

Cancer-associated fibroblasts promote angiogenesis in gastric cancer through galectin-1 expression

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Abstract

Background Galectin-1, an evolutionarily conserved glycan-binding protein with angiogenic potential, was recently identified as being overexpressed in cancer-associated fibroblasts (CAFs) of gastric cancer. The role of endogenous CAF-derived galectin-1 on angiogenesis in gastric cancer and the mechanism involved remain unknown.

Methods Immunohistochemical staining was used to investigate the correlation between galectin-1 and vascular endothelial growth factor (VEGF) and CD31 expression in gastric cancer tissues and normal gastric tissues. Galectin-1 was knocked down in CAFs isolated from gastric cancer using small interfering ribonucleic acid (RNA), or overexpressed using recombinant lentiviruses, and the CAFs were co-cultured with human umbilical vein endothelial cells (HUVECs) or cancer cells. Subsequently, proliferation, migration, tube formation, and VEGF/VEGF receptor (VEGFR) 2 expression were detected. The role of CAF-derived galectin-1 in tumor angiogenesis *in vivo* was studied using the chick chorioallantoic membrane (CAM) assay.

Results Galectin-1 was highly expressed in the CAFs and was positively associated with VEGF and CD31 expression. In the co-culture, high expression of galectin-1 in the CAFs

increased HUVEC proliferation, migration, tube formation, and VEGFR2 phosphorylation and enhanced VEGF expression in gastric cancer cells. The CAM assay indicated that high expression of galectin-1 in the CAFs accelerated tumor growth and promoted angiogenesis. In contrast, galectin-1 knockdown in the CAFs significantly inhibited this effect.

Conclusion CAF-derived galectin-1 significantly promotes angiogenesis in gastric cancer and may be a target for angiostatic therapy.

Keywords Angiogenesis · Cancer-associated fibroblasts · Galectin-1 · Gastric cancer

Introduction

Accumulating evidence indicates that solid cancer progression is produced by the evolving crosstalk between cancer cells and activated stromal cells [1–3]. The normal stroma contains few fibroblasts, but there is a sharp increase in fibroblast-like cells within the activated stroma surrounding cancer cells, and it plays an important role in tumor formation [4–6], and contributions in the process of their structural and functional are beginning to emerge. Cancer cells themselves can also activate the adjacent stromal cells to create a favorable and supportive environment for tumor progression [7–10]. Fibroblasts within the tumor stroma, called cancer-associated fibroblasts (CAFs) [1, 10], play a significant role in promoting gastric cancer growth and progression [7, 11, 12]. Contrary to resting fibroblasts, CAFs have an activated phenotype known as myofibroblasts, which can be identified by their expression of both vimentin and α -smooth muscle actin (α -SMA) [13], and have been recognized as functional fibroblasts in gastric cancer development [2, 3, 12, 14]. CAFs communicate among themselves, but also with cancer cells and inflammatory and

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immune cells directly through cell contact and indirectly through paracrine/exocrine signaling, proteases, and modulation of extracellular matrix (ECM) [15]. This complex communications network is essential for establishing the proper microenvironment to support tumorigenesis, angiogenesis, growth, invasion, and metastasis of gastric cancer [16, 17]. Such CAFs may contain abundant prognostic information and are a source of many potential new tumor biomarkers, which are useful for diagnosing and evaluating the prognosis of gastric cancer [3, 18]. Therefore, CAFs are a key decisive factor in the malignant progression of gastric cancer and may be identified as an potential predictor and therapeutic target for patients with gastric cancer [1].

Angiogenesis is crucial for the survival and growth of solid tumors because the establishment of new microvessels may provide nutrition for cancer cells proliferation [1, 19, 20]. Fibroblasts are associated with all stages of cancer cell progression, and their production of growth factors, chemokines, and ECM facilitates the angiogenic recruitment of endothelial cells (ECs) [1]. In breast cancer, CAFs may promote angiogenesis through elevated stromal cell-derived factor-1 (SDF-1/CXCL12) secretion [21]. In intestinal tumors, the expression of cyclooxygenase-2 in CAFs results in elevated prostaglandin E2 levels that stimulate cell surface receptor EP2, followed by induction of vascular endothelial growth factor A (VEGFA), epidermal growth factor-like growth factor, amphiregulin, and hepatocyte growth factor, all of which lead to the promotion of angiogenesis [22–24]. Recently, Guo et al. [7] indicated that stromal fibroblasts activated by tumor cells induced VEGFA expression and consequently promoted angiogenesis in mouse gastric cancer. Although it has been shown that the promotion of angiogenesis is an important function of CAFs during tumorigenesis, the underlying mechanisms are remain poorly understood.

Galectin-1, a member of the galectin family of β -galactoside-binding proteins, is a homodimer of 14-kDa subunits possessing two β -galactoside binding sites [25]. Galectin-1 has a variety of biological functions, including supporting cancer cell invasion and metastasis formation [26], promoting tumor angiogenesis [27], and protecting tumors from host immune responses [28]. Recently, it was shown that galectin-1 is strongly expressed in the CAFs of gastric cancer and is related with tumor invasiveness and progression, and short patient survival [29]. Despite the fact that galectin-1 is expressed in CAFs of gastric cancer, and both galectin-1 and CAFs play a role in angiogenesis, the potential association of endogenous galectin-1 and CAFs in angiogenesis in gastric cancer has not been yet characterized.

In this study, we compared the relationship between galectin-1, VEGF, and CD31 expression in gastric cancer tissue and normal gastric tissue, and investigated the effect on angiogenesis following altered galectin-1 expression levels in normal fibroblasts and CAFs isolated from primary gastric

cancer with the aim of understanding the mechanisms of CAF promotion of angiogenesis in the gastric cancer microenvironment.

Materials and methods

Human samples

To analyze galectin-1, VEGF, and CD31 expression, specimens from a total of 47 patients with gastric cancer (26 men and 21 women) who had undergone radical gastrectomy at the Clinical Medical College, Yangzhou University (Subei People's Hospital of Jiangsu Province, Yangzhou, China) were studied. The patients' median age is 53 years (range, 35–78 years). Normal gastric tissue (15 cases) served as the control group. All specimens were evaluated histologically according to the World Health Organization criteria. Tumor stages were assessed according to the tumor-nodes-metastasis stage of the International Union Against Cancer classification.

Ethics statements

All patients provided written informed consent for their participation in the study, which was approved by the Ethical Committee of Subei People's Hospital of Jiangsu Province. All copies of the Letter of Information and Consent of the participants have been stored in our laboratory, and each patient received one copy of the letter. The Ethical Committee of Subei People's Hospital of Jiangsu Province approved this consent procedure, which was recorded in the Consent Form of the Ethical Committee.

Cells and culture conditions

Two gastric fibroblast types were used: CAFs of primary gastric cancer established from a 54-year-old man with gastric carcinoma who underwent distal gastrectomy, and primary human normal gastric fibroblast cells (NGF) isolated from the gastric wall of a 65-year-old man with duodenal cancer. The primary culture was performed as previously described [30]: primary tissues were excised under aseptic conditions and minced with forceps and scissors. The tumor tissue was cultivated in Dulbecco's modified Eagle's medium (DMEM; Nikken, Kyoto, Japan) with 10 % heat-inactivated fetal calf serum (Life Technologies, Inc., Grand Island, NY, USA), 100 IU/mL penicillin (ICN Biomedicals, Costa Mesa, CA, USA), 100 mg/L streptomycin (ICN Biomedicals), and 0.5 mM sodium pyruvate (Cambrex, Walkersville, MD, USA), and incubated in humidified incubators at 37 °C in 5 % CO₂ in air. The fibroblast types were confirmed by cell

morphology and immunohistochemical staining for α -SMA [31]. Cells from passage 3 to 10 were used for all assays.

In addition, the human gastric cancer cell lines BGC823 and SGC7901, and human umbilical vein endothelial cells (HUVECs; HUV-EC-C; ATCC CRL-1730; ATCC Manassas, VA, USA) were also maintained in DMEM (Nikken) supplemented with 10 % heat-inactivated fetal bovine serum (Life Technologies) and penicillin/streptomycin.

Immunohistochemical staining and evaluation

Tissue samples were fixed by immersion in 4 % paraformaldehyde overnight at 4 °C, embedded in regular paraffin wax, and cut into 4- μ m sections. Immunohistochemical staining for galectin-1, VEGF, and CD31 was performed as previously described [17]. Briefly, tissue sections were deparaffinized and rehydrated in phosphate-buffered saline. Following antigen retrieval with target retrieval solution, endogenous peroxidase activity was blocked by incubation with 0.3 % hydrogen peroxide, and then sections were blocked in 10 % normal goat serum. Sections were then incubated with mouse monoclonal anti-galectin-1 antibody (sc-166618; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-VEGF antibody (sc-7269; Santa Cruz Biotechnology), or anti-CD31 antibody (sc-376764; Santa Cruz Biotechnology) and anti- α -SMA antibodies (MA1-37027; Thermo Fisher Scientific, Fremont, CA, USA) overnight at 4 °C, followed by incubation with biotinylated rabbit anti-mouse immunoglobulin G (IgG; Vector Laboratories Inc., Burlingame, CA, USA), and then peroxidase-conjugated streptavidin (Boster, Wuhan, China). Slides were incubated for several minutes with diaminobenzidine for color development, and hematoxylin was used to counterstain the nuclei. For the negative controls, primary antibody was replaced with nonspecific rabbit IgG. Two experienced pathologists interpreted the immunohistochemical staining results, and the mean density of staining was calculated using Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA).

Preparation and transduction of recombinant lentiviruses

The plasmids used for preparing the recombinant lentiviruses have been described previously [25]. The galectin-1 gene fragment was excised from a human cDNA library and cloned into pHAGE-CMV-MCS-IZsGreen between the *Bam*HI and *Xho*I restriction sites [25]. Galectin-1-specific oligonucleotides [32, 33] (galectin-1 siRNA #1, 5'-CCGGGCTGCCAGATGGATACGAATCTCGAGATTCGTATCCATCTGGCAGCTTTTTTGG-3'; galectin-1 siRNA #2, 5'-CCGGCAGCAACCTGAATCTCAAACCTCGAGTTTGAGATTCAGGTGCTGTTTTTGG-3') or scrambled oligonucleotide (5'-CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG-3') were ligated into the

pLKO.1-puro vector. Recombinant lentiviruses were constructed in accordance with the manufacturer's instructions and used for the transduction experiments. For viral infection, CAFs were incubated for 6 h with virus supernatant diluted in culture medium supplemented with 8 μ g/mL polybrene. After infection, galectin-1-overexpressing CAFs were selected through green fluorescent protein expression using a FACSCalibur flow cytometer; CAFs expressing the galectin-1 short hairpin RNA knockdown plasmid were selected by culturing in media containing 2 μ g/mL puromycin. Galectin-1 expression was confirmed in whole-cell extracts using western blotting and quantitative reverse transcription polymerase chain reaction. The studied groups were as follows: NGF, CAF, galectin-1-downregulated CAFs (down), and galectin-1 overexpression CAFs (over).

5-Ethynyl-20-deoxyuridine cell proliferation assay

HUVECs were purchased from ATCC and co-cultured with NGFs, CAFs, and down and over CAFs in Transwell chambers. Proliferation was assessed by immunostaining for 5-ethynyl-20-deoxyuridine (EdU; Ruibo Biotech, Guangzhou, China) or Ki-67 (Lab Vision, Fremont, CA, USA) according to the manufacturer's instructions. Briefly, 70–80 % confluent cells were treated with 0.25 % trypsin and 0.02 % ethylenediaminetetraacetic acid solution, centrifuged, and resuspended in DMEM supplemented with 10 % fetal bovine serum and antibiotics. The cells were then seeded in 96-well plates (100 μ L/well) at an initial density of 2.5×10^4 cells/mL. After 24-h incubation, 100 μ L EdU (50 μ mol/L) was added to each well and incubated for another 4 h. The supernatant was then removed and cells were fixed using a standard formaldehyde fixation protocol. After washing several times with phosphate-buffered saline containing 0.5 % Triton X-100, the cells were stained with 100 μ L Apollo[®] for 30 min. The cells were washed, mounted in standard mounting media, and imaged using fluorescence microscopy. DNA was stained with 10 μ g/mL Hoechst 33342 (100 μ L/well) for 20 min and visualized using fluorescence microscopy. Five groups of confluent cells were randomly selected from each sample image. EdU-positive cells were obtained from the image, and the relative positive ratio was calculated from the average of the five group values [34].

In vitro migration assay

HUVEC migration through Matrigel was determined using 6-well Corning Transwell chambers (8.0- μ m pore size with a polycarbonate membrane) in accordance with the manufacturer's instructions. First, the lower compartment was implanted with 2×10^5 fibroblasts for 12 h. After the fibroblasts had adhered, the medium was changed to serum-free medium. During this time, the upper chambers were coated with diluted

Matrigel (1 mg/mL; 356243; BD Biosciences, Bedford, MA, USA) and incubated at 37 °C in 5 % CO₂ for 3 h. After trypsinization, cells were suspended in serum-free medium at 2×10^5 cells/well and immediately placed onto the upper compartment. After 24-h incubation, non-migrated cells were removed from the upper surface of the membrane by wiping with cotton-tipped swabs. Cells on the lower surface of the membrane were stained with 0.1 % crystal violet for 10 min and photographed. The crystal violet was then bleached using 500 μ L 33 % acetic acid. Absorbance at 570 nm was determined using a microtiter plate reader. To avoid galectin-1 binding to serum proteins, serum-free medium was used in all lectin stimulation experiments, as indicated.

HUVEC tube formation assay

The HUVEC tube formation assay was carried out as described previously [35]. The formation of capillary-like structures was assessed in a 24-well plate using growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA). For this procedure, HUVECs (2×10^4 /well) were plated on top of Matrigel (280 μ L/well) and treated with supernatant derived from NGFs, CAFs, down or over CAFs, or cells cultured under hypoxic or normoxic conditions. After 24 h, cells were visualized under high magnification ($\times 200$). The total tube area was quantified as the mean pixel density obtained from image analysis of five random microscopic fields.

Western blotting analysis

Western blotting was performed as previously described [36]. Briefly, each group of cells were lysed in sodium dodecyl sulfate (SDS) buffer, and 100 μ g total cellular protein was separated on 10 % or 10–20 % gradient SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and incubated with mouse anti-galectin-1 antibody (sc-166618; Santa Cruz Biotechnology), anti-VEGF antibody (sc-7269; Santa Cruz Biotechnology), or anti-VEGFR2 antibody (sc-504; Santa Cruz Biotechnology) overnight at 4 °C. After incubation with peroxidase-conjugated rabbit anti-mouse IgG antibody (Cell Signaling Technologies, Beverly, MA, USA), proteins were visualized using an ECL reagent system (GE Healthcare, Chalfont St. Giles, UK). α -Tubulin was used as the loading control. VEGFR2 activation was determined by immunoblotting cell extracts with anti-phosphorylated VEGFR2 antibodies (pTyr1214; Calbiochem, San Diego, CA, USA).

Chick embryo chorioallantoic membrane assay

Angiogenic activity was assayed using a chick embryo chorioallantoic membrane (CAM) assay as described previously [37], using a 4-day-old chick embryo in the shell

(Comparative Medicine Centre, Yangzhou University). The experimental sample, SGC7901 gastric cancer cells, was mixed with NGFs, CAFs, and over or down CAFs. A 1 cm \times 1 cm window was made in the shell to create a pocket exposing the CAM. A filter disc impregnated with 0.9 % NaCl solution or 30 μ g/10 μ L sample compounds was then placed on the CAM, and 72 h later at 37 °C, the CAMs were fixed with 4 % paraformaldehyde and the number of vessels in each group was counted under high magnification ($\times 100$). Each experimental group included six eggs, and experiments were repeated three times. For tumor growth, CAMs were fixed 7 days after placement of the compounds. Tumor volumes were calculated using a standard formula: width² \times length/2 [38].

Statistical analysis

Values are expressed as the mean \pm standard deviation of at least three independent determinations. One-way analysis of variance and *t* tests were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA) to compare differences between groups. The Pearson correlation test was used to determine the relationship between individual markers. All *p* values were two-sided, and *p* values ≤ 0.05 were considered statistically significant.

Results

CAF-derived galectin-1 expression was positively related with CD31 and VEGF expression in gastric cancer

To evaluate the correlation between CAF-derived galectin-1 expression and tumor angiogenesis in gastric cancer, immunohistochemical staining for galectin-1, VEGF, and CD31 was performed. Galectin-1, VEGF, and CD31 staining intensity, indicating protein expression, significantly increased gradually from the normal gastric tissue (15 cases), high-grade intraepithelial neoplastic gastric tissue (18 cases) to the gastric cancer tissues (47 cases; Fig. 1). Galectin-1 staining was near negative in the normal gastric tissues, and there was weak staining in the mesenchyme of high-grade intraepithelial neoplastic gastric tissues (Fig. 1a1, b1, f). However, a significant correlation was found between galectin-1 staining and the degree of differentiation of gastric cancer, where galectin-1 staining in poorly differentiated tissues was markedly stronger than that in well- or moderately differentiated tissues, and galectin-1 staining in moderately differentiated tissues was much higher than that in well-differentiated tissues (Fig. 1c1–e1, f). The finding also applied to the VEGF and CD31 staining intensity (Fig. 1c2–e2, c3–e3, f), which was primarily used to demonstrate the presence of EC tissue and to assess tumor angiogenesis. Immunohistochemistry

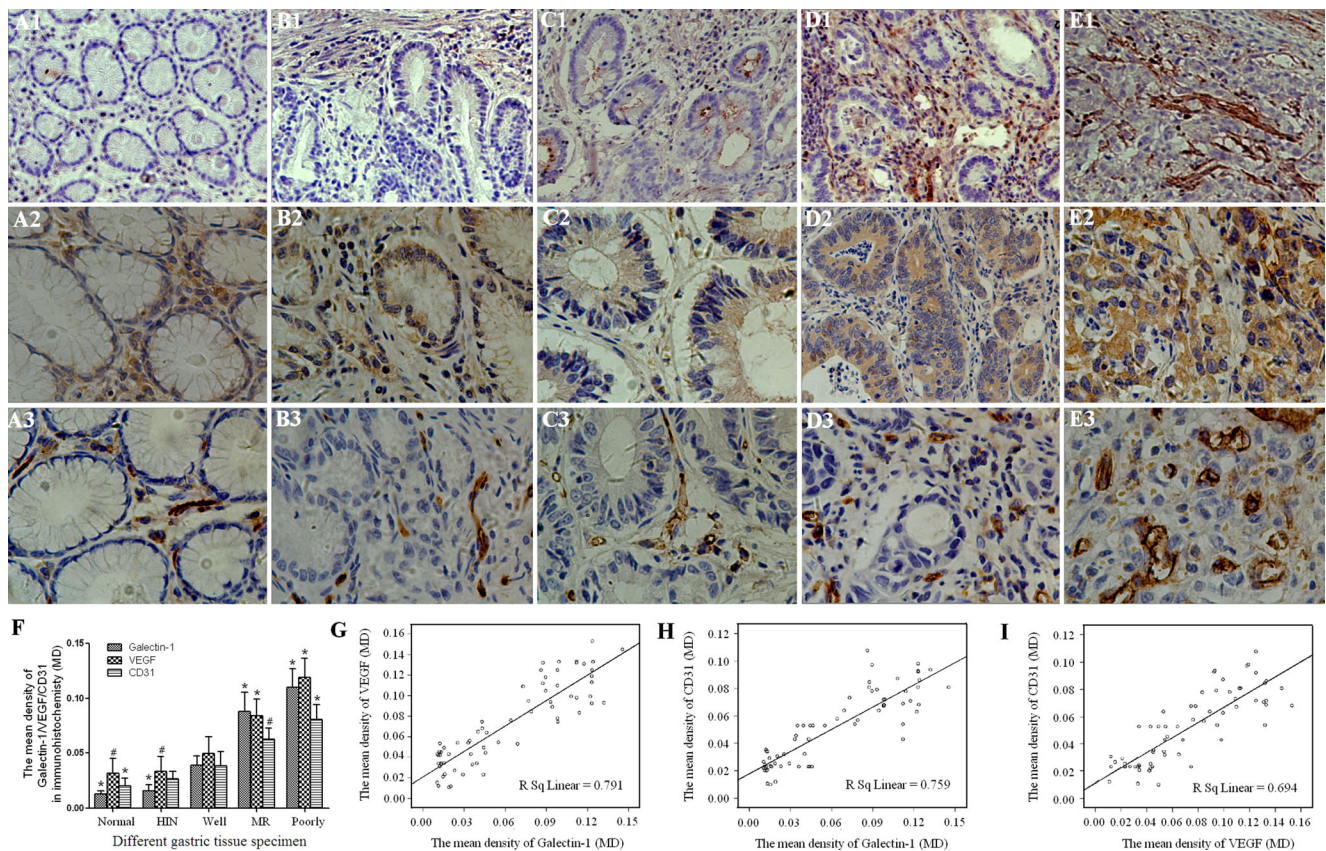


Fig. 1 Immunohistochemistry staining for galectin-1 (1), VEGF (2), and CD31 (3) expression in gastric tissues. **a** Normal gastric tissue. **b** High-grade intraepithelial neoplastic gastric tissue. **c–e** Well-differentiated, moderately differentiated, and poorly differentiated gastric cancer tissue, respectively. **f** Quantification of mean density of galectin-1, VEGF, and CD31 expression in gastric tissues. * $p < 0.01$ vs. well, # $p < 0.05$ vs. well. *HIN* high-grade intraepithelial neoplastic gastric tissue, *Well* well-

differentiated gastric cancer, *MR* moderately differentiated gastric cancer, *Poorly* poorly differentiated gastric cancer. Original magnification: $\times 200$. **g** Correlation analysis of the mean density of galectin-1 and VEGF. **h** Correlation analysis of the mean density of galectin-1 and CD31. **i** Correlation analysis of the mean density of CD31 and VEGF

determined that VEGF was mainly expressed in the tumor cell cytoplasm, while CD31 was expressed in the stromal area around the tumor cells. Pearson correlation analyses of the individual markers indicated strong positive correlations between the expression of galectin-1 and VEGF ($r^2 = 0.791$, $p < 0.001$), galectin-1 and CD31 ($r^2 = 0.759$, $p < 0.001$), and VEGF and CD31 ($r^2 = 0.694$, $p < 0.001$; Fig. 1g–i). These results suggest that CAF-derived galectin-1 promotes tumor angiogenesis by enhancing VEGF expression in gastric cancer.

Galectin-1 was highly expressed in α -SMA-positive CAFs

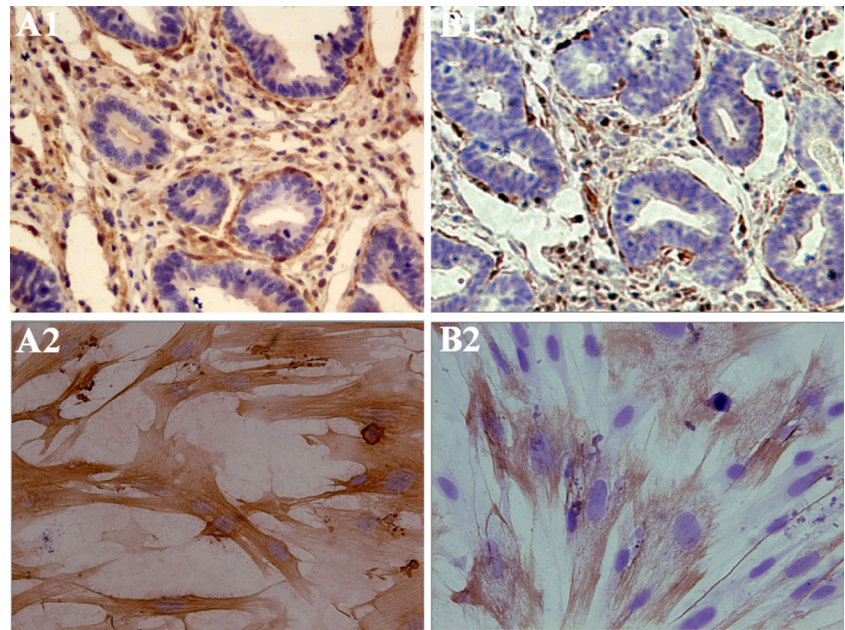
To identify the location of galectin-1 in gastric cancer tissues, we performed immunohistochemical staining on serial sections of gastric cancer tissues and freshly isolated CAFs for galectin-1 and α -SMA, a CAF marker [2, 4]. The cancer cells in the histological sections were negative for both α -SMA and galectin-1 expression; however, the majority of mesenchymal cells surrounding the cancer cells expressed α -SMA (Fig. 2a1), and galectin-1 was strongly expressed in the α -

SMA-positive regions (Fig. 2b1). To confirm that galectin-1 was expressed in the CAFs, we isolated and cultured fibroblasts from the same gastric cancer tissue. After passage 2, immunohistochemical staining indicated strong expression of both α -SMA and galectin-1 in the same original fibroblasts, which was consistent with the results of histological staining (Fig. 2a2, b2). These findings indicate that galectin-1 is highly expressed in such CAFs.

CAF-derived galectin-1 promoted HUVEC proliferation and migration

We demonstrated that galectin-1 is upregulated gastric CAFs. EdU cell proliferation assay and Transwell migration assay were performed to evaluate the effect of CAF-derived galectin-1 on the proliferation and migration ability of HUVECs, respectively. HUVECs were co-cultured with NGFs, CAFs, and down and over CAFs for 24 h. There was much higher proliferation and migration in HUVECs co-cultured with CAFs and over CAFs compared to HUVECs

Fig. 2 Expression of galectin-1 and α -SMA in gastric cancer tissues and freshly isolated CAFs. **a** α -SMA. **b** Galectin-1. **1** Gastric cancer tissues, **2** CAFs. Original magnification: $\times 200$



co-cultured with NGFs, and co-culture with over CAFs induced significantly higher HUVEC proliferation and migration than co-culture with CAFs (Fig. 3). The opposite was true when HUVECs were co-cultured with down CAFs, which confirmed that the effect was specifically induced by galectin-1. These studies strongly suggest a role for endogenous galectin-1 expressed by CAFs in EC biology.

CAF-derived galectin-1 was required for HUVEC tube formation

The role of CAF-derived galectin-1 in angiogenesis activity *in vitro* was evaluated by tube formation following co-culture of HUVECs and gastric fibroblasts. Compared to NGFs, CAFs induced significant tube formation in the HUVECs (Fig. 3a3, b3, g). Moreover, after 24-h co-culture of HUVECs with over or down CAFs, there was more tube formation in the former, while there was tortuous and irregular growth of tubes in the latter, suggesting insufficient vascular formation guidance (Fig. 3c3, d3, g). In addition, the effect of CAF-derived galectin-1 was abrogated in the presence of 10 mM lactose, a competitive inhibitor of galectin-1. These studies suggest that angiogenesis in gastric cancer might be attributed to CAF-expressed endogenous galectin-1.

CAF-derived galectin-1 induced VEGFR2 phosphorylation and stimulated VEGF-independent tumor angiogenesis

As CAF-derived galectin-1 has stronger HUVEC tube formation activity, we wanted further insight into the role of galectin-1 during angiogenesis *in vitro*. In co-culture of

SGC7901 and BGC823 gastric cancer cells with gastric fibroblasts, CAFs increased VEGF expression in the gastric cancer cells more than the NGFs did (Fig. 4). Furthermore, over CAFs stimulated more prominent VEGF expression in the gastric cancer cells than CAFs did, while VEGF expression declined following co-culture with down CAFs (Fig. 4), suggesting the role of CAF-derived galectin-1 in VEGF expression.

It is well known that VEGF binding to VEGFR mediates angiogenesis, cell migration, and tumorigenicity [39]. High galectin-1 concentrations can induce VEGFR2 phosphorylation, but not VEGFR1 or VEGFR3 phosphorylation [40]. Therefore, we co-cultured different gastric fibroblasts and HUVECs, and found that co-culture with over CAFs significantly induced VEGFR2 phosphorylation in the HUVECs. However, co-culture with down CAFs reduced VEGFR2 phosphorylation in the HUVECs (Fig. 4), suggesting the role of CAF-derived galectin-1 in VEGFR2 phosphorylation.

Taken together, these data show that CAF-derived galectin-1 increases VEGF expression in tumor cells and enhances VEGFR2 phosphorylation in ECs, which potentiate VEGF activities in ECs.

CAF-derived galectin-1 accelerated angiogenesis and was a target for angiostatic therapy

The role of CAF-derived galectin-1 in tumor angiogenesis *in vivo* was studied using the CAM assay. To compare tumor angiogenesis and growth in the presence or absence of galectin-1, NGFs, CAFs, and down and over CAFs mixed with SGC7901 cells were injected into CAMs. Three days after injection, palpable tumors developed in all CAMs, and

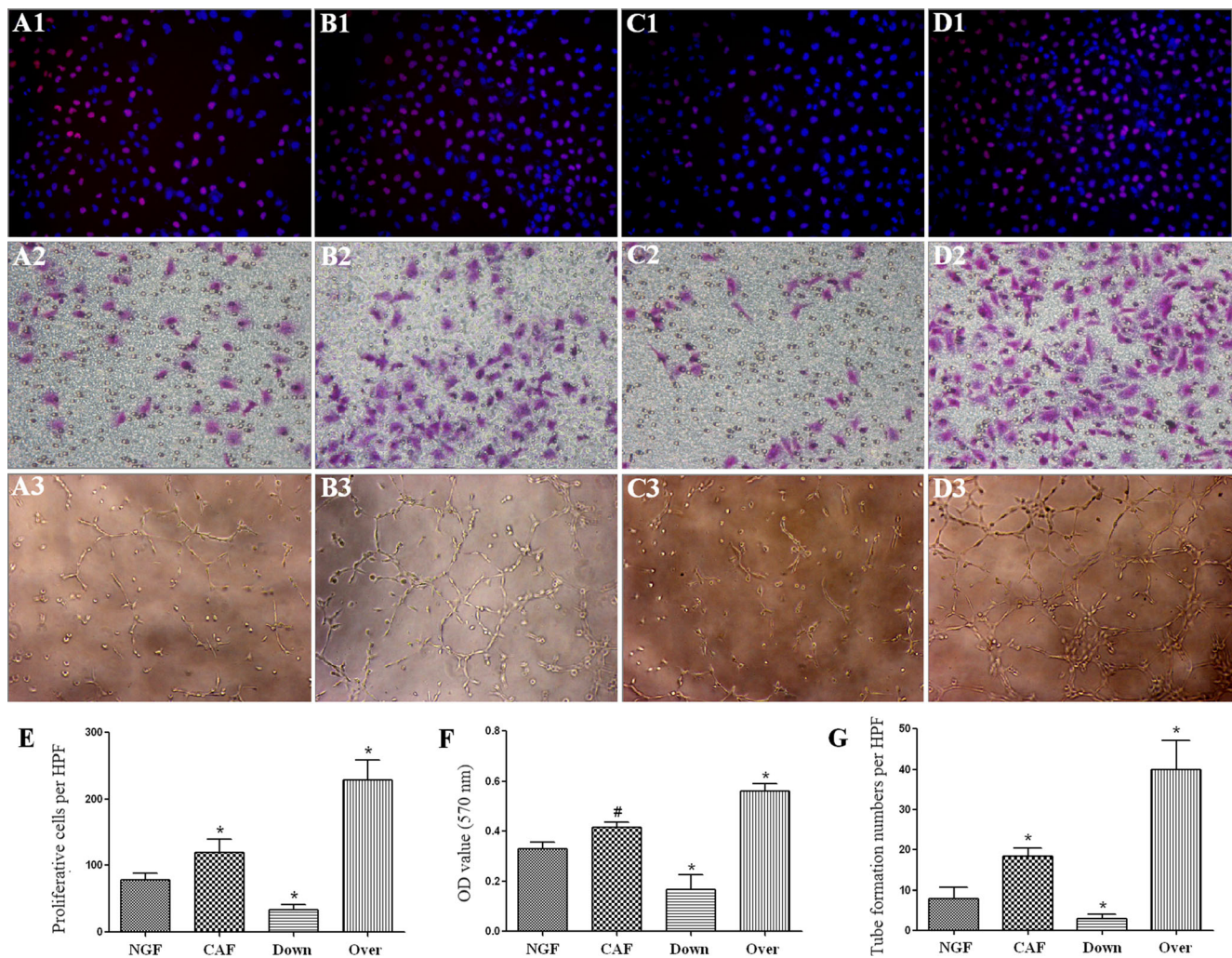


Fig. 3 Galectin-1 expression in gastric myofibroblasts co-cultured with HUVECs. **1** EdU cell proliferation assay. **2** Transwell migration assay. **3** HUVEC tube formation assay. **a–d** HUVECs co-cultured with NGFs, CAFs, and down and over CAFs. Original magnification, $\times 200$. **e** Quantification of proliferative cells in each group ($\times 200$ magnification). **f** Optical density (OD) value of cells in each group at 570 nm. **g**

Quantification of tube formation numbers in each group ($\times 200$ magnification). * $p < 0.01$ vs. NGF, # $p < 0.05$ vs. NGF. NGF normal gastric fibroblast, CAF cancer-associated fibroblast, Down galectin-1-downregulated CAFs, Over galectin-1 overexpression CAFs, HPF high-power field

were slightly larger to the naked eye in the CAF and over groups, suggesting that CAF-derived galectin-1 might initiate tumor cell proliferation. Subsequently, tumor growth and angiogenic branching was significantly increased in over group compared to the NGF and CAF groups. However, tumor growth and tumor angiogenesis were significantly abrogated in down group. One week after injection, the tumor volume and weight in the over group was much more greater than that of the NGF and CAF groups, but the tumor volume and weight of the down group were significantly lower compared with the NGF and CAF groups (Fig. 5f, g). Quantification of angiogenic branching revealed a significantly high number of blood vessels in the over group compared with the NGF and CAF groups, and much fewer blood vessels in the down group compared with the NGF and CAF groups (Fig. 5a1–d1, a2–d2, e). As expected, immunohistochemical analysis revealed

high expression of galectin-1 and CD31 in the over group and low expression in the down group (Fig. 5c3–c4, d3–d4). The results strongly suggest that the accelerated tumor progression in the CAMs largely results from increased angiogenesis by CAF-derived galectin-1, and that galectin-1 may be a target for angiostatic therapy.

Discussion

Gastric cancer growth and the formation of metastasis depend on the induction of new blood vessels, while tumor angiogenesis is uncontrolled and immature. Tumor angiogenesis is a complex process that depends on many angiogenic factors, one of the most important of which is VEGF [41]. The present study demonstrates that CAF-derived galectin-1 is an

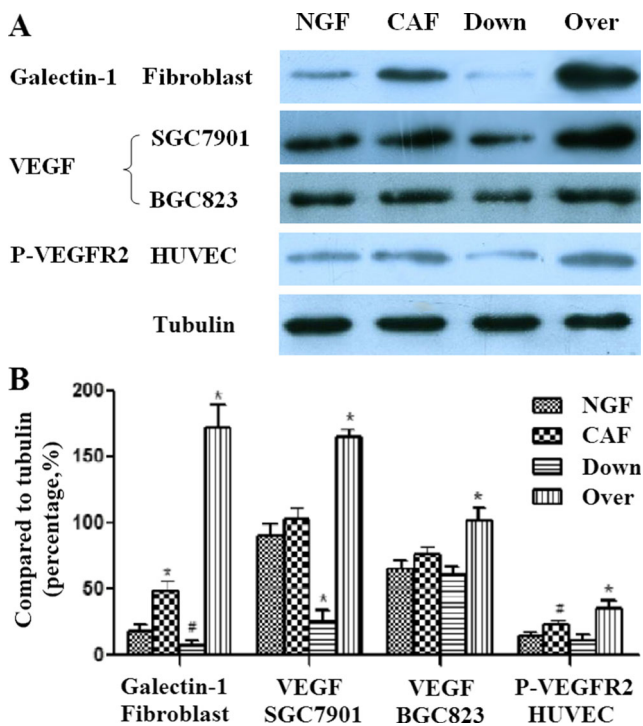


Fig. 4 Western blot analysis of the levels of proteins of interest. **a** Shown are galectin-1, VEGF, and phosphorylated (P)-VEGFR2 levels in fibroblasts, SGC7901 and BGC823 gastric cancer cells, and HUVECs. **b** Quantification of every protein compared to tubulin. * $p < 0.01$ vs. NGF, # $p < 0.05$ vs. NGF

important agent in tumor angiogenesis, where it increased VEGF expression in gastric cancer cells and enhanced VEGFR2 phosphorylation in ECs, and that targeting galectin-1 in gastric cancer microenvironment is an attractive anti-angiogenic therapeutic strategy.

Progress in gastric cancer has been identified as the product of evolving crosstalk between cancer cells and the surrounding tumor stroma [7]. The tumor stroma comprise ECM and a collection of stromal cells that includes fibroblasts, myofibroblasts, smooth muscle cells, and inflammatory cells. α -SMA-positive stromal fibroblasts, known as myofibroblasts or activated fibroblasts, are essential in the development of gastric cancer and in creating an environment that is permissive for tumor growth, angiogenesis, and invasion [4]. CAFs are an important stromal cell type with distinct properties [2, 42], influencing the tumor microenvironment by releasing ECM proteins, proteases, cytokines, and chemokines [43]. The promotion of angiogenesis is an important function of CAFs during tumorigenesis, and angiogenesis is a key mechanism that supports tumor development by providing nutrients and oxygen [44]. Via a multipronged approach, CAFs control tumor angiogenesis direct and indirect [45]. First, CAFs can make existing ECs to proliferate and migrate, or recruit endothelial progenitor cells to form blood vessels *de novo*, a process called vasculogenesis. Consequently, CAFs are often found at the leading edge of a tumor, where

tumor–host interactions take place [45, 46]. Second, CAFs have a repertoire of secreted proangiogenic growth factors, including VEGF, basic fibroblast growth factor, transforming growth factor-beta, platelet-derived growth factors, hepatocyte growth factor, connective tissue growth factor, and interleukin-8, and combined with other proangiogenic growth factors in the tumor, myofibroblast-derived proangiogenic factors can coordinate the angiogenic balance in favor of tumor angiogenesis [1, 45, 47, 48]. In addition, CAF secretion of SDF1/CXCL12 stimulates the recruitment of Sca1+/CD31+ endothelial progenitor cells and the CXCR4 receptor to enhance angiogenesis in the primary tumor [21, 49]. Moreover, prostaglandin E2 stimulates intestinal myofibroblast expression of VEGFA, epidermal growth factor-like growth factor, and amphiregulin, leading to the promotion of angiogenesis as well as epithelial proliferation [23]. Our immunohistochemistry results indicated that α -SMA-positive CAFs had strong expression of galectin-1, which was positively associated with VEGF and CD31 expression, and suggested that CAF-derived galectin-1 promotes tumor angiogenesis in gastric cancer by enhancing VEGF expression. Although it has been shown that CAFs play important roles in angiogenesis in gastric cancer, the mechanisms underlying CAFs and the promotion of angiogenesis are still not fully understood [7].

Galectin-1 plays a role in a variety of biological functions, including cell–cell and cell–matrix interactions, and cell growth [50]. Recently, several studies showed galectin-1 has potent proangiogenic properties [27, 51, 52] and plays an important role in tumor angiogenesis within a complex multi-step process. It was observed that tumors can promote angiogenesis via endogenous galectin-1 [52, 53], which transmits the proangiogenic signal by augmenting VEGFRs and H-RAS [39, 52]. Rather than directly binding to the ligand binding site, galectin-1 can also bind to neuropilin-1 and activate VEGFR2 signaling, a major transducer of VEGF signals in ECs, and modulate vascular EC migration, promoting angiogenesis [40]. Furthermore, galectin-1 recovered gestation in a model of reduced vascular expansion by regulating angiogenesis and blood vessel function [54]. In addition, galectin-1 can stimulate ECs directly [40, 52] and directly enhance the expression of classic proangiogenic factors (e.g., ANG, CXCL16, heparin-binding epidermal growth factor) and proteins involved in matrix remodeling (e.g., matrix metalloproteinase-9, tissue inhibitor of matrix metalloproteinases 1), and can also strengthen VEGF bioavailability *in vivo* by reducing soluble VEGFR1, which indirectly promotes angiogenesis [54, 55]. Our preliminary results indicate that CAF-derived galectin-1 may contribute to steps further downstream of the angiogenesis cascade, as endogenous galectin-1 promotes EC proliferation, migration, and tube formation. Next, we found that CAF-derived galectin-1 accelerated tumor angiogenesis by increasing VEGF expression in gastric cancer cells and enhanced VEGFR2 phosphorylation in ECs. The opposite

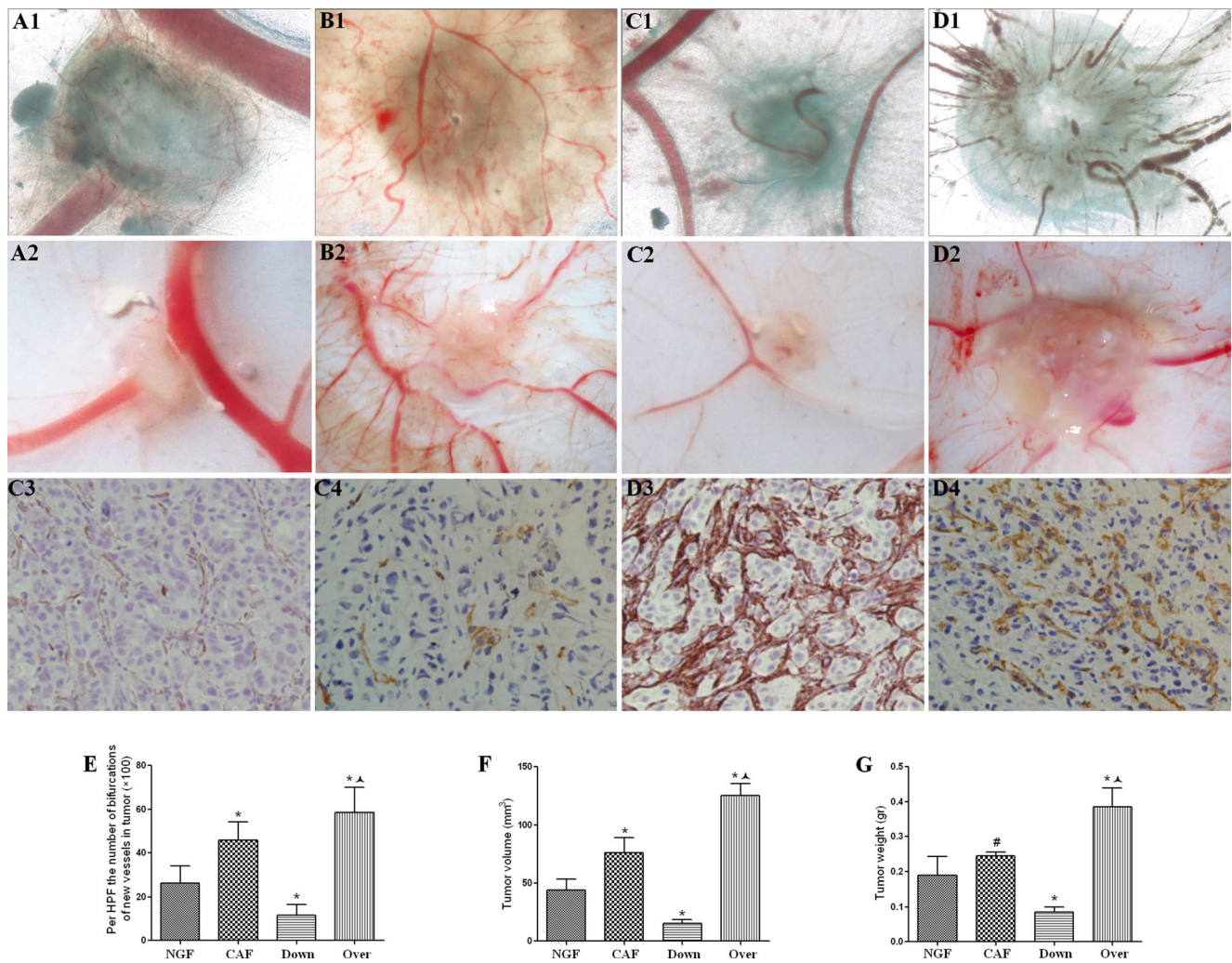


Fig. 5 Effects of CAF-derived galectin-1 on establishment and angiogenesis of tumors in CAMs. SGC7901 gastric cancer cells were co-cultured with NGFs (a), CAFs (b), and down CAFs (c) or over CAFs (d). 1 Micrographs of angiogenic branching points of tumors in each group. Original magnification: $\times 200$. 2 Tumor establishment in each group. 3 Immunohistochemical staining for galectin-1. Original

magnification, $\times 100$. 4 Immunohistochemical staining for CD31. Original magnification, $\times 100$. e Quantification of angiogenic branching in each group. f Quantification of tumor volume in each group. g Quantification of tumor weight in each group. * $p < 0.01$ vs. NGF, # $p < 0.05$ vs. NGF, $\Delta p < 0.01$ vs. CAF

was observed when galectin-1 was knocked down in the CAFs, which were then co-cultured with HUVECs or gastric cancer cells. Following inhibition of endogenous galectin-1 expression, CAFs decreased HUVEC proliferation, migration, and tube formation activity compared with HUVECs co-cultured with over CAFs, and resulted in vascular defects and tumor growth in CAM as well. Taken together, our study indicates that CAF-derived galectin-1 acts as a proangiogenic agent and interacts with the extracellular environment and gastric cancer cells.

Tumorigenesis is a complex multi-step process involving not only genetic and epigenetic changes in the tumor cell but also selective supportive conditions of the deregulated tumor microenvironment [19]. The tumor microenvironment is also an extremely complex milieu, primarily characterized by

immunosuppression, abnormal angiogenesis, and hypoxic regions [56]. Recently, galectin-1 overexpression was widely documented in several tumor types and/or in the stroma of the tumor microenvironment. The expression of galectin-1 is regulated by hypoxia-inducible factor-1, which plays a vital role in protumorigenic within the tumor microenvironment. There is evidence that galectin-1 has multiple functions at the different steps of tumor progression. In particular, galectin-1 promotes tumor escape from immunity by suppressing T cell-mediated cytotoxic immune responses [25], facilitates tumor cell growth, invasion, and metastasis by interacting with cytokine and growth factors, and promotes tumor angiogenesis [50, 57]. These results suggest that galectin-1 inhibition may lead to direct antiproliferative effects in cancer cells as well as antiangiogenic effects in tumors. One

of the major challenges of targeting galectin-1 is translating current knowledge into the design and development of effective galectin-1 inhibitors in cancer therapy [50].

In conclusion, the present results demonstrate the promoting effect of CAF-derived galectin-1 on gastric tumorigenesis and angiogenesis both in vitro and in vivo. The results suggest that novel pharmacological approaches targeting galectin-1 may be promising drug candidates for the development of gastric cancer chemoprevention therapies, although they are rare.

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Conflicts of interest None

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