

Inhibition of Tumor Growth and Angiogenesis by SP-2, an Anti-Lectin, Galactoside-Binding Soluble 3 Binding Protein (LGALS3BP) Antibody

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Abstract

Accumulating evidence indicates that serum and tissue levels of lectin, galactoside-binding soluble 3 binding protein (LGALS3BP), a secreted glycoprotein, are elevated in human cancers. Recently, we have identified LGALS3BP as a factor capable of stimulating angiogenesis of microvascular endothelial cells *in vitro* as well as *in vivo*. However, the potential therapeutic implications of LGALS3BP function blockade have not been explored yet. Here, we tested the ability of an anti-LGALS3BP mouse monoclonal antibody, SP-2, to antagonize LGALS3BP-induced angiogenesis and tumor growth. The antibody was found to inhibit endothelial cell tubulogenesis induced by either conditioned medium of breast cancer and melanoma cells or human recombinant LGALS3BP. In addition, SP-2 inhibited phosphorylation of FAK and its recruitment to membrane sites as well as AKT and ERK phosphorylation promoted by LGALS3BP. When used *in vivo*, the antibody restrained LGALS3BP-stimulated angiogenesis and growth of tumor xenografts. Furthermore, the combination of SP-2 and low-dose bevacizumab was more effective than either agent alone. Taken together, these results lead to consideration of SP-2 as a promising candidate for LGALS3BP-targeted therapy. *Mol Cancer Ther*; 13(4); 916–25. ©2014 AACR.

Introduction

Lectin, galactoside-binding soluble 3 binding protein (LGALS3BP; also known as 90K or Mac-2 BP) is a large glycoprotein that forms oligomers of 1,000 to 1,500 kDa in the extracellular milieu and promotes cell adhesion to matrix proteins (1). Over the past decade, considerable attention has been paid to the potential role of LGALS3BP in the development and progression of human cancer. Immunohistochemical and gene expression analysis showed significantly high levels of LGALS3BP in different types of human malignancies (2). Furthermore, clinical studies have revealed that high serum or tumor tissue levels of LGALS3BP were associated with a shorter survival in patients with breast carcinoma (3, 4), lymphoma (5), pleural mesothelioma (6), and non-small cell lung carcinoma (7). Despite these evidences, little is known regarding the mechanism(s) underlying LGALS3BP activ-

ity in cancer. Recently, we identified LGALS3BP as a novel proangiogenic factor capable of inducing VEGF in human breast cancer cells and promoting angiogenesis by a direct stimulation of endothelial cells (8).

SP-2 is a murine monoclonal antibody recognizing the lectin-binding domain of LGALS3BP (9, 10). Recently, SP-2 was found to reduce LGALS3BP-induced tube formation in Matrigel by endothelial cells (8). In the present study, we tested the ability of SP-2 to inhibit LGALS3BP-stimulated angiogenesis and tumor growth.

Our findings highlight the potential therapeutic benefits of anti-LGALS3BP treatment.

Materials and Methods

Cell lines and culture

The human cancer cell lines MDA-MB-231 (breast) and SKOV-3 (ovary) were purchased in 2012 from the American Type Culture Collection; these cell lines were authenticated with short tandem repeat profiling. The cutaneous melanoma cell lines IR-8 were kindly provided in 2010 by Dr. Carlo Leonetti (Regina Elena National Cancer Institute, Rome, Italy); the melanoma cell line, MEL-8863 was kindly provided in 2010 by Dr. Enrico Proietti (Istituto Superiore di Sanità, Rome, Italy); for these cell lines, no authentication was done by the authors. MDA-MB-231, SKOV-3, and MEL-8863 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen), whereas IR-8 cells were maintained in RPMI-1640 medium (Invitrogen). All media were supplemented with 10% heat-inactivated FBS (Invitrogen), L-glutamine, and

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antibiotics (Sigma Aldrich Corporation). The human umbilical vein endothelial cells (HUVEC) were purchased in 2012 from Lonza and maintained in EBM-2 Medium (Lonza) containing 10% heat-inactivated FBS supplemented with EGM-2 singlequots (Lonza).

All cells were maintained at 37°C in a humidified chamber with 95% air and 5% CO₂.

The generation of MDA-MB-231 and IR-8 cells silenced for LGALS3BP (shLGALS3BP) or not (shCTR), was performed in 2012 as previously described (8). For collection of the conditioned medium, cells were grown until 80% confluent, washed with PBS, and cultured in serum-free medium for additional 24 to 48 hours. Conditioned medium was collected, centrifuged at 1,200 rpm for 15 minutes, aliquoted under sterile conditions and frozen at -80°C until use.

All cell lines were maintained in liquid nitrogen and before each experiment were passaged for fewer than 4 weeks after resuscitation. Moreover, all cell lines were mycoplasma free, as assessed by Hoechst 33342 Fluorescent Stain (Thermo Scientific) and PCR analysis.

Recombinant LGALS3BP

Recombinant human LGALS3BP was immunoaffinity purified (11) from serum-free supernatant of human embryonic kidney EBNA-293 cells (Invitrogen) transfected with LGALS3BP cDNA (1), as previously described (8). SDS-PAGE showed a major band (90%) migrating at approximately 97 kDa. The endotoxin level of the final preparation was <5 EU/μg, as evaluated by the LAL (Lymulus Amebocyte Lysate) test (Clongen Labs).

Anti-LGALS3BP antibody

The antibody SP-2 was generated by immunizing mice with proteins secreted into culture medium of human breast cancer cells (9). Hybridoma cells were cultured in a benchtop BioFlo 3000 bioreactor (New Brunswick Scientific) using serum-free BD Cell MAb Medium (Becton Dickinson). During a three-week growth period, cell proliferation and antibody production were monitored once a week. Medium was collected, centrifuged at 1,200 rpm for 5 minutes to remove cell debris and concentrated using Vivaflow-200 membrane (Sartorius Stedim Biotech). The antibody was purified on a Protein-G column (Biovision) and dialyzed against PBS. The antibody was found about 95% pure, as judged by Coomassie blue staining of SDS-PAGE.

Confocal microscopy

HUVEC (5 × 10⁴ cells/well) were seeded on glass coverslips and allowed to grow for 24 hours at 37°C in 5% CO₂. Cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature, permeabilized with 0.25% Triton X-100 for 5 minutes and blocked with 0.1% bovine serum albumin for 1 hour at room temperature. Coverslips were then incubated for 2 hours at room temperature with anti-αvβ3 (Millipore Corporation), or anti-P-FAK Tyr 397 (Cell Signaling Technology) as primary antibodies, followed by Alexa-Fluor conjugated

secondary antibodies (Molecular Probes, Life Technologies). Fluorescein isothiocyanate (FITC)/TRITC-labeled phalloidin was used to visualize actin cytoskeleton and DRAQ5 (Vinci Biochem) to visualize nuclei. Images were acquired with a Zeiss LSM 510 meta-confocal microscope (Zeiss) using 488, 543, and 633-nm lasers. Detector gain voltages and pinhole were set at the beginning of the experiment and maintained constant during the acquisition of all samples.

Tubulogenesis assay

Capillary tube formation (tubulogenesis) assay was performed as described (12). HUVECs were suspended in either EBM-2 medium or conditioned medium of shCTR or shLGALS3BP cells and seeded at 5 × 10⁴ cells per well in Cultrex BME (Matrigel, Trevigen) coated chamber slides. Human recombinant VEGF (R&D Systems) was used as positive control. After incubation at 37°C for 4 hours, four photographs for each individual well were taken at different locations and analyzed by ImagePro Plus software (Media Cybernetics). Quantitative determination of tube formation was performed by counting the number of branch points.

Matrigel plug assay

The Matrigel plug assay was performed as described (13) with minor modifications. Female athymic CD-1^{nu+/nu+} mice (6 weeks old; 25 g each; Charles River Laboratories) were injected subcutaneously with plugs made of 0.5 mL Matrigel containing LGALS3BP (60 μg/mL) or human VEGF (200 ng/mL), as a positive control, or PBS. Mice implanted with plugs containing LGALS3BP were randomly divided into 2 groups and injected intraperitoneally with SP-2 (15 mg/kg) or PBS on alternate days for a total of 4 injections.

In other experiments, mice were implanted subcutaneously with 0.5 mL Matrigel containing either 5 × 10⁵ MDA-MB-231 cells or PBS; starting the day after implantation, animals harboring MDA-MB-231 cells were injected intraperitoneally on alternate days with either SP-2 (15 mg/kg), bevacizumab (Roche, 2 mg/kg) or a combination of SP-2 (15 mg/kg) and bevacizumab (2 mg/kg) for a total of 4 injections.

Six animals per group were used. Ten days after implantation, animals were sacrificed, plugs removed, photographed, and their hemoglobin content assayed using the Drabkin method (ref. 14; Drabkin reagent kit 525, Sigma Aldrich Corporation).

Western blotting

Cells were lysed with radioimmunoprecipitation buffer containing protease and phosphatase inhibitors (Sigma Aldrich Corporation). Lysates were clarified by centrifugation at 14,000 rpm for 15 minutes at 4°C, subjected to 10% SDS-PAGE and Western blotting using specific antibodies against rabbit P-FAK Tyr³⁹⁷, P-AKT Ser⁴⁷³, P-ERK1/2 Thr²⁰²/Tyr²⁰⁴ (Cell Signaling Technology) or mouse actin (Sigma Aldrich Corporation).

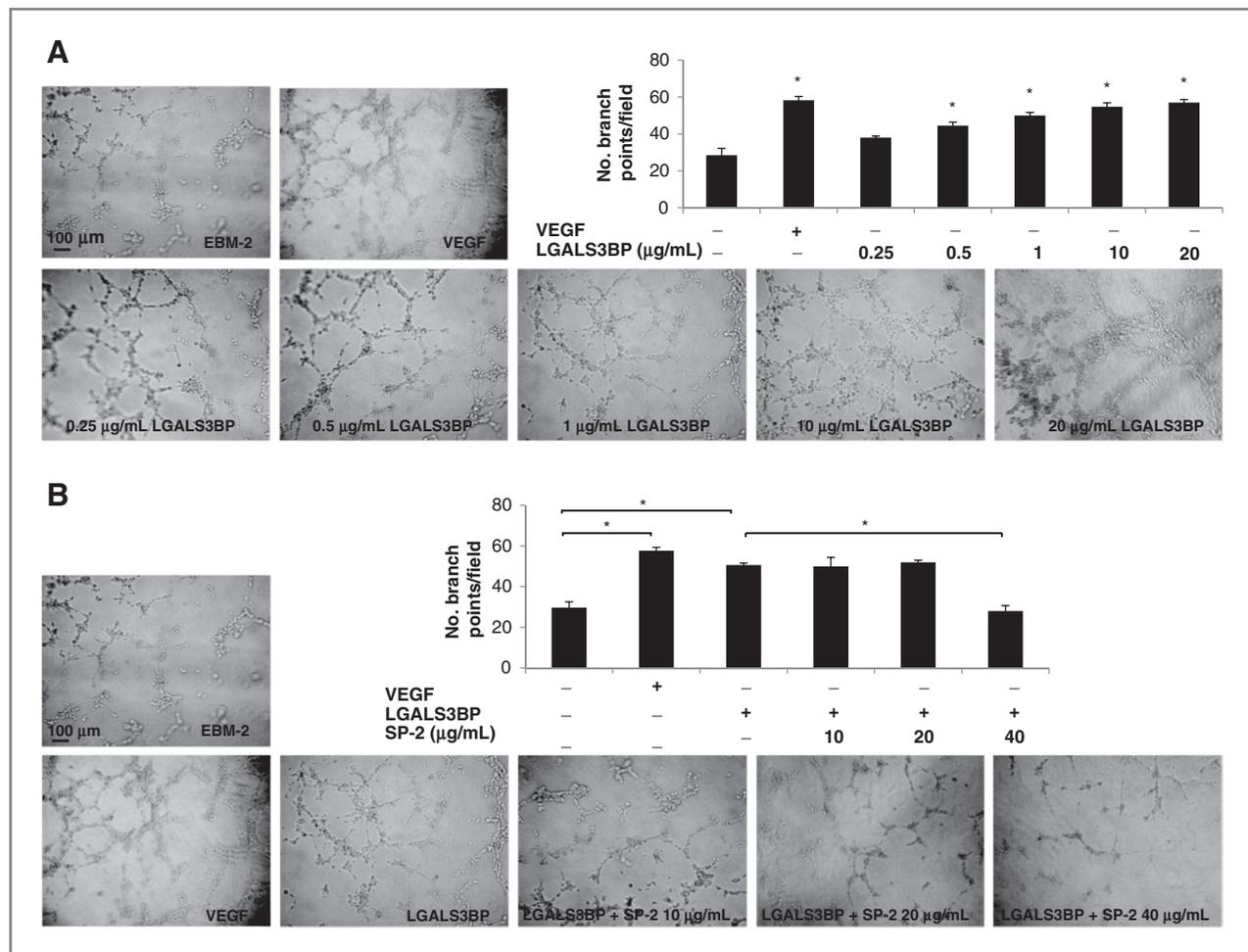


Figure 1. SP-2 inhibits tube formation induced by recombinant LGALS3BP. A, HUVEC (5×10^4 cells) in EBM-2 medium were seeded on Matrigel-coated chamber slides and exposed to recombinant LGALS3BP at the indicated concentrations (0.25 to 20 µg/mL; A), or recombinant LGALS3BP (1 µg/mL; B) in the absence or presence of SP-2 at the concentrations of 10, 20, 40 µg/mL. VEGF (50 ng/mL) was used as a positive control. Pictures show representative phase-contrast photographs of capillary-like structures taken after 4 hours from seeding. Histograms show quantification of tube formation. Data represent mean \pm SD of three independent experiments. *, $P < 0.05$.

Incubation was performed overnight at 4°C. After washing with PBS containing 0.1% Tween-20, blots were incubated with HRP-conjugated IgG secondary antibodies (Bio-Rad) at room temperature for 2 hours and developed with a chemiluminescence detection system (Perkin-Elmer).

Tumor xenografts

The following human tumor cell lines were implanted subcutaneously into the right flank of athymic female CD-1^{nu+/nu+} mice (6 weeks old; 25 g each; Charles River Laboratories): MDA-MB-231 (breast cancer), SKOV-3 (ovarian cancer), IR-8 and MEL-8863 (melanoma). When tumors became palpable (approximately 50–100 mm³), animals implanted with the different cell lines (each at 5×10^6 cells) were randomly divided into two groups of 10 animals: one group was treated intraperitoneally with SP-2 and the other with a mouse IgG (as a control group), both at the dose of 15 mg/kg twice a week.

In experiments evaluating the combination of SP-2 with bevacizumab, animals were implanted with MDA-MB-231 cells and randomly divided into four groups (10 animals each). Starting 3 days after cell implantation, animals were injected intraperitoneally twice a week with either SP-2 (15 mg/kg), bevacizumab (2 mg/kg), a combination of both, or mouse IgG (15 mg/kg; as a control group). Tumor volume was monitored twice a weekly with a caliper and calculated using the following formula: tumor volume (mm³) = (length \times width²)/2. Animal studies were approved by the local Institutional Animal Ethics Committee.

Immunohistochemistry

Cryostat sections (6 µm) of tumor xenografts were fixed in ice-cold acetone for 10 minutes. Blood vessels were identified by incubating sections with a mixture of rat antibodies, one against mouse CD31 (PECAM-1, BD Pharmingen) and the other against mouse CD105 (endoglin). A

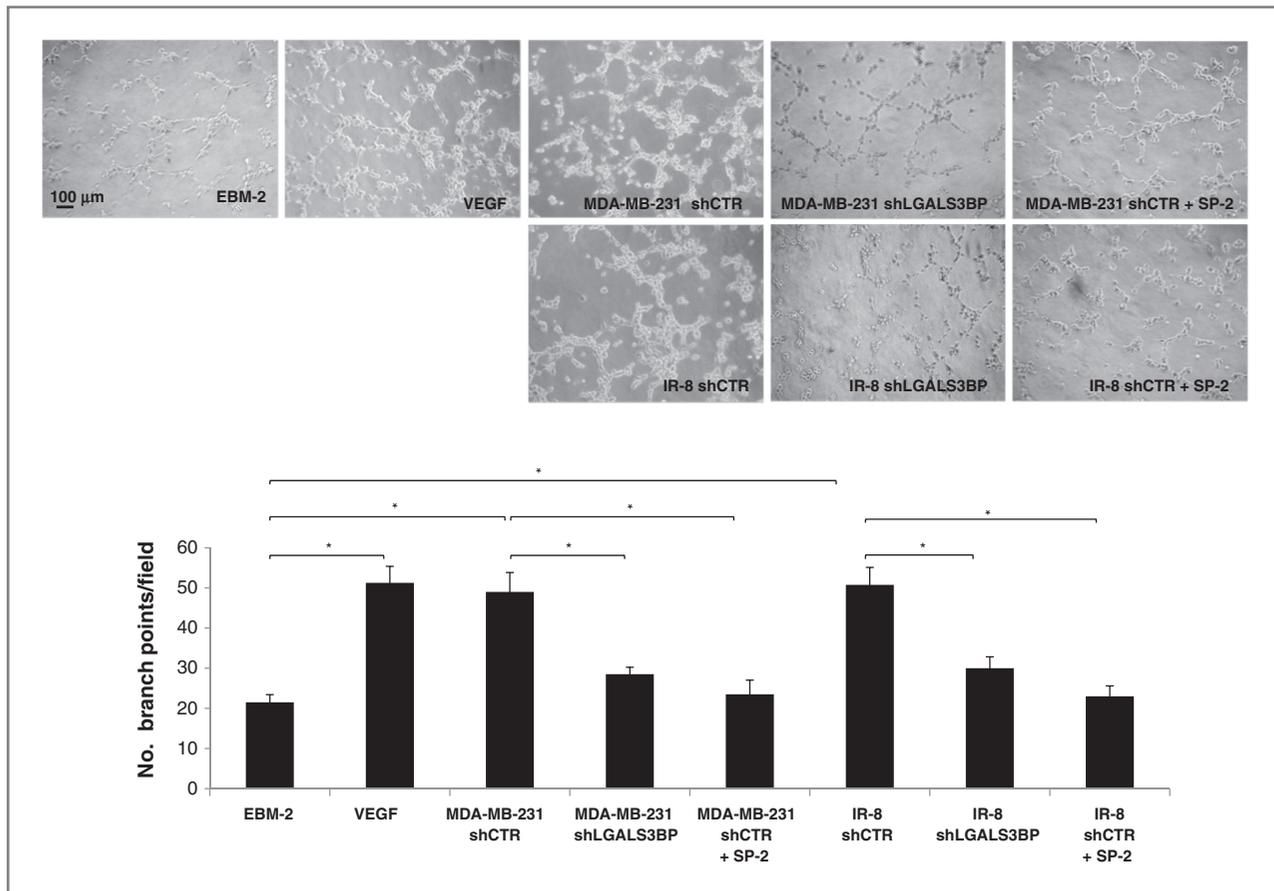


Figure 2. SP-2 inhibits tube formation induced by conditioned medium (CM) of MDA-MB-231 breast cancer and IR-8 melanoma cells. HUVECs suspended in either EBM-2 medium, or conditioned medium of shCTR cells, or shLGALS3BP cells, or shCTR cells treated with SP-2 (40 $\mu\text{g}/\text{mL}$), were seeded at 5×10^4 cells per well in Matrigel-coated chamber slides. VEGF (50 ng/mL) was used as a positive control. Pictures show representative phase-contrast photographs of capillary-like structures taken after 4 hours from seeding. Histograms show quantification of tube formation. Data represent mean \pm SD of three independent experiments. *, $P < 0.001$.

goat anti-rat IgG was used as a secondary antibody (Jackson IR). Following incubation with avidin–biotin–alkaline phosphatase complex (ABC-AP, Thermo Scientific), the reaction was visualized using Vulcan Fast RED (Biocare Medical) as a chromogen. Slides were counterstained with CAT hematoxylin (Biocare Medical). Positive controls were stained in parallel. Omission of the primary antibody was used as a negative control. Immunoreactive microvessels were counted by one pathologist (R. Lattanzio) at $400\times$ magnification (0.17 mm^2) in 4 different areas characterized by the greatest number of intratumoral microvessels (hotspots) and averaged for each tumor.

Statistical analysis

Data are expressed as mean \pm SEM and differences among groups were determined using one-way ANOVA analysis. Mean differences in vessel counts between tumors from animals treated with SP-2 or mouse IgG were compared with the use of the paired t test. The 0.05 level of probability was used as the criterion of significance. Mann–Whitney test was used to compare the efficacy of treatments in xenograft experiments.

Results

SP-2 inhibits tube formation induced by LGALS3BP

We previously reported that human recombinant LGALS3BP was able to enhance tube formation by a direct stimulation of HUVECs (8). Here, we confirm and extend this finding by showing that tube formation was stimulated at a protein concentration as low as 0.5 $\mu\text{g}/\text{mL}$ (Fig. 1A). SP-2 (40 $\mu\text{g}/\text{mL}$) inhibited LGALS3BP-induced tube formation by approximately 50% (Fig. 1B). SP-2 alone showed any effect on tube formation (data not shown).

To evaluate whether native LGALS3BP was also effective, tube formation assay was performed in the presence of conditioned medium of two human tumor cell lines (MDA-MB-231 breast cancer and IR-8 melanoma) expressing high LGALS3BP levels. In both cases, an increase in tubes number was observed compared with the EBM-2 medium alone (Fig. 2). In the presence of SP-2, the number of tubes was as low as that observed when HUVEC were exposed to conditioned medium from shLGALS3BP cells (Fig. 2). These results indicate that both recombinant and native LGALS3BP are similarly effective in promoting tube formation by HUVEC and that SP-2 neutralizes this effect.

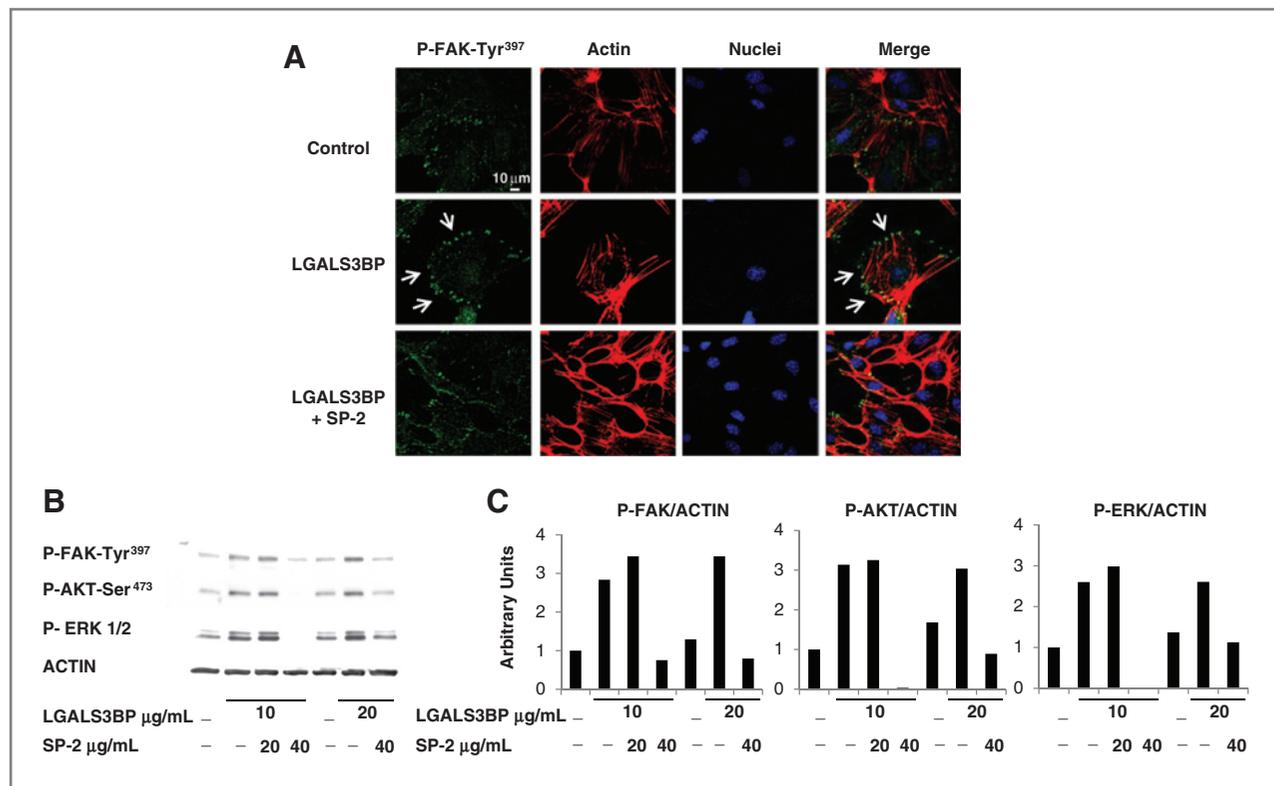


Figure 3. SP-2 inhibits LGALS3BP-induced cell signalling activation. **A**, HUVEC (5×10^4 cells/well) were seeded on glass coverslips and maintained in EBM-2 medium in the absence (control) or presence of LGALS3BP (10 $\mu\text{g/mL}$) alone or in combination with SP-2 (40 $\mu\text{g/mL}$) for 10 minutes. Cells were then fixed and processed for immunofluorescence. Staining of P-FAK Tyr³⁹⁷ (green), actin (red), and nuclei (blue) was observed using a confocal microscope. Representative photographs are shown. Arrows indicate P-FAK membrane recruitment. **B**, HUVECs were exposed to LGALS3BP (10 or 20 $\mu\text{g/mL}$) alone or in the presence of SP-2 (20 or 40 $\mu\text{g/mL}$) for 10 minutes. Cell lysates were subjected to Western blot analysis to detect FAK, AKT, ERK phosphorylation. Actin was used as an internal loading control. Representative Western blots are shown. **C**, histograms represent band intensity ratio of P-FAK/actin, P-AKT/actin, and P-ERK/actin, respectively, as measured by Image J software.

SP-2 counteracts LGALS3BP-induced FAK activation, $\alpha\text{v}\beta 3$ membrane clustering, and actin remodeling

Confirming previous findings (8), exposure of HUVEC to recombinant LGALS3BP resulted in enhanced FAK phosphorylation and translocation to membrane sites (Fig. 3A). Here, we further demonstrate that LGALS3BP induced phosphorylation of FAK, AKT, and ERK (Fig. 3B and C) and that these effects were markedly reduced when cells were coexposed to SP-2 (Fig. 3A–C). Promotion of tube formation by recombinant LGALS3BP may involve binding of the protein to its endogenous ligand, galectin-3, followed by crosslinking and clustering of integrin $\alpha\text{v}\beta 3$ on the surface of endothelial cells (15). This hypothesis was confirmed by results of Fig. 4 showing that exposure of HUVEC to recombinant LGALS3BP was accompanied by $\alpha\text{v}\beta 3$ membrane clustering and promotion of lamellipodia formation; this effect was inhibited by coincubation with SP-2.

FAK activation by integrins is involved in actin remodeling and endothelial cell migration (16). Therefore, we sought to determine whether SP-2 could antagonize LGALS3BP-induced actin remodeling. Exposure of HUVEC to LGALS3BP was associated with actin membrane recruitment in lamellipodia, whereas the pres-

ence of these structures decreased when HUVEC were coincubated with SP-2 (Fig. 4).

The *in vivo* antiangiogenic effect of SP-2 is potentiated by bevacizumab

Previously, we found that silencing of LGALS3BP in MDA-MB-231 cells impaired breast cancer cell to induce blood vessels formation *in vivo* (8). To explore the inhibitory effect of SP-2 on LGALS3BP-induced angiogenesis *in vivo*, we first tested the ability of recombinant LGALS3BP to directly stimulate blood vessel formation in the Matrigel plug assay. To this end, nude mice were injected subcutaneously with Matrigel mixed with different concentrations of LGALS3BP or PBS. As shown in Fig. 5A, LGALS3BP increased blood vessel formation in a dose-dependent fashion, reaching similar effect to that induced by VEGF as evaluated by determining the hemoglobin content of the plugs.

To assess the effect of SP-2, nude mice bearing plugs containing LGALS3BP (30 μg) were injected intraperitoneally with SP-2 (15 mg/kg) or PBS on alternate day for a total of 4 injections. As shown in Fig. 5B, plugs derived from SP-2-treated animals exhibited a reduced angiogenic response compared with plugs derived from PBS-treated mice.

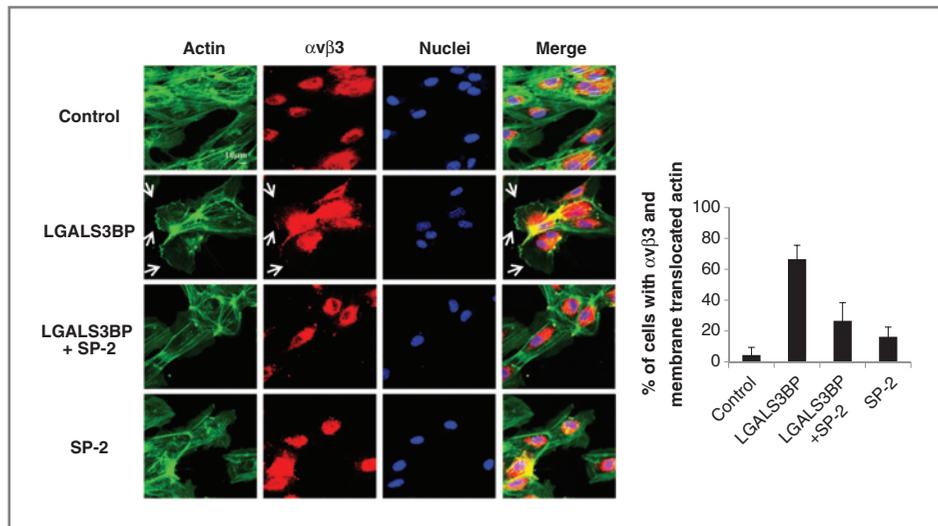


Figure 4. SP-2 counteracts LGALS3BP-induced $\alpha\beta3$ membrane clustering and actin membrane recruitment. HUVECs (5×10^4 cells/well) in EBM-2 medium were seeded on glass coverslips and treated with LGALS3BP (10 $\mu\text{g}/\text{mL}$) with or without SP-2 (40 $\mu\text{g}/\text{mL}$) for 15 minutes. Cells were fixed and processed for confocal microscopy analysis to detect $\alpha\beta3$ integrin (red), actin (green), and nuclei (blue) localization. Representative photographs are shown. Arrows indicate actin membrane recruitment (image on the left), $\alpha\beta3$ membrane clustering (image in the middle), and their colocalization in merged image. Each bar of the histogram represents percentage of cells with $\alpha\beta3$ clustering and actin membrane recruitment for the indicated treatments.

These data demonstrate that SP-2 is able to inhibit LGALS3BP-induced formation of blood vessel *in vivo*, supporting the hypothesis that the antibody may function as an efficient antiangiogenic agent.

To compare the effect of SP-2 with that of bevacizumab, nude mice implanted with plugs containing MDA-MB-231 cells which secrete high levels of both LGALS3BP and VEGF (8) were injected intraperitoneally with SP-2 (15 mg/kg), bevacizumab (2 mg/kg) or a combination of SP-2 and bevacizumab. The dose of bevacizumab (2 mg/kg) was chosen as the one sufficient to almost completely abolish angiogenesis induced by standard doses (100 ng) of VEGF in preliminary experiments (data not shown). As shown in Fig. 5C, the antiangiogenic effect of SP-2 was comparable with that of bevacizumab. Remarkably, the combination of SP-2 and bevacizumab was more effective than either agent alone, resulting in a nearly complete inhibition of angiogenesis.

SP-2 restrains tumor growth and its effect is potentiated by bevacizumab

To evaluate the effect of SP-2 on tumor growth, we employed tumor xenograft models grown in nude mice. Compared with the IgG control group, the administration of SP-2 (15 mg/kg i.p. twice a week) resulted in a significant delay in tumor growth (395 mm^3 vs. 85 mm^3 ; $P < 0.007$ for MDA-MB-231 and 411 mm^3 vs. 233 mm^3 ; $P < 0.043$ for IR-8; Fig. 6A and B).

The same effects were seen in tumor xenografts derived from ovarian cancer SKOV-3 cells and melanoma MEL-8863 cells (Supplementary Fig. S1).

Moreover, tumors from SP-2-treated animals showed reduced angiogenesis compared with control group, as estimated by counting the number of CD31/CD105-pos-

itive microvessels (17.3 vs. 22 for MDA-MB-231; 13.2 vs. 16.1 for IR-8; Fig. 6C and D).

To evaluate whether treatment with SP-2 plus low-dose bevacizumab leads to better tumor inhibition compared with SP-2 or bevacizumab treatment alone, we injected MDA-MB-231 cells into nude mice. Starting 3 days from cell implantation, mice were injected intraperitoneally with SP-2 (15 mg/kg), bevacizumab (2 mg/kg), SP-2 plus bevacizumab or with mouse IgG (15 mg/kg), as a control. Compared with the IgG control group, SP-2-treated mice showed significantly reduced tumor volumes (600 mm^3 vs. 287 mm^3 ; $P < 0.005$); the low-dose bevacizumab-treated mice did not show significantly reduced tumor volumes (600 mm^3 vs. 358 mm^3 ; $P = 0.057$) and tumor volumes for mice treated with SP-2 plus bevacizumab were reduced significantly (600 mm^3 vs. 169 mm^3 ; $P < 0.001$). Furthermore, mice injected with SP-2 plus bevacizumab had significantly reduced tumor volumes compared with SP-2-treated mice (169 mm^3 vs. 287 mm^3 ; $P = 0.048$; Fig. 6E).

Discussion

Tumor angiogenesis is an integral part of solid tumor development resulting from the imbalance between proangiogenic and antiangiogenic factors (17). However, much more remains to be understood about the molecular nature of unidentified factors that have the ability to promote angiogenesis in the development of human cancers.

LGALS3BP has an emerging role in tumor progression and development of metastasis (18). Elevated expression levels of LGALS3BP have been found in the large majority of solid human tumors. Several groups have reported that

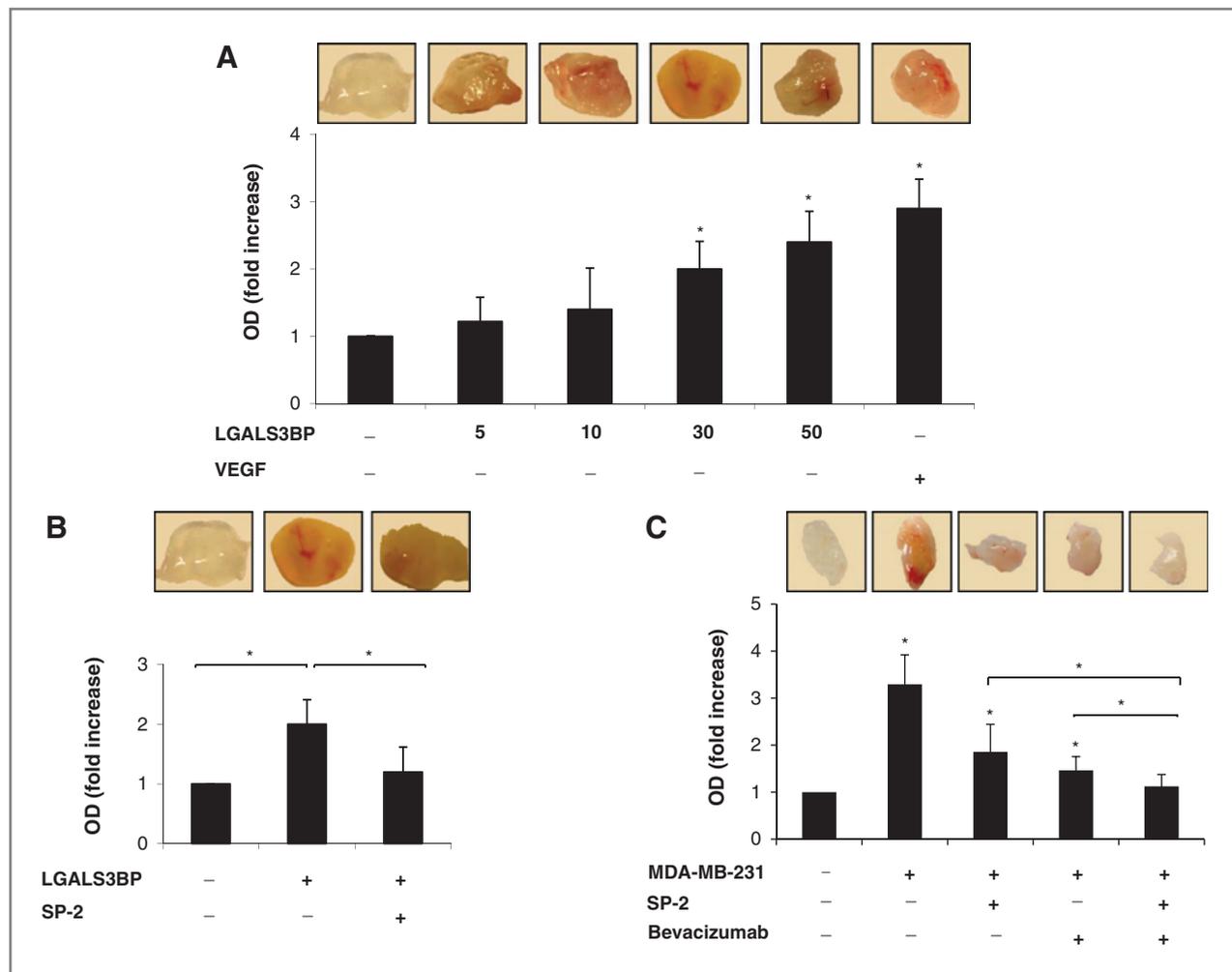


Figure 5. The antiangiogenic effect of SP-2 *in vivo* is potentiated by bevacizumab. **A**, aliquots (0.5 mL) of Matrigel containing either LGALS3BP, VEGF (as a positive control), or PBS, were injected subcutaneously into nude mice. Six mice per group of treatment were used. Seven days after implantation, mice were sacrificed and plugs removed and photographed. Top, representative photographs of Matrigel plugs. Bottom, quantification of blood vessel formation through measurement of hemoglobin content normalized to the plug weight. Results are expressed as fold increase relative to plugs containing PBS \pm SEM of at least three independent experiments. *, $P < 0.05$. **B**, aliquots (0.5 mL) of Matrigel containing either LGALS3BP (30 μ g) or PBS were injected subcutaneously into nude mice (6 mice/group). Starting the day after implantation, animals implanted with LGALS3BP-containing plugs were injected intraperitoneally with SP-2 (15 mg/kg) or PBS on alternate days for a total of 4 injections. The day after the last injection, mice were sacrificed, and plugs removed. Top, representative photographs of Matrigel plugs. Bottom, quantification of blood vessel formation through measurement of hemoglobin content normalized to the plug weight. Each bar represents fold increase relative to plugs containing PBS \pm SEM of at least three independent experiments; *, $P < 0.05$. **C**, aliquots (0.5 mL) of Matrigel containing either MDA-MB-231 cells (5×10^5) were injected subcutaneously into nude mice (6 mice/group). Starting the day after implantation, animals were injected intraperitoneally on alternate days for a total of 4 injections with SP-2 (15 mg/kg), bevacizumab (2 mg/kg), a combination of SP-2 (15 mg/kg) and bevacizumab (2 mg/kg) or PBS. The day after the last injection, mice were sacrificed, plugs removed, photographed, and their hemoglobin content assayed using the Drabkin method (14). Top, representative images of Matrigel plugs. Bottom, hemoglobin content of the plugs normalized to plug weight. Each bar represents fold increase relative to plugs containing PBS \pm SEM of at least three independent experiments. *, $P < 0.05$.

high LGALS3BP levels in serum or tumor tissue of patients with cancer correlate with a poor survival or a more advanced disease in breast cancer (4), lung cancer (7), prostatic cancer (19), hepatocarcinoma (20), melanoma (21), and non-Hodgkin lymphoma (5).

Recently, we have found that LGALS3BP secreted by human breast cancer cells functions as a proangiogenic factor through a dual mechanism, that is, induction of VEGF by tumor cells and stimulation of endothelial cells tubulogenesis, in a VEGF-independent manner (8).

In this study, we demonstrated that SP-2, a monoclonal antibody directed against LGALS3BP, was able to antagonize LGALS3BP-induced endothelial cells tubulogenesis *in vitro* and angiogenesis *in vivo*. Moreover, in Matrigel plugs containing MDA-MB-231 human breast cancer cells, SP-2 was able to inhibit angiogenesis to the same extent of bevacizumab. Noteworthy, the addition of SP-2 to bevacizumab resulted in a greater angiogenesis suppression than either agent alone. This observation indicated that, in MDA-MB-231 model, the

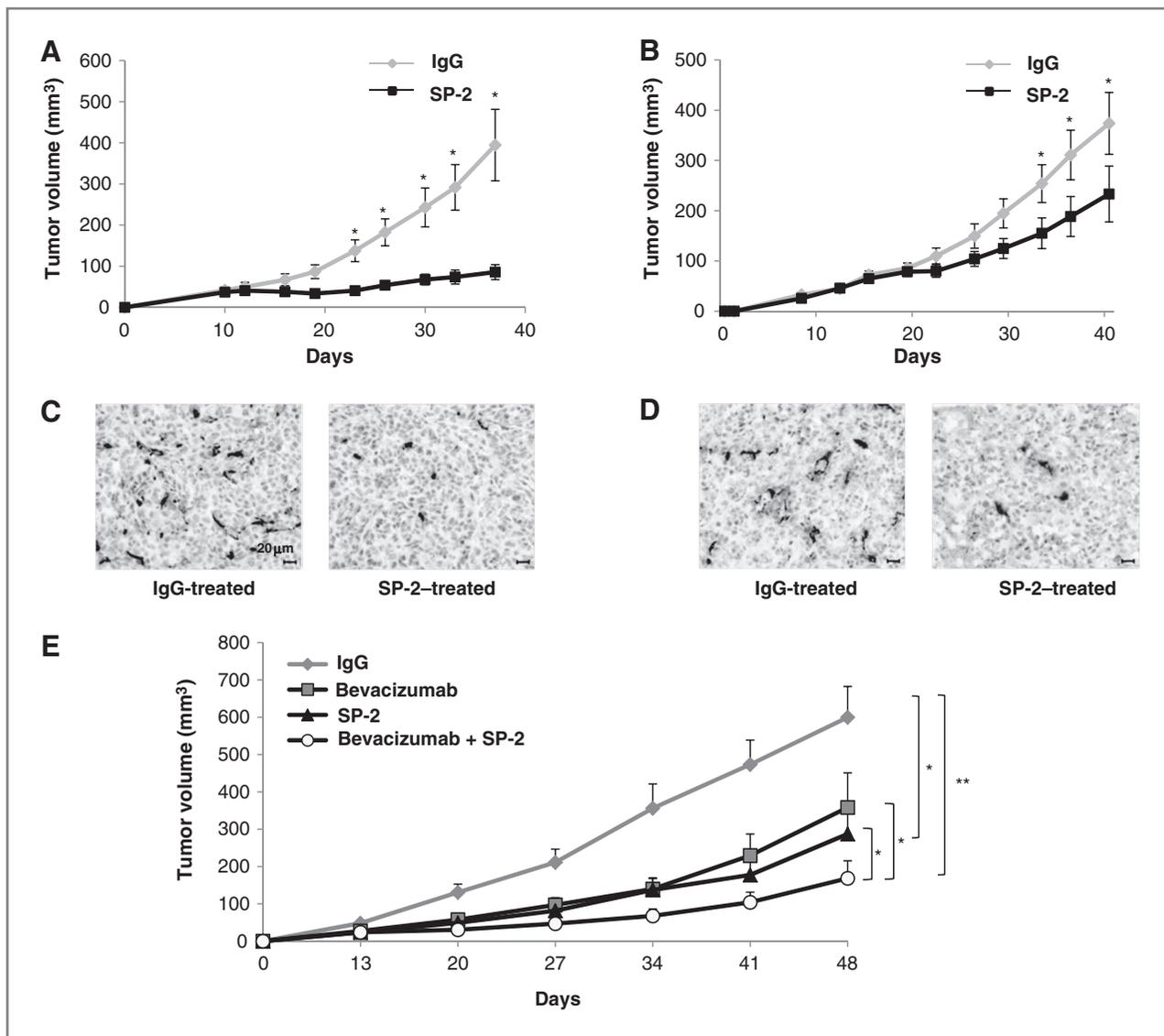


Figure 6. SP-2 restrains tumor growth and its effect is potentiated by bevacizumab. Xenografts were established by subcutaneous implantation of 5×10^6 of MDA-MB-231 cells (A) or IR-8 cells (B) into nude mice. When tumors became palpable (approximately 50–100 mm³), animals were injected intraperitoneally with SP-2 (15 mg/kg) or mouse IgG (15 mg/kg, as a control) twice a week. Tumor volume was monitored by a caliper and calculated according to the formula: tumor volume (mm³) = (length \times width²)/2. Ten mice per group of treatment were used; *, $P < 0.05$ by the Mann-Whitney test. MDA-MB-231 (C) or IR-8 (D) tumor xenografts were excised 6 to 8 weeks after cell implantation and analyzed for CD31/CD105 expression by immunohistochemistry. The representative images show intratumor microvessels at 400 \times magnification (0.17 mm²) from IgG or SP-2 treated mice. E, xenografts were established by subcutaneous implantation of 5×10^6 of MDA-MB-231 cells into nude mice. The third day after cell injection, animals were randomly divided into four groups (10 animals each) and injected twice a week with a mouse IgG (15 mg/kg), SP-2 (15 mg/kg), bevacizumab (2 mg/kg), or a combination of SP-2 and bevacizumab. Tumor volume was monitored by a caliper and calculated according to the formula: tumor volume (mm³) = (length \times width²)/2. Ten mice per group of treatment were used; *, $P < 0.05$; **, $P < 0.001$ by the Mann-Whitney test.

angiogenesis is mainly due to secreted LGALS3BP and VEGF, whose activities are inhibited by SP-2 and bevacizumab, respectively. The molecular mechanism underlying the proangiogenic effects of LGALS3BP is not completely understood. In a previous article (8), we proposed that extracellular LGALS3BP docks galectin-3 molecules resulting in cross-linking and clustering of integrins on the surface of endothelial cells. This leads to activation of FAK-mediated signaling pathways that

modulate the angiogenic cascade. Here, we show that LGALS3BP induces clustering of integrin $\alpha v \beta 3$ and actin recruitment in lamellipodia, processes that are necessary for functional connection between focal adhesion and actin cytoskeleton needed to drive cell migration (16). Furthermore, enhanced phosphorylation of AKT and ERK was observed following exposure of HUVEC to LGALS3BP. All these effects were inhibited by SP-2. Because this antibody recognizes a

conformational epitope of the lectin binding domain of LGALS3BP (10), it could be argued that SP-2 hampers formation of LGALS3BP-galectin-3 complex, preventing integrin clustering at the surface of endothelial cells.

The effect of SP-2 on tumor growth was also assessed in this study. Although the antibody did not affect tumor cell growth *in vitro* (data not shown), it led to a significant growth delay in several xenograft models, including melanoma, breast, and ovarian carcinoma.

The therapeutic activity of SP-2 is particularly relevant in melanoma, which is often resistant to currently available therapies and frequently overexpresses LGALS3BP (21). Thus, potential future therapeutic application of SP-2 might be explored in this malignancy.

Of note, tumors from SP-2-treated animals displayed a reduced number of blood vessels as evidenced by CD31/CD105 staining, indicating that the antibody targets tumor vasculature *in vivo*.

LGALS3BP expression detected by SP-2 was stronger in tumor cells, while it was little or not observed in normal human tissues. On these substrates, the antibody produced a cytoplasmic staining pattern limited to myoepithelial cells of the mammary gland, proximal tubules, and median layer of blood vessels of the kidney, some ducts, and acinar structures of the pancreas, and keratinocytes of the skin with absence of positivity of the cardiac muscle (Natali and colleagues, in preparation), thus suggesting low levels of toxicity if the antibody is administered to humans.

In current cancer therapy, particular attention has been devoted to antiangiogenic drugs, such as anti-VEGF antibody (bevacizumab) and VEGF receptor tyrosine kinase inhibitors (sunitinib, sorafenib, and pazopanib; ref. 22). However, the benefits of these agents appear to be transitory, as drug resistance, tumor regrowth, and extensive

vascular recovery, rapidly develop once the therapy is terminated (23–25). It would be quite interesting to investigate whether LGALS3BP plays an active role in these evasion responses.

Our data indicate that SP-2 halted tumor growth and angiogenesis. More importantly, SP-2 has activity comparable with that of bevacizumab, and when these agents are given in combination, they were more effective in suppressing angiogenesis and tumor growth, thus pointing out new possibilities for therapeutic intervention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Molecular Cancer Therapeutics

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