leukocytes or tumor cells to endothelia. A null cell line, COS-7, was used to test the hypothesis that COS-7^{LYVE-1(+)} cells can alter their interactions with tumor cells. The established HS-578T cell line was used as a model for tumor cell adhesion, since this tumor cell line contains abundant HA on its surface. COS-7^{pEGFP-N1} cells or untreated COS-7 cells allowed only minimal adherence of HS-578T cells, while COS-7^{LYVE-1(+)} cells showed strong adhesion of HS-578T cells

FIGURE 4 NEAR HERE

(Fig. 4). In addition, pretreatment of HS-578T cells with *Streptomyces* hyaluronidase decreased the adhesion to COS-7^{LYVE-1(+)} cells

FIGURE 5 NEAR HERE

(Fig. 5). These results demonstrated that LYVE-1 may contribute to the adhesion of HS-578T cells to COS-7 cells, which might be partly mediated by HA.

Discussion

This study suggests a role for LYVE-1 in the regulation of adhesion of tumor cells to COS-7 cells *in vitro*. When COS-7 cells were transfected with LYVE-1, a rapid and significant change was observed in the adherence of COS-7^{LYVE-1(+)} cells with a high HA expressing breast cancer cell line, HS-578T, while the COS-7^{pEGFP-N1} cells or the parental nontransfected COS-7 cells, did not exhibit this alteration under similar conditions. This adhesion could be partially inhibited by a brief treatment with *Streptomyces* hyaluronidase, an enzyme that specifically degraded hyaluronan. These findings suggest that

FIGURE 3. Human HS-578T cells and COS-7 cells organize HA-dependent pericellular matrices. A, C: The particle-exclusion assay was used to demonstrate the presence of the HA-dependent pericellular matrix, which was apparent as a clear zone between the HS-578T and red blood cells, but not between COS-7 cells and red blood cells. B, D: The same field as in A and C about 5 minutes after *Streptomyces* hyaluronidase was added to the culture dish. The hyaluronidase degraded the HA coats, allowing the red blood cells to contact the HS-578T cells and COS-7 cells directly. Bar = 30 μ m.

LYVE-1, specifically located in the lymph vessels, is involved in the adhesion of tumor cells to COS-7 cells through binding HA on the tumor cell surface.

Breast cancer is a common cancer affecting women [21]. It spreads primarily via the lymphatics, but the mechanism by which tumor cells enter, interact with, and transport within lymphatic vessels is poorly understood due to the lack of specific markers for the lymphatic endothelium. The development of specific markers, such as the lymphatic hyaluronan receptor LYVE-1, has allowed many new experimental studies of tumor lymphatics to be initiated [22, 23], and has expanded the study of tumor metastasis since the blood vasculature has been the primary system studied in metastasis.

Tumor cell arrest and the formation of stable adhesive interactions between tumor cells and endothelial cells are crucial steps in the metastatic process. Our hypothesis was that LYVE-

1, through interaction with HA on the tumor cell surface, could influence the adhesion of tumor cells to lymphatic endothelial cells and allow the tumor cells to metastasize into the lymph systems or be transported within the lymph vessels. Previous research indicated that LYVE-1 could not bind HA *in vitro*, and suggested that LYVE-1, like CD44, may become active after appropriate unmasking *in vivo*. LYVE-1, expressed on COS-7 cells, could bind HA, but, when expressed on other cells, was unable to bind HA. COS-7 cells were considered as naked cells, since few HA binding molecules are present on the cell surface to prevent the adhesion of LYVE-1. Other cells may have other HA receptors (for example, CD44) to interfere with LYVE-1.

In this study, COS-7 cells were transfected with LYVE-1 to model lymphatic endothelial cells, and COS-7^{LYVE-1(+)} cells were able to bind Texas Red-HA as reported. Meanwhile, the

FIGURE 4. Effect of LYVE-1 on HS-578T cell adhesion to COS-7 cells. A: The number of HS-578T cells adhered to COS-7 cells transfected with or without LYVE-1 was evaluated by counting 5 random views of fluorescence microscope images as described in Materials and Methods. HS-578T cells appear as blue spheres (a, b and c) on top of the confluent COS-7 cells (d, e and f), which are attached to the culture plate. GFP-LYVE-1 in cells is shown in g, and the GFP control in cells is shown in h. Bar = 200 μ m. B: Quantitative evaluations of the data from HS-578T cell adhesion on the monolayer of COS-7 cells are shown. Each assay was performed in triplicate. Results are shown as the mean $\pm$ S.E. of triplicate wells from a typical experiment and are expressed as fold increase relative to control pEGFP-N1 vector transfected COS-7 cells.

* $P < 0.01$ versus indicated transfected pEGFP-N1 vector control COS-7 cells.

FIGURE 5. *LYVE-1 mediates the adhesion of HS-578T cells mainly because of its interaction with HA on the cell membrane.* **A:** COS-7 cells transfected with LYVE-1 were grown to confluence on glass coverslips in 24-well plates. A suspension of HS-578T cells pre-treated with or without *Streptomyces* hyaluronidase (30 U/ml) was added onto the monolayer of COS-7 cells. After 15 min of incubation at 37°C, cells were washed, paraformaldehyde-fixed, photographed and counted. HS-578T cells appear as blue spheres (a, b) on top of the confluent COS-7 cells (c, d), which are attached to the culture plate. GFP-LYVE-1 in cells is shown in e and f. Bar = 200 μ m. **B:** Quantitative evaluations of the data from HS-578T cell adhesion on the monolayer of COS-7^{LYVE-1(+)} cells are shown. Each assay was performed in triplicate. Results are shown as the mean $\pm$ S.E. of triplicate wells from a typical experiment and are expressed as a decrease relative to untreated HS-578T cells.

* $P < 0.01$ versus *Streptomyces* hyaluronidase untreated control.

highly HA expressing breast carcinoma cell line, HS-578T, was chosen to adhere to COS-7^{LYVE-1(+)}, COS-7^{pEGFP-N1}, and non-transfected COS-7 cells to explore the function of tumor cell adhesion to lymphatic endothelium cells, as mediated by LYVE-1. Transfection of LYVE-1 into COS-7 cells was found to promote the adhesion of HS-578T cells to COS-7^{LYVE-1(+)} cells, possibly by binding the HA on the tumor surface, which was consistent with the observation that LYVE-1 could bind immobilized and soluble HA [7]. We also found that *Streptomyces* hyaluronidase could reduce, but not prevent, the adhesion between COS-7^{LYVE-1(+)} cells and HS-578T cells. Thus, it appears that LYVE-1 mediates the adhesion of tumor cells through its surface HA. *Streptomyces* hyaluronidases could not prevent the adhesion by LYVE-1, which may be explained by incomplete deletion of HA on the tumor surface. Consistent with previous work by Kirsir Rilla *et al.*, hyaluronan and the exclusion space were reduced, but not eliminated by *Streptomyces* hyaluronidases. The inability of *Streptomyces* hyaluronidases to completely prevent adhesion by LYVE-1 might also be explained by the ongoing synthesis of HA, making it difficult to obtain an equilibrium state. It is also possible that LYVE-1 might bind to additional ligands and additional studies are needed to explore this possibility. Moreover, previous studies have reported that LYVE-1 undergoes cytokine-induced endocytosis, independent of HA uptake, under inflammation conditions ⁽²⁵⁾. A recent study ⁽²⁶⁾ suggests that HA homeostasis is unperturbed in LYVE-1^{-/-} mice under normal physiological conditions. Thus, there may be some complicated mechanisms involved in the regulation of LYVE-1 function *in vivo*.

In conclusion, our study provides evidence that LYVE-1 has a specific biological function and is not merely a useful tracing marker for the lymphatics. The results revealed the possible role of LYVE-1 in the mediation of the adhesion of tumor cells to lymphatic vessels by means of binding to the tumor cell surface HA. These results suggest that LYVE-1 might represent a potential pharmacological target for the inhibition of tumor metastasis through lymph systems; however, as no experimental procedures have been explored with regard to lymphatic endothelial cells, it could not be confirmed that LYVE-1 is directly involved in the tumor cell metastasis in the lymphatic vessels. Further work in this area is ongoing in our laboratory.

Acknowledgments

This research was supported by the Shanghai Committee of Science and Technology (No.10411950500).

List of Abbreviations

LYVE-1	Lymphatic vessel endothelial hyaluronan receptor
HA	hyaluronan
GFP	green fluorescent protein
TR-HA	Texas red-labeled HA

References

- Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. *N Engl J Med.* 1991; 324: 1-8.
- Chu JS, Lee WJ, Chang TC, Chang KJ, Hsu HC. Correlation between tumor angiogenesis and metastasis in breast cancer. *J Formos Med Assoc.* 1995; 94: 373-8.
- Kato T, Kameoka S, Kimura T, *et al.* Angiogenesis as a predictor of long-term survival for 377 Japanese patients with breast cancer. *Breast Cancer Res Treat.* 2001; 70: 65-74.
- Fox SB, Leek RD, Weekes MP, Whitehouse RM, Gatter KC, Harris AL. Quantitation and prognostic value of breast cancer angiogenesis: comparison of microvessel density, Chalkley count, and computer image analysis. *J Pathol.* 1995; 177: 275-83.
- Saleh M, Stacker SA, Wilks AF. Inhibition of growth of C6 glioma cells in vivo by expression of antisense vascular endothelial growth factor sequence. *Cancer research.* 1996; 56: 393-401.
- Kim KJ, Li B, Winer J, *et al.* Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature.* 1993; 362: 841-4.
- Banerji S, Ni J, Wang SX, *et al.* LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *The Journal of cell biology.* 1999; 144: 789-801.
- Wigle JT, Oliver G. Prox1 function is required for the development of the murine lymphatic system. *Cell.* 1999; 98: 769-78.
- Breiteneder-Geleff S, Soleiman A, Kowalski H, *et al.* Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. *Am J Pathol.* 1999; 154: 385-94.
- Nibbs RJ, Kriehuber E, Ponath PD, *et al.* The beta-chemokine receptor D6 is expressed by lymphatic endothelium and a subset of vascular tumors. *Am J Pathol.* 2001; 158: 867-77.
- Irjala H, Johansson EL, Grenman R, Alanen K, Salmi M, Jalkanen S. Mannose receptor is a novel ligand for L-selectin and mediates lymphocyte binding to lymphatic endothelium. *J Exp Med.* 2001; 194: 1033-42.
- Skobe M, Hawighorst T, Jackson DG, *et al.* Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med.* 2001; 7: 192-8.

13. Mattila MM, Ruohola JK, Karpanen T, Jackson DG, Alitalo K, Harkonen PL. VEGF-C induced lymphangiogenesis is associated with lymph node metastasis in orthotopic MCF-7 tumors. *Int J Cancer*. 2002; 98: 946-51.
14. Zhang L, Underhill CB, Chen L. Hyaluronan on the surface of tumor cells is correlated with metastatic behavior. *Cancer research*. 1995; 55: 428-33.
15. Itano N, Sawai T, Miyaishi O, Kimata K. Relationship between hyaluronan production and metastatic potential of mouse mammary carcinoma cells. *Cancer research*. 1999; 59: 2499-504.
16. Collis L, Hall C, Lange L, Ziebell M, Prestwich R, Turley EA. Rapid hyaluronan uptake is associated with enhanced motility: implications for an intracellular mode of action. *FEBS Lett*. 1998; 440: 444-9.
17. Evanko SP, Angello JC, Wight TN. Formation of hyaluronan- and versican-rich pericellular matrix is required for proliferation and migration of vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 1999; 19: 1004-13.
18. Knudson W, Bartnik E, Knudson CB. Assembly of pericellular matrices by COS-7 cells transfected with CD44 lymphocyte-homing receptor genes. *Proc Natl Acad Sci U S A*. 1993; 90: 4003-7.
19. Simpson MA, Wilson CM, Furcht LT, Spicer AP, Oegema TR, Jr., McCarthy JB. Manipulation of hyaluronan synthase expression in prostate adenocarcinoma cells alters pericellular matrix retention and adhesion to bone marrow endothelial cells. *The Journal of biological chemistry*. 2002; 277: 10050-7.
20. Clarris BJ, Fraser JR. On the pericellular zone of some mammalian cells in vitro. *Exp Cell Res*. 1968; 49: 181-93.
21. Forbes JF. The incidence of breast cancer: the global burden, public health considerations. *Semin Oncol*. 1997; 24: S1-20-S1-35.
22. Gao F, Lu YM, Cao ML, Liu YW, He YQ, Wang Y. Expression and quantification of LYVE-1 in human colorectal cancer. *Clin Exp Med*. 2006; 6: 65-71.
23. Cunnick GH, Jiang WG, Gomez KF, Mansel RE. Lymphangiogenesis quantification using quantitative PCR and breast cancer as a model. *Biochem Biophys Res Commun*. 2001; 288: 1043-6.
24. Kohda D, Morton CJ, Parkar AA, et al. Solution structure of the link module: a hyaluronan-binding domain involved in extracellular matrix stability and cell migration. *Cell*. 1996; 86: 767-75.
25. Johnson LA, Prevo R, Clasper S, Jackson DG. Inflammation-induced uptake and degradation of the lymphatic endothelial hyaluronan receptor LYVE-1. *The Journal of biological chemistry*. 2007; 282: 33671-80.
26. Gale NW, Prevo R, Espinosa J, et al. Normal lymphatic development and function in mice deficient for the lymphatic hyaluronan receptor LYVE-1. *Mol Cell Biol*. 2007; 27: 595-604.