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ORIGINAL ARTICLE

# LGALS3BP, lectin galactoside-binding soluble 3 binding protein, induces vascular endothelial growth factor in human breast cancer cells and promotes angiogenesis

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**Abstract** Elevated serum or tissue levels of lectin galactoside-binding soluble 3 binding protein (LGALS3BP) have been associated with short survival and development of metastasis in a variety of human cancers. However, the role of LGALS3BP, particularly in the context of tumor–host relationships, is still missing. Here, we show that LGALS3BP knockdown in MDA-MB-231 human breast cancer cells leads to a decreased adhesion to fibronectin, a reduced transendothelial migration and, more importantly, a reduced expression of vascular endothelial growth factor (VEGF). Production of VEGF, that was restored by exposure of silenced cells to recombinant LGALS3BP, required an intact PI3k/Akt signaling. Furthermore, we show that LGALS3BP was able to directly stimulate HUVEC tubulogenesis in a VEGF-independent, galectin-3-dependent manner. Immunohistochemical analysis of human breast cancer tissues revealed a correlation among LGALS3BP expression, VEGF expression, and blood vessel density. We propose that in addition to its prometastatic role, LGALS3BP secreted by breast cancer cells functions critically as a pro-angiogenic factor through a dual mechanism, i.e by induction of tumor VEGF and stimulation of endothelial cell tubulogenesis.

**Keywords** Angiogenesis · VEGF · LGALS3BP · Extracellular matrix · Galectin-3

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## Introduction

The development of human cancer is a multistep process characterized by the accumulation of progressive genetic alterations that confer to tumor cells the ability to survive, proliferate, and metastasize. Once they have expanded to a critical level, tumor cells find a way to promote new vasculature development, a process known as tumor angiogenesis in order to progress and metastasize [1]. This "angiogenic switch" can occur at different stages of tumor progression and is dictated by an imbalance between factors that either induce or oppose

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angiogenesis in favor of the former [2]. Vascular endothelial growth factor (VEGF) represents the prototypical pro-angiogenic factor [3]. Although hypoxia appears to be the main driver of tumor angiogenesis, growth factors and inflammation are able to boost VEGF production through paracrine or autocrine mechanisms [4, 5]. However, much more remains to be understood about the molecular nature of unidentified factors that are also believed to have the ability to promote angiogenesis in the development of a variety of human cancers.

LGALS3BP (also known as 90 K or Mac-2 BP) is a large oligomeric glycoprotein composed of 90-kDa subunits that was originally identified as a tumor-secreted antigen [6] and as a ligand of the lactose-specific S-type lectin, galectin-3 (formerly Mac-2) [7]. Over the past decade, considerable attention has been focused on a potential role of LGALS3BP in the development of human cancer. Immunohistochemical and gene expression analysis showed significantly higher levels of LGALS3BP in different types of human malignancies [8]. Furthermore, clinical studies have revealed that elevated serum or tumor tissue levels of LGALS3BP are associated with a shorter survival in patients with breast carcinoma [9, 10], lymphoma [11], pleural mesothelioma [12], and non-small cell lung carcinoma [13].

Taken together, these data indicate that expression levels of LGALS3BP may serve as a prognostic indicator. Nonetheless, little is known regarding LGALS3BP activity in human cancers.

In the current study, we tested the hypothesis that LGALS3BP acts as a pro-angiogenic factor. We find and report for the first time that LGALS3BP induces VEGF expression in human breast cancer cells by activation of the PI3k/Akt pathway. Furthermore, we show that LGALS3BP directly acts on endothelial cells, promoting angiogenesis in vitro and in vivo in a VEGF-independent, galectin-3-dependent manner.

## Materials and methods

### Cell lines and culture

The estrogen receptor (ER)-negative human breast cancer cell lines MDA-MB 231 and SK-BR-3 and the ER-positive MCF-7 and T47D were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) with 10 % heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), L-glutamine, and antibiotics (Sigma Aldrich Corporation, St. Louis, MO, USA). The human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Lonza, Basel, Switzerland) and maintained in EBM-2 medium containing 10 % heat-inactivated FBS supplemented with EGM-2 SingleQuots (Lonza, Basel, Switzerland).

For transfection of HUVEC, siRNA duplexes directed against human galectin-3 or nontargeting siRNA duplex or siRNA duplexes against human VEGF (Thermo Scientific, Waltham, MA, USA) were used according to the manufacturer's instructions. SiRNA duplexes against LGALS3BP were obtained from Qiagen (Qiagen, Hilden, Germany).

For collection of the conditioned media (CM), cells were grown until 70 % confluent. The next day, the medium was replaced with serum-free medium after washing with phosphate-buffered saline (PBS). The medium was collected 24–48 h later and centrifuged at 1,200 rpm for 15 min to remove cells and debris.

### LGALS3BP gene knockdown

A 21-nucleotide sequence corresponding to nucleotide 2216-2236 of human LGALS3BP mRNA (NCBI Accession NM-005567.3) or a 21-nucleotide sequence with no significant homology to any mammalian gene sequence serving as a non-silencing control (OligoEngine, Hercules, CA, USA) was inserted into the pSUPER.retro.puro (OligoEngine, Hercules, CA, USA). After transformation into DH5α competent cells (Invitrogen, Carlsbad, CA, USA), the recombinant plasmids were confirmed by PCR amplification, restriction enzymes digestion, and DNA sequencing. The generation of knockdown cells was performed as previously described [14].

### Production of recombinant LGALS3BP

Recombinant LGALS3BP was immunoaffinity-purified from free supernatant of human embryonic kidney EBNA-293 cells (Invitrogen) [15] transfected with LGALS3BP cDNA [16].

### Quantitative real-time PCR

Total RNA was extracted using the RNeasy mini kit (Qiagen), quantified by optical density, and checked for quality by gel electrophoresis. RNA was reverse-transcribed with cDNA High Capacity kit (Applied Biosystem, Foster City, CA, USA) according to the manufacturer's instructions. Quantitative real-time PCR was performed using 96-well optic plates on an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The predeveloped TaqMan assay reagents of human VEGF (ID: Hs00173626_ m1) and human LGALS3BP (ID: Hs00174774_ m1) were used according to the manufacturer's instructions. Relative expression values were obtained using the Q-Gene software and normalized to the expression of GAPDH (ID: Hs0017266705g1).

### Confocal microscopy and spreading assay

MDA-MB-231 cells and HUVEC grown on coverslips were treated as indicated, fixed in 4 % paraformaldehyde for 15 min at room temperature, permeabilized with 0.25 % Triton X-100 for 5 min, and blocked with 0.1 % bovine serum albumin for 1 h at room temperature. Coverslips were then incubated for 2 h at room temperature with the indicated primary antibodies, followed by Alexa Fluor specific secondary antibodies (Molecular Probes, Life Technologies, Paisley, UK). TRITC-labeled phalloidin was used to visualize actin cytoskeleton; DRAQ5 (Vinci-Biochem, Firenze, Italy) was used to visualize nuclei. Images were acquired with the Zeiss LSM 510 meta-confocal microscope (Zeiss, Oberkochen, Germany) using 488-, 543-, and 633-nm lasers.

### Fluorescent labeling of MDA-MB-231 Cells

Serum-starved MDA-MB-231 cells were dispersed in 0.25 % trypsin, resuspended in serum-free medium, and labeled by incubation with 5 μM calcein AM (Molecular Probes) for 30 min. Cells were washed twice with PBS and resuspended in serum-free medium at a concentration of $5\times10^6$ cells/mL. Calcein-labeled cells were then used for adhesion and transmigration assays.

### Adhesion assay

Human MDA-MB-231 cells were serum starved for 18 h. Cells were washed with PBS (containing 0.1 % BSA), gently detached with 0.25 % trypsin, and plated onto 96-well culture plate precoated with 10 μg/mL of BSA, collagen IV (Millipore, Billerica, MA, USA), collagen V (Sigma Aldrich, St. Louis, MO, USA), fibronectin (Fn) (Millipore, Billerica, MA, USA), and laminin (Becton Dickinson, Franklin Lakes, NJ, USA). After incubation and washing, the adherent cells were dyed with 0.1 % crystal violet for 30 min at room temperature and solubilized with 150 μL/well of 2 % SDS. Absorbance at 550 nm was measured with a microplate reader (SpectraMax, Molecular Devices, Sunnyvale, CA, USA).

Adhesion assay of MDA-MB-231 cells to HUVEC monolayer was performed as described [17]. Attached tumor cells were observed under a fluorescent microscope and counted from ten random fields of ×200 magnification.

### Transendothelial penetration of MDA-MB-231 cells

Transendothelial penetration (TEP) of MDA-MB-231 cells on HUVEC cells was performed as described [17]. Transmigrated tumor cells were observed under a fluorescent microscope and counted from ten random fields of ×200 magnification.

### Enzyme-linked immunosorbent assay

Sandwich enzyme-linked immunosorbent assay (ELISA) was performed using kits specific for VEGF-A (Invitrogen) and LGALS3BP (Diesse, Siena, Italy) in accordance with the manufacturers' instructions.

### Tubulogenesis assay

Capillary tube formation (tubulogenesis) assay was performed as described [18] with a few modifications. HUVEC were resuspended in a serum-free EBM-2 or the indicated CMs and seeded in Matrigel-coated chamber slides at a density of $5\times10^4$cells/well. Human recombinant $VEGF_{165}$ (R&D Systems, Minneapolis, MN, USA) was used as positive control. After incubation at 37 °C for 4 h, cultures were photographed. For each individual well, three photographs were taken at different locations and analyzed by the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

### In vivo Matrigel plug assay

The Matrigel plug assay was performed as described previously [19] with some modifications. Female athymic (nu+/nu+) mice (Charles River Laboratories, Milan, Italy) were subcutaneously injected with 0.5 mL Cultrex (basic membrane extract, BME, Trevigen, Gaithersburg, MD, USA) containing $5\times10^6$ shRNA control or shRNA LGALS3BP cells. Human recombinant $VEGF_{165}$ (R&D Systems) and PBS were used as positive and negative control, respectively. Six animals per group were used. Seven days after injection, mice were sacrificed, and plugs were removed and photographed. Hemoglobin content of the plug was assayed using the Drabkin method [20] (Drabkin reagent kit 525; Sigma Aldrich, St. Louis, MO, USA). Hemoglobin content was normalized to the plug weight and the values expressed as fold increase compared to the control. The procedure involving animals and their care was conducted according to the institutional guidelines in compliance with national and international laws and policies.

### Western blotting

Cell lysates were subjected to 10 % SDS–PAGE and Western blotting using the following antibodies: rabbit anti-Ser (473)-Akt, rabbit anti-phospo-p42/44 Mapk (ERKs), rabbit Akt, rabbit anti p42/44 Mapk (all diluted 1:1,000; all from Cell Signaling Technology, Danvers, MA, USA), mouse anti-HIF-1 α (1:1,000; Becton Dickinson), and mouse

anti-actin (1:3,000; Sigma Aldrich). Specific signals were detected using an ECL kit.

### Immunohistochemistry

The study of breast cancer samples was approved by "G. D'Annunzio" University and Foundation Local Ethics Committee. Archival, paraffin-embedded breast cancer tissues were collected from 137 female patients (118 ductal and 19 lobular carcinoma) over the period from October 2007 to April 2010. Immunostaining was performed with the following primary antibodies: mouse monoclonal antibody 1A4. 22 [21] for LGALS3BP; clone JH121 (NeoMarkers, Fremont, CA, USA) for VEGF, and clone 1A10 (Ylem, Rome, Italy) for CD31/PECAM. Sections for LGALS3BP and VEGF immunolabeling were subjected to antigen retrieval by treating them with microwave (10 min) in citrate buffer, pH 6.0 and EDTA buffer, pH 8.5. For CD31/PEAM, antigen retrieval was carried out by immersing sections in boiling citrate buffer (pH 6.0) for 1 min in a domestic pressure cooker before cooling in water. Positive controls were stained in parallel. Omission of the primary antibody was used as a negative control. Staining of LGALS3BP and VEGF was expressed as the percentage of positive staining cells. The microvessel density was determined by microscopic evaluation of three different fields under ×200 magnification. The stained vessels from the three sites were then counted and averaged. A single pathologist (M.P.) selected the areas of tumor with the highest number of vessels. Areas of sclerosis or dense inflammation were avoided.

### Statistical methods

To test differences in means of cell numbers and biological activities, a two-sided *t* test was used. The correlation among expression of LGALS3BP, VEGF, and CD31/PECAM was evaluated by the Pearson Chi-square. High and low categories were obtained using the following median cutoff values: for LGALS3BP, 40 %; for VEGF, 61 %; for CD31/PECAM, 10 vessels. The SPSS version 15.0 (SPSS, Chicago, IL, USA) was used throughout, and $P<0.05$ was considered statistically significant.

## Results

### Knockdown of LGALS3BP impairs cell adhesion and spreading

To gain further insight into the mechanisms of LGALS3BP-dependent cell adhesion, expression of endogenous LGALS3BP was reduced in the human breast cancer cell line MDA-MB-231 by pSuper retro-based vectors to express stable short hairpin RNA (shRNA). LGALS3BP-specific shRNA reduced the expression of LGALS3BP mRNA and protein by 80 to 90 % (data not shown). Similar results were obtained by silencing LGALS3BP in three other human breast cancer cell lines, SK-BR-3, MCF-7, and T47D (data not shown). To test the effect of LGALS3BP knockdown on cell adhesion, cells were seeded on plates coated with collagen IV, collagen V, fibronectin, and laminin and the extent of adhesion quantified by staining cells with crystal violet. As compared to shRNA control cells, adhesion on fibronectin was significantly reduced in shRNA LGALS3BP cells (Fig. 1a). Image acquisition with laser scanning microscopy revealed that LGALS3BP knockdown cells appeared more round and less spread when plated on fibronectin, with few adhesion contacts as revealed by staining for focal adhesion kinase (FAK; Fig. 1b), suggesting an involvement of LGALS3BP in cytoskeleton rearrangement after adhesion to fibronectin. On Western blot, shRNA LGALS3BP cells plated on fibronectin showed a reduced activation of FAK and Akt as compared to shRNA control cells, whereas ERK activation was unchanged (Fig. 1c). These data indicate that LGALS3BP knockdown impairs adhesion and spreading on fibronectin of MDA-MB-231 cells, possibly through a reduced activation of FAK and Akt.

To assess the involvement of LGALS3BP in cell motility, we performed a transwell assay. A similar fraction of cells passed through the filter, independently of whether they were knocked down for LGALS3BP or not, exposed to CM of silenced or control cells, or exposed to recombinant LGALS3BP (data not shown).

### Knockdown of LGALS3BP impairs adhesion to endothelial cells and transendothelial penetration

To study the involvement of LGALS3BP in the adhesion of tumor cells to endothelium and their intravasation, calcein-labeled cells were added to HUVEC monolayers and the number of adherent cells assessed under a fluorescent microscope. In serum-free conditions, no significant difference was observed between shRNA control and shRNA LGALS3BP cells (Fig. 2a). On the other hand, when the assay was performed in the presence of CM from shRNA control cells, adhesion increased if compared to that observed in serum-free conditions, whereas it returned to the basal level in the presence of CM from shRNA LGALS3BP cells. Adhesion was not modified by the addition of recombinant LGALS3BP (10 μg/mL; Fig. 2a).

TEP of tumor cells was next explored. To this end, calcein-labeled MDA-MB-231 cells were added to HUVEC monolayers in Transwell apical chambers and the number of penetrating cells assessed under a fluorescent microscope. In the presence of serum, no difference in TEP was seen between shRNA control and shRNA LGALS3BP cells (Fig. 2b). The

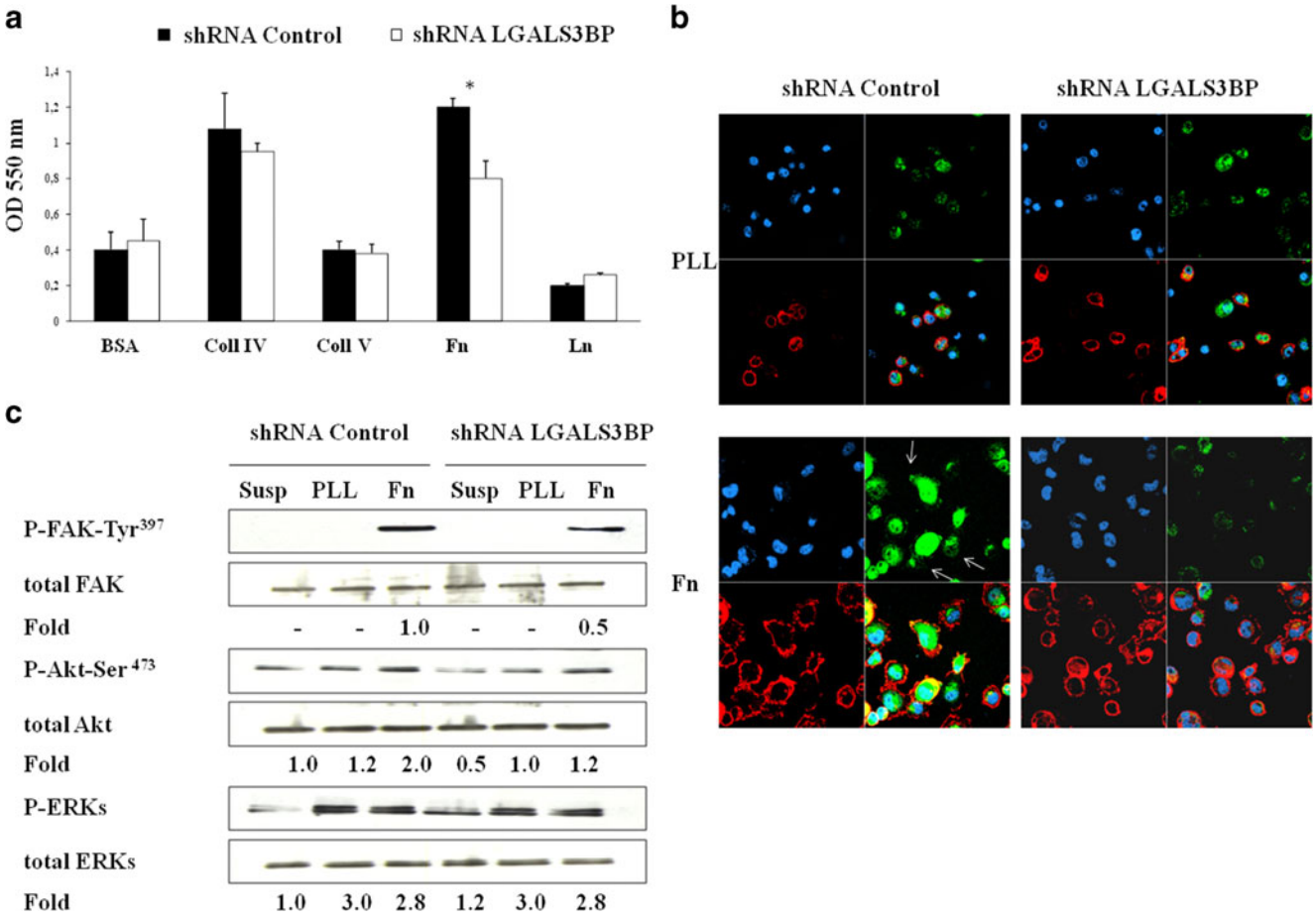


**Fig. 1** shRNA-mediated inhibition of LGALS3BP expression impairs cell adhesion and spreading. **a** Adhesion assay of shRNA control and LGALS3BP MDA-MB-231 cells on ECM proteins. *$P<0.05$ as compared to shRNA control cells. **b** Confocal microscopy images showing shRNA control and shRNA LGALS3BP MDA-MB-231 cells seeded on poly-L-lysine (*PLL*) or fibronectin (*Fn*) for 20 min. Pictures show FAK (*green*), actin (*red*), and nuclei (*blue*). **c** Western blotting of shRNA control and LGALS3BP MDA-MB-231 cells. Cells were plated on PLL or Fn-coated well for 20 min, lysed, and analyzed for the indicated proteins. Cells maintained in suspension (*Susp*) were used as control. Fold increase was calculated by densitometric scanning of the bands using ImageJ. Ratio between the level of the phosphorylated protein and the corresponding total protein level was indicated. The ratio of shRNA control cells was arbitrarily set to 1. Data are represented as mean±SEM of at least three independent experiments

presence of CM from shRNA control cells led to an increased TEP of tumor cells that reached the level of that observed in the presence of serum (Fig. 2b); CM from shRNA LGALS3BP cells did not affect transmigration. As expected, VEGF, used as a positive control, led to an increased TEP, whereas recombinant LGALS3BP failed to increase TEP. Expression of VEGF in MDA-MB-231 shRNA LGALS3BP cells was reduced by approximately 50 % both at mRNA and protein levels (Fig. 2c, d). Similar results were obtained in SK-BR-3, MCF-7 and T47D cells (Fig. S1).

## LGALS3BP stimulates VEGF production via PI3k/Akt pathway in human breast cancer cells

Given the observed downregulation of VEGF in shRNA LGALS3BP cells, we wanted to determine whether LGALS3BP could directly stimulate VEGF production. Exposure of shRNA LGALS3BP cells to the indicated concentration of LGALS3BP for 6 h significantly increased mRNA expression of VEGF (Fig. 3a). The induced transcript was functional, as demonstrated by an increased secretion of VEGF protein into the culture medium (Fig. 3b). The hypoxia inducer, cobalt chloride ($CoCl_2$), was used as a positive control for VEGF stimulation. Similar results were obtained in SK-BR-3, MCF-7, and T47D cells (Fig. S2).

To explore the possible pathway through which LGALS3BP induces VEGF, we first focused on HIF-1α, a major regulator of VEGF expression. To this end, shRNA control and shRNA LGALS3BP cells were exposed to LGALS3BP (10 μg/mL) for different times and HIF-1α levels analyzed by Western blotting. As shown in Fig. 3c, HIF-1α expression was not influenced by LGALS3BP treatment, whereas it was clearly induced after treatment of cells with $CoCl_2$ used as a positive control.

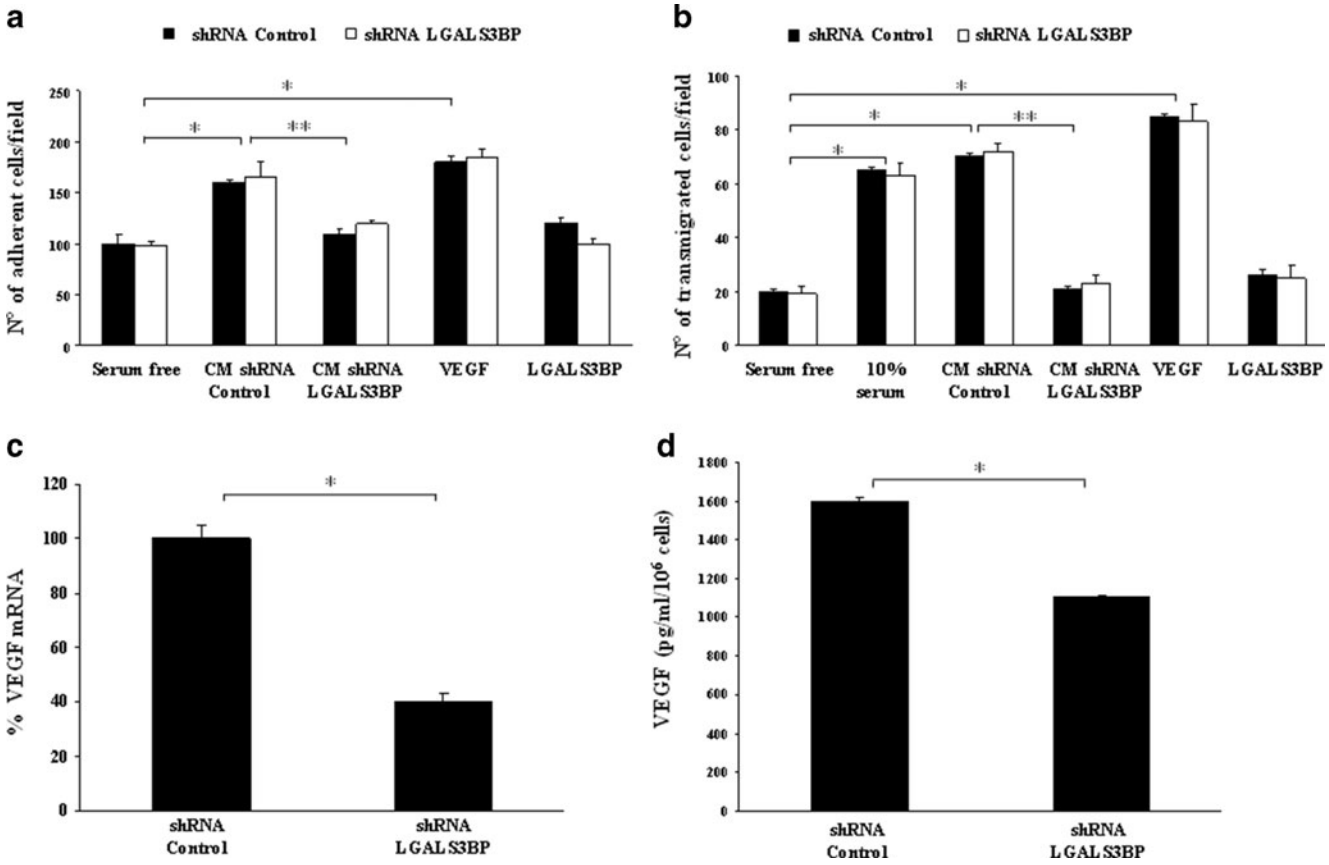


**Fig. 2** shRNA-mediated inhibition of LGALS3BP expression impairs adhesion and penetration of MDA-MB-231 cells across HUVEC. **a** Adhesion assay of MDA-MB-231 cell clones on HUVEC monolayers. Calcein-labeled cells were added to each well in serum-free medium, CM medium from shRNA control and shRNA LGALS3BP cells, VEGF (100 ng/mL), or recombinant LGALS3BP (10 μg/mL) and incubated for 2 h. The number of attached tumor cells was evaluated in ten randomly selected fields at ×200 magnification. The results are presented as the mean±SD of triplicate samples. *$P<0.005$ between serum free and the CM of shRNA control cells. **$P<0.001$ between CM of shRNA control and shRNA LGALS3BP cells. **b** Trasmigration of calcein-labeled MDA-MB-231 clones through HUVEC monolayers grown on fibronectin-coated 24-well transculture inserts. Serum-free medium, CM medium from shRNA control and shRNA LGALS3BP cells, VEGF (100 ng/mL), or human recombinant LGALS3BP (10 μg/mL) were added to the basolateral chambers. After 6 h of incubation, the apical chamber was washed extensively with PBS. The number of migrating tumor cells was evaluated by a fluorescent microscope in ten randomly selected fields at ×200 magnification. The results are presented as the mean±SD of duplicate samples and are representative of three individual experiments. *$P<0.005$ between serum free and the CM of shRNA control cells. **$P<0.001$ between CM of shRNA control and shRNA LGALS3BP cells. **c** Levels of VEGF mRNA and **d** VEGF protein in shRNA control and shRNA LGALS3BP MDA-MB-231 cells were measured by quantitative real-time PCR and ELISA, respectively. *$P<0.05$ compared to shRNA control cells. Data are represented as mean±SEM of at least three independent experiments

We then analyzed other pathways involved in VEGF production. As shown in Fig. 3c, exposure of cells to LGALS3BP (10 ug/mL) resulted in a time-dependent increase of Akt phosphorylation at $Ser^{473}$ residue, with a maximal response between 2 and 10 min. No effect of LGALS3BP on ERKs was observed. Interestingly, in cells incubated with LGALS3BP, VEGF secretion was enhanced up to 1.5-fold as compared to unstimulated cells (1,990±11 vs 1,343±37 pg/mL in ctrl); co-incubation with the PI3k/Akt inhibitor, wortmannin, completely blocked LGALS3BP-induced increase in VEGF secretion (1,990±11 vs 590±29 pg/mL; Fig. 3d). Taken together, these results strongly suggest that increased secretion of VEGF by MDA-MB-231 cells in response to LGALS3BP is mediated by the PI3k/Akt pathway.

## LGALS3BP silencing inhibits in vitro and in vivo angiogenesis

To determine whether the reduced VEGF expression induced by silencing LGALS3BP gene is biologically relevant, CMs from shRNA control and shRNA LGALS3BP cells were tested for their ability to affect in vitro HUVEC tubulogenesis. Consistent with the reduced content of VEGF, HUVEC exposed to CM from shRNA LGALS3BP cells produced less tube-like structures as compared to those exposed to CM from shRNA control cells (Fig. 4a, upper panel). These changes were quantified and found to be significant (Fig. 4a, lower panel).

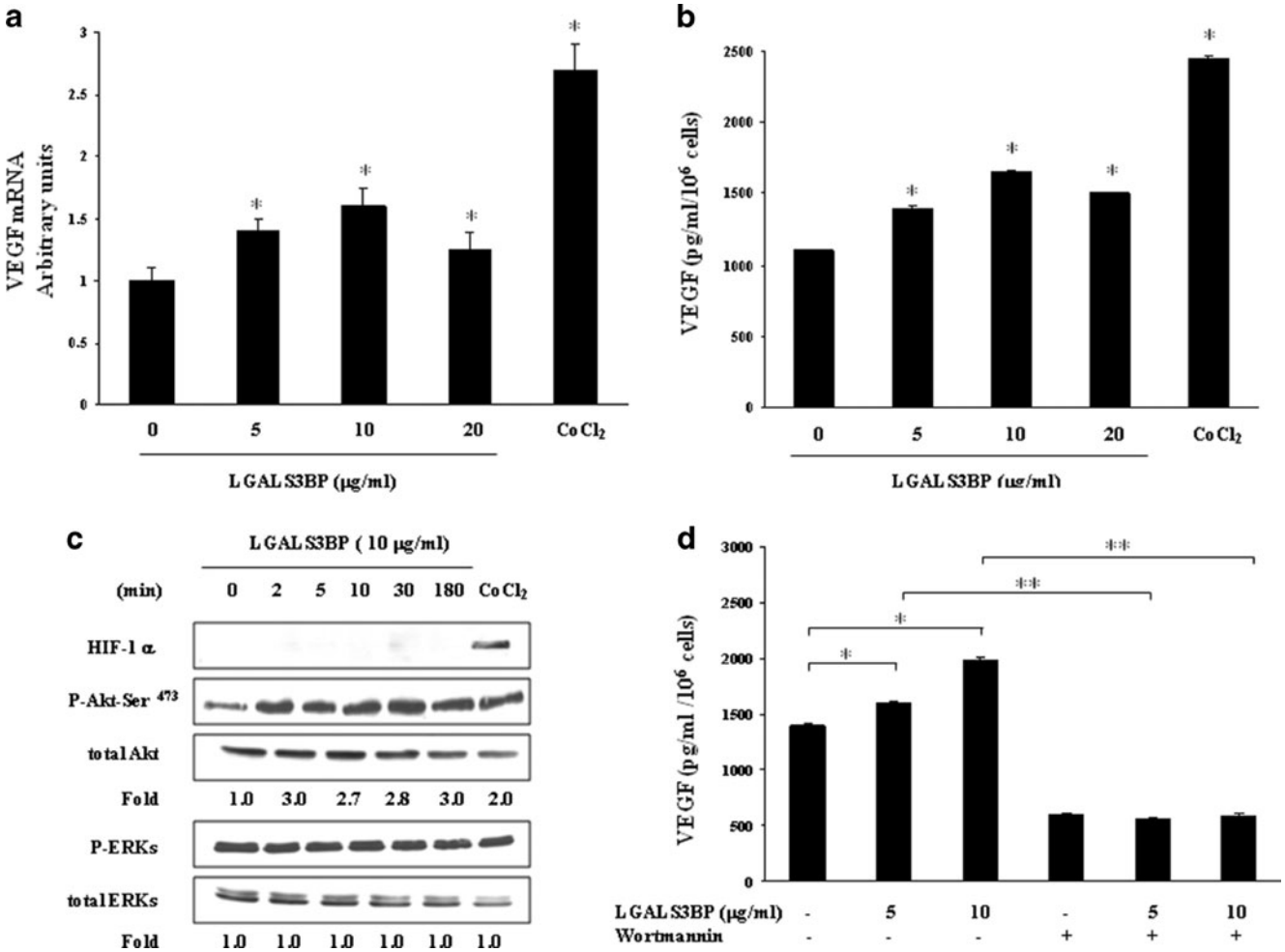


**Fig. 3** LGALS3BP stimulates VEGF production by MDA-MB-231 cells via PI3k/Akt pathway. shRNA LGALS3BP cells were exposed for 24 h with 5 and 10 µg/mL of LGALS3BP or 200 µg/mL $CoCl_2$ as a positive control. **a** Total RNAs were prepared, and VEGF mRNA levels were determined by real-time PCR using VEGF-A-specific primers. The VEGF mRNA arbitrary unit for shRNA control cells was 1.6. *$P<0.05$ compared to cells not exposed to LGALS3BP. **b** The amounts of VEGF in CM of shRNA LGALS3BP cells were quantified with VEGF Human ELISA Kit. The amounts were normalized for the number of cells at the time of media collection. Data are represented as mean±SEM of at least three independent experiments. *$P<0.05$ compared to cells not exposed to LGALS3BP. **c** Western blotting of shRNA LGALS3BP MDA-MB-231 cells treated with LGALS3BP (10 µg/mL) or $CoCl_2$ (200 µg/mL) for the indicated times. After treatment, cell lysates were harvested, and samples were subjected to Western blotting to detect the indicated protein. Fold increase was calculated by densitometric scanning of the bands using ImageJ. Ratio between the level of the phosphorylated protein and the corresponding total protein level is shown. The ratio of cells not exposed to LGALS3BP was arbitrarily set to 1. **d** The amount of VEGF secreted in CM of cells incubated for 24 h with LGALS3BP at 5 and 10 µg/mL with or without a co-incubation with the PI3k/AKT inhibitor, wortmannin (100 nM), was quantified by the VEGF Human ELISA Kit and normalized for the number of cells at the time of media collection. *$P<0.05$ compared to cells not exposed to LGALS3BP. **$P<0.001$ compared to LGALS3BP stimulated cells. Data are represented as mean±SEM of at least three independent experiments

Next, we questioned whether LGALS3BP gene knockdown could influence tumor angiogenesis in vivo. To this end, nude mice were injected subcutaneously with shRNA control or shRNA LGALS3BP MDA-MB-231 cells suspended in growth factor-reduced Matrigel. Injections of Matrigel containing PBS or VEGF were used as negative and positive control, respectively. As shown in Fig. 4b, upper panel, plugs containing shRNA LGALS3BP cells exhibited a reduced angiogenic response compared to that observed in plugs containing shRNA control cells. Quantification of the angiogenic response by determination of the hemoglobin content showed that plugs containing shRNA control cells had a significantly higher (3.4-fold) level of vascularization than those containing shRNA LGALS3BP MDA-MB-231 cells. (Fig. 4b, lower panel). These data demonstrated that LGALS3BP has the capacity to induce the formation of new blood vessel in vivo, and support the hypothesis that LGALS3BP may function as a pro-angiogenic factor.

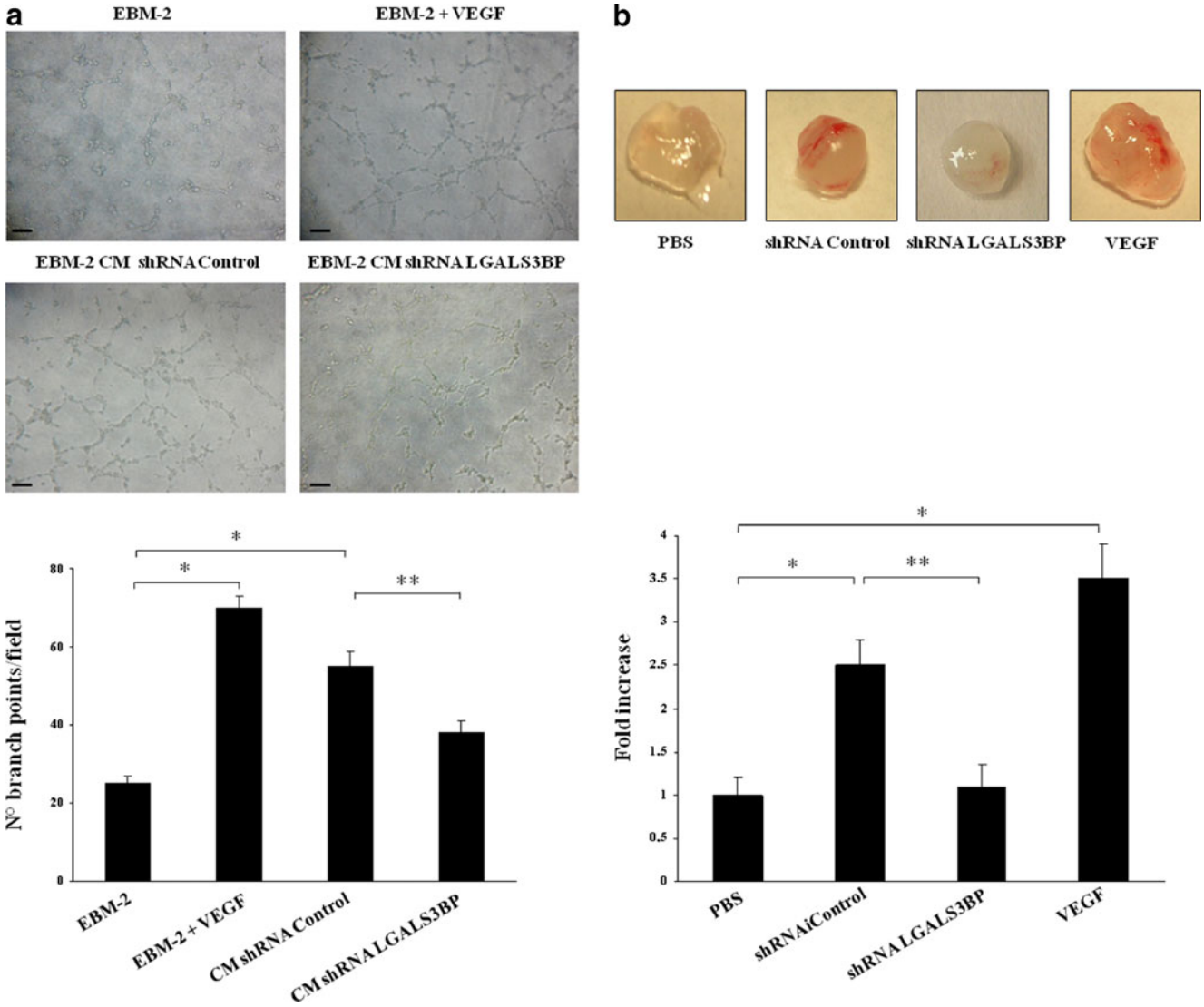


**Fig. 4** LGALS3BP silencing inhibits in vitro and in vivo angiogenesis. **a** HUVEC tubulogenesis in vitro. *Upper panel*, representative phase-contrast photographs of capillary-like tube formation of HUVEC on Cultrex incubated with EBM-2 alone, CMs of shRNA control, or shRNA LGALS3BP MDA MB-231 cells. VEGF (50 ng/mL) was used as a positive control. *Lower panel*, quantification of tube formation was performed by counting closed areas (tubes) in four different fields. Data are represented as mean±SEM from at least three independent experiments. **P*<0.01 compared to EBM-2; ***P*<0.05 compared to CM of shRNA control cells. *Scale bars*, 100 μm. **b** Matrigel plug assay of shRNA control or shRNA LGALS3BP MDA MB-231 cells. *Upper panel*, representative Matrigel plugs containing PBS, shRNA control cells, shRNA LGALS3BP cells, or VEGF. *Lower panel*, quantification of novel blood vessel formation through measurement of the hemoglobin content of the plugs. Six mice per group of treatment were used. **P* <0.004 compared to PBS; ***P*<0.05 compared to shRNA control cells. Data are represented as mean±SEM of at least three independent experiments

## Recombinant LGALS3BP stimulates endothelial cell angiogenesis in a VEGF-independent, galectin-3-dependent manner

Above, we have shown that HUVEC exposed to CM from shRNA LGALS3BP cells form less tube-like structures, probably as a result of a reduced VEGF content. However, a direct involvement of LGALS3BP needs to be ruled out. To this end, MDA-MB-231 cells were knocked down for LGALS3BP and/or VEGF by using specific siRNA and the CMs of the different silenced clones added to HUVEC to perform tube formation. As expected, in the presence of CM from siRNA VEGF or siRNA LGALS3BP cells, tube formation was reduced by about 30 % as compared to that seen with CM from siRNA control cells (Fig. 5a). The use of CM from LGALS3BP/VEGF double knocked down cells resulted in a greater reduction of tube formation. Moreover, addition of recombinant LGALS3BP to CM of double knocked down cells completely rescued tube formation (Fig. 5a), indicating a VEGF-independent effect of

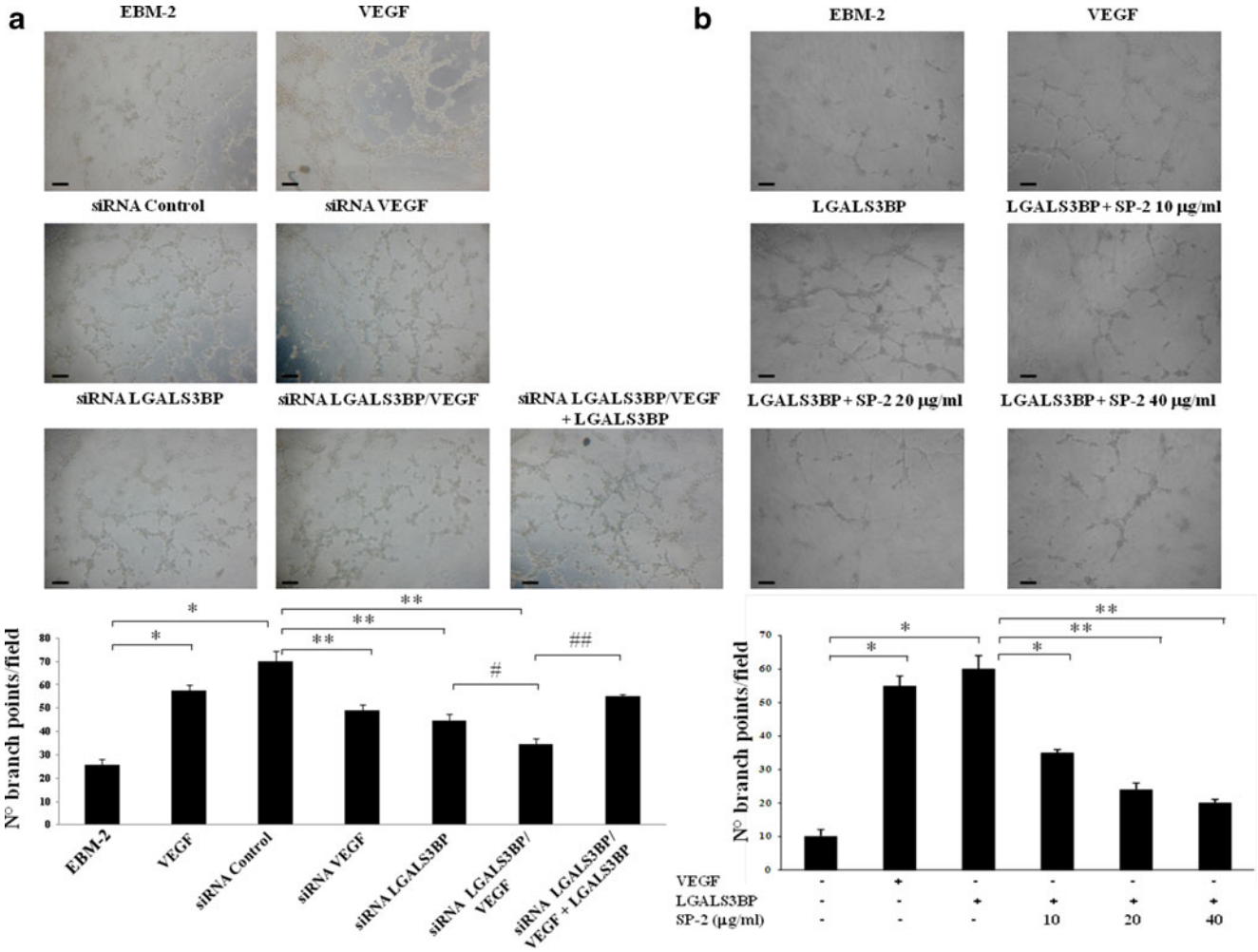


**Fig. 5** Recombinant LGALS3BP stimulates endothelial cell angiogenesis in a VEGF-independent manner. **a** HUVEC tubulogenesis in vitro. *Upper panel*, representative phase-contrast photographs of capillary-like tube formation by HUVEC on Cultrex incubated with serum-free EBM-2, CMs from siRNA control, siRNA VEGF, siRNA LGALS3BP, or siRNA VEGF/LGALS3BP MDA-MB-231 cells. VEGF (50 ng/mL) was used as a positive control. LGALS3BP (10 µg/mL) was added to the CM of siRNA VEGF/LGALS3BP cells. *Scale bars*, 100 µm. *Lower panel*, quantification of tube formation was performed by counting closed areas (tubes) in four different fields. Data are collected from at least three independent experiments. (*$P$<0.001 compared to EBM-2; **$P$<0.008 compared to CM of siRNA control cells; $^{\#}P$<0.005 compared to CM of siRNA LGALS3BP; $^{\#\#}P$<0.0001 compared to CM of siRNA LGALS3BP/VEGF). **b** HUVEC tubulogenesis in vitro. *Upper panel*, representative phase-contrast photographs of capillary-like tube formation of HUVEC on Cultrex, incubated with serum-free EBM-2 in the presence of LGALS3BP (10 µg/mL) or VEGF (50 ng/mL). To test the effect of a LGALS3BP blocking antibody, LGALS3BP was incubated with the indicated concentration of SP-2 prior to the assay. *Scale bars*, 100 µm. *Lower panel*, quantification of tube formation was performed by counting the number of closed areas (tubes) in four different fields. Data are collected from at least three independent experiments. (*$P$<0.001 compared to cells maintained in serum-free EBM-2; **$P$<0.005 compared to LGALS3BP alone)

LGALS3BP. To confirm that the effect was specific for LGALS3BP, we used an anti-LGALS3BP blocking antibody, SP-2, which was shown to inhibit LGALS3BP function (30). SP-2 dose dependently inhibited tube formation (Fig. 5b, upper panel); the effect was quantified and found to be significant (Fig. 5b, lower panel), demonstrating a LGALS3BP specific role in tube formation.

Recently, data have been presented to show that galectin-3 is required for VEGF and bFGF-mediated angiogenic response in endothelial cells [22]. Since galectin-3 is a well-known ligand of LGALS3BP, we wanted to verify whether the lectin is a necessary ligand for LGALS3BP-induced tubulogenesis. To this end, tube formation assay was performed using HUVEC knocked down for galectin-3. As expected, galectin-3 silenced cells produced a less tube-like structure as compared to control cells (Fig. S3A). Most importantly, stimulation of tubulogenesis by LGALS3BP was lost in galectin-3 knocked down cells (Fig. S3A). Furthermore, the use of a galectin-3 blocking antibody, M3-38, inhibited LGALS3BP-induced tube formation. Finally, LGALS3BP was able to induce FAK phosphorylation at tyrosine 125 (Fig S3B) and its translocation to the plasma

membrane to distinct areas of the plasma membrane, to constitute the so-called focal adhesion (Figure S3C) [22]. Altogether, the results indicate that galectin-3 is the ligand responsible for LGALS3BP-stimulated tubulogenesis of endothelial cells.

LGALS3BP is associated with VEGF expression and blood vessel density in breast cancer

To further substantiate a link between LGALS3BP and angiogenesis, we performed immunohistochemistry to stain LGALS3BP, CD31/PECAM, a marker of human vascular endothelial cells, and VEGF in 137 cases of human breast carcinoma. We found that cancers displaying a high percentage of LGALS3BP-positive cells (Fig. 6a) exhibited a high degree of VEGF expression (Fig. 6c) and vascularization (Fig. 6e). On the contrary, cancers with low LGALS3BP (Fig. 6b) showed low VEGF expression (Fig. 6d) and CD-31/PECAM density (Fig. 6f). Pearson Chi-square analysis showed that LGALS3BP expression directly correlated with VEGF expression ($P<0.009$) and blood vessel density ($P<0.0001$; not shown).

## Discussion

Tumor-associated angiogenesis is a hallmark of a growing tumor and thus serves as a rational target for cancer eradication [23, 24]. However, blocking the process of angiogenesis as an effective therapeutic modality still awaits the complete understanding of the many biochemical and molecular signals that regulate the process on one hand and the identification of inducers and inhibitors of angiogenesis on the other.

LGALS3BP is overexpressed in many cancers, and high levels are associated with distant metastasis and poor survival in patients with a variety of cancers. In the present paper, we show for the first time that LGALS3BP induces VEGF in breast cancer cells and promotes endothelial angiogenesis both in vitro and in vivo (Figs. 3 and 4). The pro-angiogenic effect of LGALS3BP appears particularly interesting in light of the observed positive correlation between the expression levels of LGALS3BP, VEGF, and blood vessel density in human breast cancer (Fig. 6). Recently, VEGF and its receptors were also involved in promoting tumor metastasis [25]. These findings could explain the phenomena reported previously that breast cancer patients with elevated serum or tumor levels of LGALS3BP have a significantly higher propensity to develop metastases and a shorter survival [9, 10].

The mechanism(s) through which LGALS3BP induce(s) VEGF in breast cancer cells is (are) presently unknown. This induction is dependent on an intact PI3k/Akt signaling pathway as it was completely blocked by a PI3k/Akt inhibitor (Fig. 3d). Several reports have documented that PI3k/Akt signaling is required for VEGF expression through HIF-1α in response to growth factor stimulation and oncogene activation [26, 27]; however, the PI3k/Akt activation was also associated to VEGF increase with a HIF-1α-independent mechanism [28]. The PI3k/Akt network plays

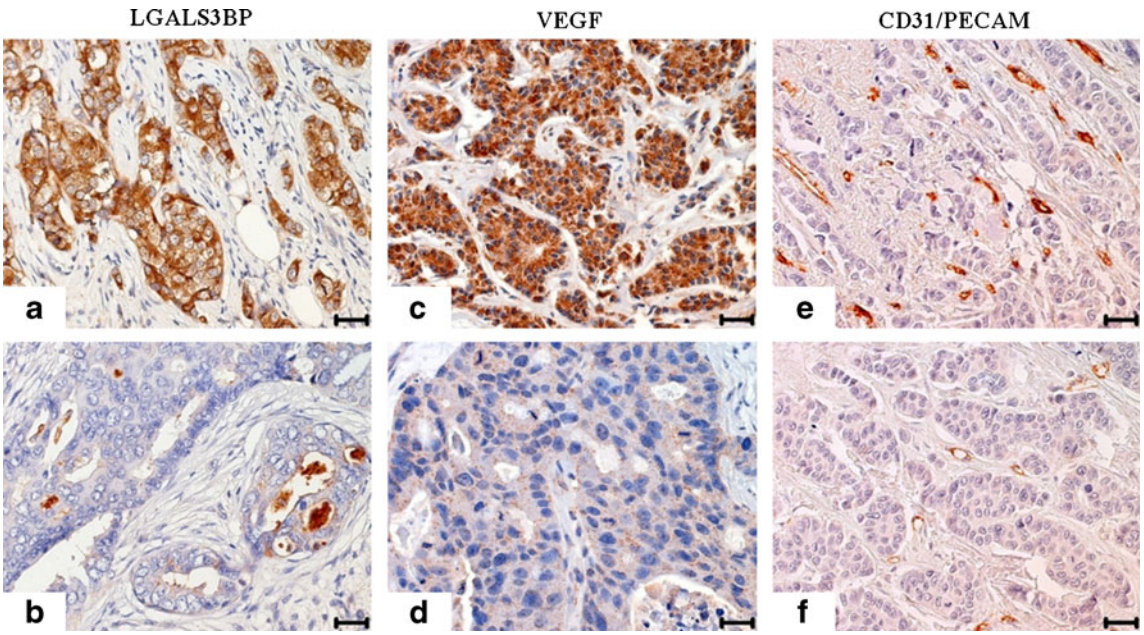


**Fig. 6** Expression levels of LGALS3BP correlate with VEGF expression and blood vessel density in human breast cancer. One hundred and thirty-seven infiltrating breast carcinomas were analyzed for the expression of LGALS3BP, VEGF, and CD-31/PECAM by immunohistochemistry. Representative photographs of high (**a**) and low/absent (**b**) immunostaining for LGALS3BP, VEGF (**c**, **d**), and CD-31 positive vessels (**e**, **f**). Original magnification ×40 (*scale bar*, 20 μm)

a key regulatory function in cell survival, proliferation, and angiogenesis; genetic aberrations found at different levels, either with activation of oncogenes or inactivation of tumor suppressors, make this pathway one of the most commonly disrupted in human breast cancer.

We add new insight in the poorly understood mechanism underlying the promoting effect of LGALS3BP on HUVEC tubulogenesis providing that this effect appears to be VEGF-independent, as it was seen after exposure to CM from VEGF-silenced breast cancer cells (Fig. 5a), or when endothelial cells themselves were knocked down for VEGF (data not shown). Interestingly, promotion of tubulogenesis by LGALS3BP was linked to galectin-3 involvement, activation of FAK, i.e., its phosphorylation and translocation toward membrane sites (Fig. S3). The involvement of galectin-3 and FAK suggests that the pro-angiogenic effect of LGALS3BP could be mediated by integrins, whose clustering is known to trigger FAK activation [29]. Galectin-3 is a member of the galectin family of mammalian lectins that has affinity for B-galactoside structures [30]. Extracellularly, the lectin is assumed to mediate cell–cell and cell–matrix interactions by binding to lactosamine-containing cell surface glycoconjugates, including LGALS3BP. Recently, galectin-3 has been involved in VEGF/bFGF-dependent angiogenesis through cross-linking and clustering of integrin $\alpha v\beta 3$ and activation of FAK [22]. The role of galectin-3–LGALS3BP interaction in the angiogenic response is substantiated by experiments in which this interaction is disrupted. We found previously that synthetic lactulose amines, which block the binding of galectins to LGALS3BP, are capable of preventing tumor cell aggregation and angiogenesis [18]. Therefore, it could be proposed that extracellular LGALS3BP docks galectin-3 molecules, and the resulting complex cross-links and clusters the integrin on the surface of the endothelial cells, thus resulting in the activation of FAK-mediated signaling pathways that modulate the angiogenic cascade.

In summary, the results of this study show that LGALS3BP is an important contributor to angiogenesis. Anti-angiogenic intervention is receiving considerable effectiveness in the suppression of cancer growth and progression, which includes monoclonal antibodies against angiogenic factors and synthetic small molecule inhibitors specific for membrane receptor kinases or intracellular mediators [23, 31]. The present findings that LGALS3BP upregulates tumor VEGF and directly promotes angiogenesis may hold promise for developing novel therapeutic agents targeting LGALS3BP that is overexpressed in a broad type of human cancers.

**Conflict of interest** None

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