Galectin-1 From Cancer-Associated Fibroblasts Promotes the Invasion and Metastasis of Gastric Cancer Through TGF-β1-Induced Epithelial-Mesenchymal Transition

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Research

Keywords: Galectin-1, Gastric cancer microenvironment, Cancer-associated fibroblasts, Epithelial-mesenchymal transition, TGF-β1/Smad signaling pathway

Posted Date: March 4th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-263927/v1

Abstract

Background The gastric cancer (GC) microenvironment has important effects on biological behaviors, such as tumor cell invasion and metastasis. However, the mechanism by which the GC microenvironment promotes GC cell invasion and metastasis is unknown. The present study aimed to clarify the effects and mechanism of galectin-1 (GAL-1, encoded by *LGALS1*) on GC invasion and metastasis in the GC microenvironment.

Methods The expression of GAL-1/ *LGALS1* was determined using western blotting, immunohistochemistry, and quantitative real-time reverse transcription PCR in GC tissues. Besides, methods including stable transfection, Matrigel invasion and migration assays, and wound-healing assays *in vitro*; and metastasis assays *in vivo*, were also conducted.

Results GAL-1 from cancer-associated fibroblasts (CAFs) induced the epithelial-mesenchymal transition (EMT) of GC cells though the transforming growth factor beta (TGF-β1)/ Sma- and mad-related protein (Smad) pathway, and affected the prognosis of patients with GC. The level of GAL-1 was high in CAFs, and treating MGC-803 and SGC -7901 cell line with the conditioned medium from CAFs promoted their invasion and metastasis abilities. Overexpression of *LGALS1* promoted the expression of TGF-β1 and induced EMT of GC cell lines. A TGF-β1 antagonist inhibited the invasion and migration of GC cells. *In vivo*, overexpression of *LGALS1* promoted GC growth and metastasis, and the TGF-β1 antagonist dramatically reversed these events.

Conclusions These findings suggested that high expression of GAL-1 in the GC microenvironment predicts a poor prognosis in patients with GC by promoting the migration and invasion of GC cells via EMT through the TGF-β1/Smad signaling pathway. The results might provide new therapeutic targets to treat GC.

Background

Gastric cancer (GC) is one of most malignant tumors of digestive tract, with a high invasive ability. Worldwide, more than 1 million new cases of GC occur every year, and the incidence and mortality of GC are the fifth and third among all malignant tumors [1]. Thus, GC seriously threatens human health, especially in East Asian countries, such as China, South Korea, and Japan[2]. Although there has been considerable progress in the diagnosis and treatment of GC, the 5-year survival rate is still less than 30% in most countries because of the difficulty of early diagnosis and the high recurrence and metastasis of GC [3]. Therefore, it is important to explore the molecular mechanism of GC invasion and metastasis and seek effective molecular markers for early diagnosis and prognosis, to improve the diagnosis and treatment of GC and improve the survival rate of patients with GC.

For a long time, tumor biologists only focused on the biological characteristics of tumor cells, ignored the decisive role of the microenvironmental components of non-tumor cells in tumorigenesis and development, which stalled the development of anti-tumor therapies. Recently, researchers found that the

tumor and its microenvironment form an integrated structure, and began to study of the effect of the microenvironment non-tumor cells on tumorigenesis and development. The tumor microenvironment is a complex and functional environment, which includes the extracellular matrix and multiple types of stromal cells, such as mesenchymal stem cells, macrophages, inflammatory cells, and cancer-associated fibroblasts (CAFs) [4]. CAFs comprise a group of highly homologous activated fibroblasts from different parts of the tumor that have unique phenotypes [5]. Recent studies have shown that CAFs are highly activated in GC tissues, and are closely associated with the malignant potential of GC, such as tumor size, tumor invasion, metastasis, metabolism, and remodeling [6]. Further studies confirmed that CAFs promote the tumorigenesis, development, invasion, metastasis, and other malignant potentials of GC cells via the secretion of various cytokines that act on GC cells [5, 7].

Galectin-1 (GAL-1), a member of the lectin family, is encoded by the *LGALS1* gene, and is characterized by its affinity for glycans containing β galactosides [8]. In the tumor microenvironment, GAL-1 is a multivalent carbohydrate binding protein that mediates malignant cellular activities by cross-linking glycoproteins [9]. For example, GAL-1 can cluster cell surface glycoproteins, form lattices and larger aggregates, and cross-link receptors thought to be involved in various mechanisms [9, 10]. GAL-1 can be activated by paracrine or autocrine sugar-dependent interactions with β-galactoside-containing glycoconjugates in the extracellular environment, and participates in tumor cell adhesion, migration, invasion, tumor-induced angiogenesis, and apoptosis via multiple interactions [11, 12]. Previous studies have shown that GAL-1 is overexpressed in a variety of malignant tumors, including GC [13]. Generally, in malignant tumor tissues with high expression of GAL-1, tumor progression is regulated by interactions with sugar complexes in the tumor microenvironment. Specifically, GAL-1 serves as a scaffold for vascular growth and vascular network formation by establishing physical connections between vascular endothelial cells and the extracellular matrix in the tumor microenvironment, which provides the necessary physical support for new vasculature [14-16] . However, how GAL-1 regulates GC invasion and metastasis in the GC microenvironment remains elusive.

The invasion and metastasis of GC is a complex multifactorial and multistep process. Many studies have found that the epithelial-mesenchymal transition (EMT) leads to downregulation of epithelial-associated markers, such as E-cadherin, and upregulation of mesenchymal markers, such as Vimentin, which plays an important role in the invasion and metastasis of GC [17]. During EMT, epithelial cells lose their polarity, reduce their contact with peripheral cells and stromal cells, and reduce intercellular interactions, which enhance cell migration and motility. Numerous signaling pathways are involved in EMT through cooperation and antagonism, such as transforming growth factor beta (TGF-β), Wnt/beta-catenin, and Ras-mitogen activated protein kinase (MAPKI). [18-20]. TGF-β is considered the most important factor that induces EMT in developmental processes and other pathological states. In some cultured epithelial cell lines, simple TGF-β stimulation can induce EMT. TGF-β signaling-mediated EMT can be achieved by classical the Sma- and Mad-related protein (Smad) pathway or non-Smad pathway. In the classical Smad pathway, TGF-β activates Smad2 and Smad3 and combines with Smad4. Then, Smad complexes will be transferred to the nucleus to mediate the inhibition or activation of target genes together with transcription factors. Meanwhile, Smad complexes can also induce the expression of microRNA in the

nucleus, which inhibits the signature protein expression of epithelial cells and promotes the expression of proteins that confer mesenchymal cell properties, which facilitating EMT [21]. TGF-β signaling-mediated non-Smad signaling pathway, can activate phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-protein kinase B (AKT)-mechanistic target of rapamycin (mTOR) signals for transcriptional regulation [22], and activated AKT can also trigger EMT by inhibiting ribonucleoprotein transcriptional regulation [23].

Our previous study showed that GAL-1 promoted the invasion, metastasis, and vasculogenic mimicry of GC via EMT [24]. However, the role of TGF-β signaling pathways in GAL-1-mediated promotion of GC EMT remains unclear. Clarifying the effects of GAL-1 on GC invasion and metastasis in GC microenvironment, and its molecular mechanism, will provide a new perspective and therapeutic targets to treat GC.

Materials And Methods

Tumor tissue samples

Patients with primary gastric adenocarcinoma (n = 127) were enrolled in the present study. None of the patients had undergone preoperative neoadjuvant radiotherapy or chemotherapy, and all patients received radical gastrectomy with D2 lymphadenectomy at the Department of Gastrointestinal Surgery, Taizhou Clinical Medical School of Nanjing Medical University (Taizhou People's Hospital) of Jiangsu province. GC tissues and matching adjacent gastric mucosa tissues for IHC were formalin-fixed and paraffin-embedded. GC tissues and matching adjacent mucosal tissues for molecular analysis were collected from 15 patients, which were stored in liquid nitrogen. Our study was approved by the Clinical Research Ethics Committee of Taizhou People's Hospital (TZRY-EC-12-068). Provided all patients with details regarding the assessment procedure, and all patients gave informed written consent.

Cell line and cell culture

The human gastric adenocarcinoma cell lines, MGC-803 and SGC-7901 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Roswell Park Memorial Institute (RPMI) medium (Thermo Fisher Scientific, Waltham, MA, USA) was used to culture the cells, The culture medium was supplemented with 10% (V/V) fetal bovine serum (FBS; Thermo Fisher Scientific), 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco, Grand Island, NY, USA). The cells were maintained at 37 °C in a humidified atmosphere containing 5% (V/V) CO_2 [24] When confluence reached 80%, the cells were passaged via trypsinization.

Lentiviral transduction

Lentiviral transduction was performed as previously described[24] . Lentiviral vectors for *LGALS1* overexpression and silencing were constructed by Genechem Co. Ltd (Shanghai, China). The short hairpin RNA (shRNA) sequences and the lentiviral vector were designed in a previous report[15]. MGC-803 or SGC-7901 cells were seeded in 6-well plates at a concentration of 5×10^4 cells per well before lentiviral transduction. Cells were transduced with lentiviral vector and 10 μg/mL polybrene (Sigma-Aldrich, St.

Louis, MO, USA) according to a multiplicity of infection of 10. The medium was replaced 12 h after transfection. Puromycin (Sigma-Aldrich) was added at 2 μg/mL to select stably transduced cell lines. After 48 h, the stably transduced cells were cultured with puromycin at 0.5 μg/mL. The transduction efficiency was confirmed under a fluorescent microscope (OLYMPUS-U-HGLGPS-IX73, Olympus, Tokyo, Japan) after 72 h, and further confirmed by qRT-PCR and WB.

Reagents and antibodies

ITD-1, a hedgehog signaling pathway inhibitor, was purchased from Macklin (1884570, Shanghai, China). The anti-GAL-1 antibody was purchased from Abcam (ab138513, Cambridge, UK), the anti-SMA-α antibody was purchased from Cell Signaling Technology (56856, Danvers, MA, USA), anti-E-cadherin (bs-10009R), anti-Vimentin (bs-0756R), anti-TGF-β1 (bs-0086R), and anti-p-Smad2/3(bs-8853R) antibodies were purchased from Bioss (Beijing, China), and the anti-glyceraldehyde phosphate dehydrogenase (GAPDH) antibody (sc-47724), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (sc-516102), and goat anti-rabbit IgG (sc-2357) antibodies were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). The dimethyl sulfoxide (DMSO) and MTT assay kit and were provided by Sigma Biotechnology (St. Louis, MO, USA).

RNA extraction and qRT-PCR

An RNeasy Mini Kit (Invitrogen, Waltham, MA, USA) was used to extract total RNA, and a Reverse transcription kit (Takara, Shiga, Japan) was used to synthesize cDNA from the RNA. A SYBR Green dye kit (Roche Diagnostics, Mannheim, Germany) was used to performed qPCR and an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) was used to analyze the reaction products. The thermocycling conditions were as follows: 95°C for 30 s; 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s. *GAPDH* was used as the reference control gene. The following primers were used: *LGALS1* (forward): GCTGAACCTGGGCAAAGACAG an GTGGCGGTTGGGGAACTT (producing a 247 bp amplicon); and *GAPDH* (forward) TGACTTCAACAGCGACACCCA) and (reverse) CACCCTGTTGCTGTAGCCAAA (producing a 121 bp amplicon).

Western blotting analysis

Total cell extracts and nuclear extracts were prepared using an extraction kit (Beyotime, Shanghai, China). Cell lysates (20 μg) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then the separated proteins were transferred to a nitrocellulose membrane (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The blots were probed with antibodies against GAL-1, E-cadherin, Vimentin, TGF-β1, p-Smad2/3, and GAPDH at a dilution of 1:2000. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin was used as the secondary antibody also at a dilution of 1:2000. The immunoreactive protein bands were visualized using the West Pico chemiluminescent Substrate (Pierce, Carlsbad, CA, USA), and densitometric image analysis software (Image Master VDS; Pharmacia Biotech, Little Chalfont, UK) was used to quantify the visualized protein bands. The level of GAPDH was used as an internal reference. All experiments were performed in triplicate.

Histological examination and IHC evaluation

Proteins were detected in human GC tissues, matching adjacent non-GC tissues, and murine lung tissues using IHC according to our previous reports [13, 24]. Primary antibodies against GAL-1 (1:200), SMA-α (1:200), E-cadherin (1:150), vimentin (1:150), TGF-β1 (1:150), and p-Smad2/3 (1:150) were incubated with the slides. The other steps and staining scores of the proteins of interest (GAL-1, SMA-α, TGF-β1, and p-Smad2/3) were the same as in our previous report [24]. E-cadherin and vimentin staining was defined as positive or negative according to the evaluation of the staining results as defined in our previous report [24].

Wound-healing assay

GC cells were cultured to a confluent monolayer (80–90 %) in a 6-well plate, scored across the cell surface to create a wound using a sterile plastic tip. The plates were washed three times with phosphate-buffered saline (PBS) to remove cellular debris, and then incubated with serum-free medium containing 10 μg/mL mitomycin C to block proliferation. The wound was photographed at 0 and 48 h. All

assays were performed in triplicate.

Cell viability assay

The viability of the cells was determined using the MTT assay. Cells were seeded into a 96-well flat bottom plate at 5×10^3 cells/well. The cells were cultured overnight at 37 °C with 5% CO_2 in a humidified atmosphere and then treated with ITD1 at various concentrations (5, 10, 15, and 20 μM). After 24 h, 20 μL of MTT was added to each well and incubated for 4 h. The supernatant was then removed, and 150 μL of DMSO was added to dissolve the formazan crystals. The absorbance was measured at 490 nm using a microplate reader (Model 550; Bio-Rad, Hercules, CA, USA).

Cell invasion and migration assays

The invasive and migratory ability of GC cells were measured using 24-well Transwell units with polycarbonate filters (pore size, 8.0 μm; Corning, Corning, NY, USA). The upper Transwell inserts were first coated with or without 100 μl of Matrigel basement membrane (BD Biosciences, San Diego, CA, USA) and 100 μL of serum-free RPMI medium, then 1×10^5 cells were seeded on the upper Transwell inserts. The lower chamber was loaded with Medium (600 μL) containing 10% FBS as a chemoattractant. Cells were allowed to migrate or invade at 37 °C for 24 h, then the non-invasive or non-migratory cells were removed. The filters were fixed using 4% paraformaldehyde, and the cells were stained with a 0.05% crystal violet solution. Six fields were selected randomly to count the cells under a microscope at 100× magnification for each sample. All assays were performed in triplicate.

Animal models

Five-week-old male athymic mice were purchased from the Comparative Medicine Centre of Yangzhou University (Yang Zhou, JiangSu, China) and used to construct subcutaneous GC implantation model and lung metastasis model of GC. These experiments were approved by the Ethics Committee of Yang Zhou University (YZU-EC-JS2742). A laminar flow cabinet under pathogen-free conditions was provided to breed the mice. MGC-803 cells (wild-type control), LGALS1-overexpressing (OE-LGALS1) MGC-803 cells, and OE-LGALS1 MGC-803 treated with ITD1 were separately inoculated into the right flank or the tail vein of the athymic mice (2×10^6 cells/mouse; n = 6). The mice in the subcutaneous model group were sacrificed on day 21 and the mice in the lung metastasis model group were sacrificed on day 50. The subcutaneous GC tumors or the lung were harvested for hematoxylin and eosin (H&E) staining, WB and immunohistochemical staining.

Statistical analysis

SPSS 20.0 (IBM Corp, Armonk, NY, USA) was used to conduct the statistical analysis. Means ± standard error were used to express the continuous variables, one-way analysis of variance (ANOVA) and Dunnett's t test was used for comparisons between groups based on the normal distribution of the data. Pearson or Spearman's correlation coefficients were used to determine the relationship between two variables. Values of $P < 0.05$ were regarded as statistically significant.

Results

1. GAL-1/ *LGALS1* is overexpressed in human GC tissues, which is derived from CAFs and promotes lymph node metastasis in GC

To confirm that *LGALS1* is associated with the malignant behavior of GC, we initially performed quantitative real-time reverse transcription PCR (qRT-PCR) to evaluate *LGALS1* mRNA expression in 15 matched gastric cancer tissues (GCT) and non- gastric cancer tissues (NGCT). We observed a significant difference in *LGALS1* mRNA expression between GCT and NGCT: GCT exhibited significantly higher levels of *LGALS1* mRNA than that in NGCT ($P < 0.01$; Fig. 1A). We then examined GAL-1 protein levels in 15 pairs of GCT and NGCT using western blotting (WB), the results of which were consistent with the qRT-PCR results, demonstrating higher levels of GAL-1 in GCT than in NGCT ($P < 0.01$; Fig. 1B). Besides, the expression of GAL-1 was detected in GCT and in adjacent NGCT of 127 patients with GC using immunohistochemistry (IHC), Image Pro Plus (Media cybernetics, San Diego, CA, USA) was used to evaluated the digital images of IHC. The median IHC scores of GAL-1 in GCT and NGCT were 78.29 (9.51–186.24) and 31.09 (5.89–123.45), respectively. We observed a significant difference in GAL-1 expression between GCT and NGCT(Fig. C and Fig. D; $P < 0.01$), and we found that GAL-1 was mainly expressed by stroma tissue of GC (Fig. 1C) . To further confirm which cells produced GAL-1/ *LGALS1*, we used IHC to detect alpha smooth muscle actin (SMA-α) in GCT from 127 patients with GC. We found that GAL-1 was derived from cancer-associated fibroblasts (CAFs) with a SMA-α protein positive phenotype (Fig. 1C). Moreover, we found that GC cells expressed low or no GAL-1 (Fig. 1C). Surprisingly, we found that the GAL-1 was highly expressed in GC cells from metastatic lymph node tissues when detected using

IHC (Fig. 1E). GAL-1 expression correlated positively with SMA-α expression in GCT (r = 0.963, $P < 0.01$; Fig. 1F). We classified the GAL-1 IHC scores as positive and negative using Receiver Operating Curve (ROC) statistics, and found that the lymph node metastasis rate of the GAL-1 positive group was significantly higher (64/86) than that in the GAL-1 negative group (7/41) ($P < 0.01$; Fig. 1G).

CAF-derived GAL-1/*LGALS1* promotes the invasion and metastasis ability of GC cell lines *in vitro*.

To further clarify the origin of GAL-1/*LGALS1*, we cultured CAFs from human GC tissue, and GC cell lines (MGC-803, BGC-823, AGS and SGC-7901), *in vitro* and investigated the GAL-1 levels in all cells using WB, which confirmed that GAL-1 was strongly expressed in CAFs, with lower levels in GC cells (Fig. 2A). Meanwhile, we performed qRT-PCR to evaluate the expression of *LGALS1* mRNA in all cell lines, the results of which were consistent with those of WB (Fig. 2B). MGC-803 cells and SGC-7901 were then treated with conditioned medium (CM) from CAFs (CM-CAFs) for 72 h, and then the expression of GAL-1 and *LGALS1* mRNA were monitored by WB and qRT-PCR. Compared with untreated MGC-803 and SGC-7901 cells, we found that treatment of MGC-803 and SGC-7901 cells with CM-CAFs significantly increased the GAL-1 protein and *LGALS1* mRNA levels (Fig. 2C and 2D). We observed that the proliferation of MGC-803 and SGC-7901 cells were increased when treated with CM-CAFs for 48 h, and the proliferation effect was abolished when the medium contained 10 μg/mL mitomycin C (Fig. 2E). As shown in Figure 2F, MGC-803 and SGC-7901 cells treated with CM-CAFs exhibited a significantly enhanced migration capacity compared with that of the wild-type (untreated) control; the fold changes in migration are shown in Fig 2G ($P < 0.01$). Transwell assays showed that MGC-803 and SGC-7901 cells treated with CM-CAFs had increased cell invasion and migration abilities ($P < 0.01$, Figure 2H and 2I).

GAL-1/*LGALS1* promotes EMT in GC, and EMT promotes lymph node metastasis of GC

To determine whether GAL-1/*LGALS1* promotes the invasion and migration abilities of GC cells via EMT, we examined the EMT-related biomarkers Vimentin and E-Cadherin in GC tissue using IHC. We identified E-cadherin-positive staining in GC cells and Vimentin staining in tumor stroma cells in 85 cases, indicating that EMT had not occurred (Fig. 3A). In 42 cases, the expression of Vimentin was positive in both tumor stroma cells and GC cells, and E-cadherin was decreased in those samples, indicating that EMT had occurred (Fig. 3B). The EMT rate in the GAL-1 positive group was 41.86% (36/86), while that in the GAL-1 negative group was 14.63% (6/41), suggesting that GAL-1 promotes significantly EMT in GC ($P < 0.05$, Fig. 3C). In addition, we found that GC cases with EMT were more prone to lymph node metastasis: There were 30 cases with lymph node metastasis among 42 patients with GC with EMT (71.43%, 30/42), while there were 41 cases with lymph node metastasis among 85 cases of GC without EMT (48.24%, 41/85) ($P < 0.05$, Fig. 3D). GC cells with or without EMT in metastatic lymph nodes are shown in Fig. 3E and 3F.

GAL-1/*LGALS1*activates the TGF-β/Smad signaling pathways in GC tissues

To explore the mechanism by which GAL-1/ *LGALS1* promotes EMT in GC, we examined the protein levels of GAL-1, TGF-β1, and phosphorylated (p)-Smad2/3 in GCT and NGCT using IHC. Representative images

of IHC for GAL-1, TGF-β1, and p-Smad2/3 protein levels in GCT and NGCT are shown in Fig 4A. Significant differences in TGF-β1 and p-Smad2/3 levels were observed between GCT and NGCT (all $P < 0.01$; Fig. 4B and 4C). For TGF-β1, the median IHC score for was 108.26 (8.741–234.56) in GC tissues, 127.22 (56.67-234.56) in GAL-1-positive GC tissues, and 43.47 (8.741-97.12) in GAL-1-negative GCT. The TGF-β1 IHC scores in GAL-1-positive GCT were significantly higher than that in GAL-1-negative GCT ($P <0.01$; Fig. 4D). The TGF-β1 IHC scores correlated positively with the GAL-1 IHC scores in GC tissues ($r = 0.97$; $P < 0.01$; Figure 4E).

GAL-1/*LGALS1* induces EMT through TGF-β/Smad signaling pathways *in vitro*

To determine whether GAL-1 induces EMT through activation of TGF-β/Smad signaling in GC, MGC-803 and SGC-7901 cells were transfected with LV-*LGALS1*-RNAi and LV- *LGALS1*-OE to silence or overexpress *LGALS1*, respectively, as described previously [15]. Western blotting showed that MGC-803 overexpressing *LGALS1* (OE-*LGALS1*) had increased Vimentin levels and decreased E-cadherin levels compared with non-transfected MGC-803 cells (wild-type), and the levels of TGF-β1 and p-Smad2/3 also increased in MGC-803 OE-*LGALS1* cells (all $P < 0.01$, Fig. 5A). The results in SGC-7901 cells were consistent with those in MGC-803 cells (all $P<0.01$, Fig. 5B). When *LGALS1* was silenced in MGC-803 cells, the level of E-cadherin increased, and the levels of Vimentin, TGF-β1, and p-Smad2/3 decreased (all $P < 0.01$, Fig. 5C). Similar results were observed in SGC-7901 cells (all $P < 0.01$, Fig. 5D).

GAL-1/*LGALS1* promotes the migration and invasion of GC cells *in vitro* through TGF-β/Smad signaling pathways

To investigate whether GAL-1/*LGALS1* promotes the migration and invasion of GC cells via activation of the TGF-β/Smad pathway, the TGF-β/Smad signaling pathway specific antagonist, ITD1, was used to explore the relationship between GAL-1-induced migration and invasion of GC cells in GC and TGF-β/Smad pathway activation. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay indicated that 10 μM ITD1 did not affect the numbers of MGC-803 and SGC-7901 cells at 48 h. OE-*LGALS1* increased the number of cells; however, the proliferation effect was abolished using 10 μg/mL mitomycin C. OE-*LGALS1* MGC-803 cells exhibited significantly enhanced migration compared with the wild-type control (WC) and negative control-transfected MGC-803 cells (OE-con) ($P < 0.01$, Fig. 6A). However, the migration capacity was abolished when the medium contained 10 μM ITD1($P < 0.01$, Fig. 6A). The fold changes in migration are shown in Fig 6B.

To confirm that GAL-1 promotes migration and invasion *in vitro*, we repeated these experiments in SGC-7901 cells. OE-*LGALS1* SGC-7901 cells showed an enhanced migration capacity compared with WC and OE-con SGC-7901 and 10 μM ITD1 abolished the migration capacity (Fig. 6C and 6D). As shown in Figure 6E, Transwell assays demonstrated increased invasiveness of MGC-803 and SGC-7901 cells transfected with LV-*LGALS1*-OE, and the addition of 10 μM ITD1 abolished this capacity for invasion.

GAL-1/*LGALS1* promotes GC cell metastasis *in vivo* through the TGF-β/Smad signaling pathway

Through clinical analysis and *in vitro* experiments, we demonstrated that GAL-1/*LGALS1* promotes GC migration, invasion, and metastasis by an EMT-mediated process via the TGF-β/Smad signaling pathway. Subcutaneous GC implantation and lung metastasis models in athymic BALB/c mice (n = 6/group) were generated to further elucidate whether GAL-1/*LGALS1* promotes GC growth and metastasis through EMT induced by activating TGF-β/Smad signaling. At 21 days after subcutaneous GC implantation, tumors in the *LGALS1* overexpression group (OE-*LGALS1*) were larger and heavier than those in the WC group, and the tumors formed by OE-*LGALS1* MGC-803 cells treated with ITD1 were significantly smaller and lighter than those in the WC group (Figure 7A, B and C; $P < 0.01$). From day 12 onward, the tumor volume in the OE-*LGALS1* group was significantly higher compared with that in the WC group, and the volumes of the tumors in the OE-*LGALS1* MGC-803 cells treated with ITD1 were significantly lower ($P < 0.01$ and $P < 0.05$; Fig. 7B). Western blotting showed that the levels of Vimentin in the OE-*LGALS1* group were increased, and the levels E-cadherin were decreased, which suggested that *LGALS1* overexpression promoted EMT. Compared with the WC group, TGF-β1 and p-Smad2/3 levels were elevated in the OE-*LGALS1* group ($P < 0.01$, Fig. 7D). However, ITD1 treatment reduced the levels of p-Smad2/3 significantly ($P < 0.01$, Fig. 7D), and inhibited EMT in the implanted GC cells (Figure 7D) .

Larger pulmonary metastases were found in all the mice in the OE-*LGALS1* group after fifty days in the lung metastasis models. Four cases of pulmonary metastasis were found in the WC group, while the ITD1-treated OE-*LGALS1* group did not have any pulmonary metastases ($P = 0.002$; Figure 7E). Pulmonary metastases in the OE-*LGALS1* group and the WC group were identified using hematoxylin and eosin (H&E) staining (Figure 7F). IHC staining was used to examine the TGF-β/Smad signaling markers in the pulmonary metastases, which indicated that GAL-1, Vimentin, TGF-β1, and p-Smad 2/3 levels were increased significantly and the E-cadherin level was decreased in the pulmonary metastases tissues of the OE-*LGALS1* group (Figure 7G). These results suggested that GAL-1 plays an important role in GC metastasis and EMT, and that TGF-β/Smad signaling pathway potentially contributes to this process.

Discussion

The growth and metastasis of a malignant tumor is a complex and multi-step process. Recent studies show that a variety of growth factors and cytokines released by tumor stromal cells create a microenvironment suitable for tumor growth, which promotes tumor proliferation, growth, invasion, and escape from immune surveillance, leading to distant metastasis [25]. GAL-1 is a multivalent carbohydrate-binding protein that regulates the activity of malignant tumor cells by cross-linking glycoproteins in the tumor microenvironment [26]. GAL-1 is synthesized on cytoplasmic ribosomes with a prototypical acetylated N-terminus but no signal peptide [27], then GAL-1 is transported from the nucleus to the medial side of the cell membrane and secreted into the extracellular tumor microenvironment [27, 28] In extracellular environment, GAL-1 has high affinity with β-galactosides [27], and regulates homotypic aggregation of cancer cells by interacting with cell surface sugar complexes [8]. Furthermore, GAL-1 can mediate the adhesion of tumor cells to the extracellular matrix [12]. Laminin, fibronectin, and other glycoproteins present in the basement membrane provide the necessary binding sites for GAL-1 to crosslink cells with the extracellular matrix, thus GAL-1 regulates cancer cell adhesion during metastasis

through glycoproteins [12]. Conversely, GAL-1 inhibits adhesion between tumor cells and the extracellular matrix by competitive binding of matrix glycoproteins or cell surface sugar complexes [29]. This suggests another role of GAL-1, the regulation of tumor cell isolation, which allows tumor cells to detach from the primary site and migrate to secondary sites [29]. In the extracellular matrix, GAL-1 regulates the adhesion between tumor cells and the extracellular matrix, regulates the binding of the extracellular matrix to glycoproteins, and enhances the activities of proteolytic enzymes, thereby promoting the metastasis of tumor cells [12].

GAL-1 has been found in multiple tumor cells, including melanoma, lung cancer [30], pancreatic cancer [31], bladder cancer [32], thyroid cancer [33], cervical cancer [34], and colorectal cancer [35]. In the present study, we showed that the GAL-1 protein and *LGALS1* mRNA levels in GC tissue were significantly higher than those in NGCT, suggesting that GAL-1/*LGALS1* is associated with the malignant biological behavior of GC. We also observed that the GAL-1 in GC tissue is secreted by SMA-α-positive CAFs in the tumor microenvironment. WB and qRT-PCR also confirmed that the high expression of GAL-1 in CAFs. Intriguingly, GC cells showed low or no GAL-1 expression in GCT, but GC cells in metastatic lymph nodes showed high expression of GAL-1. Moreover, we treated GC cells with conditioned medium from CAFs cells, which increased GAL-1 expression in MGC803 and SGC-7901 cell lines, and promoted the invasion and metastasis of GC cell lines significantly. This indicated that GAL-1/*LGALS1* promotes GC cells to acquire a metastatic phenotype and promotes GC cell metastasis. However, how GAL-1/ *LGALS1* in the microenvironment regulates the invasion and metastasis of GC remains mostly unknown.

Many studies showed that EMT is a crucial step in the invasion and metastasis of malignant tumors [20]. Therefore, we hypothesized that GAL-1/*LGALS1* promotes the invasion and metastasis of GC through EMT. To test this hypothesis, we examined the EMT-related biomarkers Vimentin and E-Cadherin in GC and metastatic lymph nodes with IHC, and found that the level of GAL-1 was related to EMT in GCT. In addition, we found that GC with EMT were more prone to lymph node metastasis.

The TGF-β family plays a vital role in EMT regulation [23]. The results of our IHC test also showed that the levels of TGF-β1 and p-Smad 2/3 in GC tissue were significantly higher than those in gastric mucosal tissue, and the level of TGF-β1 in GCT with GAL-1 positive expression was significantly higher than that in GAL-1 negative expression tissues. Moreover, the expression of GAL-1 in GCT correlated positively with TGF-β1 expression. These results suggested that GAL-1 in the GC microenvironment potential activates the TGF-β/Smad signaling pathway .

TGF- β1 is the prototypic member of a large family of functionally and structurally related proteins, which includes its close relatives TGF-β2 and TGF-β3, and growth and differentiation factors (GDF), Mullerian-inhibiting substance (MIS), and bone morphogenetic proteins (BMPs) [36]. In the 1980s, TGF- β1 was discovered as a secreted factor that induced the growth of normal rat kidney cells in soft agar. TGF-β1 transmits signals from cell surface receptors to the nucleus, and the Smads family of proteins play key roles in this process. The Smads family are divided into receptor-regulated (R-) Smads (R-SMAD1, -2, -3, -5 and -8), the common (Co-) Smads (Smad4), and inhibitory I-Smads (Smad -6 and -7). R-Smads interact

with, and become phosphorylated, by activated TGF-β type I receptor kinases. Co-Smads form heteromeric complexes with activated R-SMADs, while I-Smads antagonize canonical Smad signaling [37]. In the case of the canonical Smad pathway, TGF- β1 exerts its cellular effects through cell surface TGF-β type I and type II receptors. TGF- β1 binds to type II receptors, which recruit and phosphorylate type I receptors, and the type I receptors rephosphorylate R-Smad proteins (Smad2 and Smad3), which bind to coSmad (Smad4). The R-Smad/coSmad complex aggregates in the nucleus as a transcription factor and cooperates with other transcription regulators to modulate target gene expression; therefore, Smad2/3 act as intracellular transcriptional effectors of TGF- β family receptor signaling.

In the present study, IHC examination of GC tissue showed that the GAL-1 levels in the GC microenvironment correlated positively correlated with TGF- β1 in GCT, and GCT with high galectin expression had significantly higher levels of p-Smad2/3. The levels of TGF- β1 and p-Smad2/3 increased in GC cell lines overexpressing *LGALS1*, which underwent EMT and showed enhanced invasion and migration abilities. The overexpression of *LGALS1* promoted subcutaneous tumor growth and lung metastasis in nude mice. This subcutaneous growth and lung metastasis was inhibited in nude mice when the cell lines were treated with ITD1, a specific inhibitor of the TGF-β signaling pathway. This further confirmed that GAL-1/*LGALS1* promotes the invasion and metastasis of GC through the TGF-β/Smad signaling pathway.

TGF-β receptors are widely expressed in human cells and mediate various biological phenomena, such as embryonic development, organogenesis, immune surveillance, and tissue repair. Alterations in TGF-β signaling lead to many diseases, including cancer. At the early stage of a malignant tumor, TGF-β signaling acts to inhibit tumorigenesis by inducing apoptosis of premalignant cells. Meanwhile, when cancer cells acquire oncogenic mutations, they become resistant to TGF-β-induced apoptosis. TGF-β can induce tumor cells to undergo EMT, leading to metastasis and chemotherapy resistance [38].

EMT-induced changes in tumor cells and the microenvironment are the main factors that help cancer stem cells to escape from the primary site [39]. Previous studies have found that TGF-β, chemokine 4/12, interleukin-6, and tumor necrosis factor-α in the tumor microenvironment can enhance EMT. EMT results in more epithelial growth factors being secreted by tumor cells and leads to an acidic, hypoxic, and high interstitial fluid pressure state in the microenvironment, which activates CAFs [40]. In the present study, we found that activated CAFs in GC secreted GAL-1, which can activate the TGF-β/Smad signaling pathway and promote EMT in GC. Therefore, this represents a positive feedback loop that ultimately promotes the invasion and metastasis of GC.

The clinical specimen analysis and the *in vitro* and *in vivo* experiments all confirmed that GAL-1 can promote the invasion and metastasis of GC through the TGF-β/Smad signaling pathway. However, the mechanism by which GAL-1/*LGALS1* in GC microenvironment activates the TGF-β/Smad signaling pathway in GC cells remains elusive. Further experiments are required to reveal its specific molecular mechanism to provide new targets for targeted therapy of GC.

Conclusion

Taken together, the results of the present study suggest that GAL-1, derived from CAFs in the GC microenvironment, promotes GC cells to acquire a metastatic phenotype, leading to lymph node metastasis. TGF-β/Smad signaling pathway components are activated in GC by GAL-1, resulting in EMT, which controls the initiation of invasion and metastasis in GC. These findings provided evidence that the microenvironment and the GAL-1/TGF-β/Smad pathway plays important roles in invasion and metastasis in GC. This provides novel insights into the mechanisms underlying invasion and metastasis in GC, and might contribute towards the identification of a therapeutic target for GC.

However, further study is required to explore the mechanism of GAL-1/*LGALS1*-mediated activation of TGF-β/Smad signaling. Taking the microenvironment of GC as the breakthrough point for the treatment of GC, inhibition of this signaling pathway might reverse the EMT of GC and improve therapeutic outcomes for patients with refractory GC.

Abbreviations

CAFs: Cancer-associated fibroblasts; EMT: Epithelial-mesenchymal transition;GAL-1:Galectin-1; GC: Gastric cancer; GCT: Gastric cancer tissues; TGF-β:transforming growth factor beta; Smad: Sma- and mad-related protein

Declarations

Acknowledgements

We thank Wei Xiao, FUxing Liu and Xiaowei Zhu for their technical help. We would like to thank the native English speaking scientists of Elixigen Company (Huntington Beach, California) for editing our manuscript.

Funding

This work was supported in part by Natural Science Foundation of Jiangsu province [grant numbers BK20201230], Taizhou fifth phase 311 talent training project [grant numbers RCPY202022].

Availability of data and materials

Not Applicable

Authors' contributions

XLY conceived and designed the experiments. XLY, JW, XJZ and ZYC performed the experiments. CJH, YXJ, and WXT analyzed the data. JW,XJZ ,TRZand XHS helped in sample collection. XLY wrote the paper. XLY supervised the whole experimental work. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no conflicts of interest.

Consent for publication

Not Applicable

Ethics approval and consent to participate

This clinical study was approved by the Clinical Research Ethics Committee of Taizhou People's Hospital (TZRY-EC-12-068), all patients consented to participate in our study. The animal experiments were approved by the Ethics Committee of Yang Zhou University (YZU-EC-JS2352).

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A *et al.* Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians2021 Feb 4.
2. Sano T. Gastric cancer: Asia and the world. Gastric cancer : official journal of the International Gastric Cancer Association and the Japanese Gastric Cancer Association2017 Mar;20(Suppl 1):1-2.
3. Diaz MDP, Icaza G, Nunez L, Pou SA. Gastric Cancer Mortality Trends in the Southern Cone: Disentangling age, period and cohort patterns in Argentina and Chile. Scientific reports2020 Jan 30;10(1):1526.
4. Roma-Rodrigues C, Mendes R, Baptista PV, Fernandes AR. Targeting Tumor Microenvironment for Cancer Therapy. International journal of molecular sciences2019 Feb 15;20(4).
5. Bu L, Baba H, Yoshida N, Miyake K, Yasuda T, Uchihara T *et al.* Biological heterogeneity and versatility of cancer-associated fibroblasts in the tumor microenvironment. Oncogene2019 Jun;38(25):4887-901.
6. Ma Z, Chen M, Yang X, Xu B, Song Z, Zhou B *et al.* The Role of Cancer-associated Fibroblasts in Tumorigenesis of Gastric Cancer. Current pharmaceutical design2018;24(28):3297-302.
7. Yan Y, Wang LF, Wang RF. Role of cancer-associated fibroblasts in invasion and metastasis of gastric cancer. World journal of gastroenterology2015 Sep 7;21(33):9717-26.
8. Astorgues-Xerri L, Riveiro ME, Tijeras-Raballand A, Serova M, Neuzillet C, Albert S *et al.* Unraveling galectin-1 as a novel therapeutic target for cancer. Cancer treatment reviews2014 Mar;40(2):307-19.
9. Belardi B, O'Donoghue GP, Smith AW, Groves JT, Bertozzi CR. Investigating cell surface galectin-mediated cross-linking on glycoengineered cells. Journal of the American Chemical Society2012 Jun 13;134(23):9549-52.
10. Brewer CF, Miceli MC, Baum LG. Clusters, bundles, arrays and lattices: novel mechanisms for lectin-saccharide-mediated cellular interactions. Current opinion in structural biology2002 Oct;12(5):616-

23.

11. Fettis MM, Farhadi SA, Hudalla GA. A chimeric, multivalent assembly of galectin-1 and galectin-3 with enhanced extracellular activity. Biomaterials science2019 Apr 23;7(5):1852-62.
12. Tamai R, Kobayashi-Sakamoto M, Kiyoura Y. Extracellular galectin-1 enhances adhesion to and invasion of oral epithelial cells by Porphyromonas gingivalis. Canadian journal of microbiology2018 Jul;64(7):465-71.
13. You X, Wang Y, Wu J, Liu Q, Chen D, Tang D *et al.* Prognostic significance of galectin-1 and vasculogenic mimicry in patients with gastric cancer. OncoTargets and therapy2018;11:3237-44.
14. Wei J, Li DK, Hu X, Cheng C, Zhang Y. Galectin-1-RNA interaction map reveals potential regulatory roles in angiogenesis. FEBS letters2021 Jan 22.
15. You X, Wu J, Wang Y, Liu Q, Cheng Z, Zhao X *et al.* Galectin-1 promotes vasculogenic mimicry in gastric adenocarcinoma via the Hedgehog/GLI signaling pathway. Aging2020 Nov 10;12(21):21837-53.
16. Andersen MN, Ludvigsen M, Abildgaard N, Petruskevicius I, Hjortebjerg R, Bjerre M *et al.* Serum galectin-1 in patients with multiple myeloma: associations with survival, angiogenesis, and biomarkers of macrophage activation. OncoTargets and therapy2017;10:1977-82.
17. Ma S, Kong S, Gu X, Xu Y, Tao M, Shen L *et al.* As a biomarker for gastric cancer, circPTPN22 regulates the progression of gastric cancer through the EMT pathway. Cancer cell international2021 Jan 11;21(1):44.
18. Wei Y, Zhang F, Zhang T, Zhang Y, Chen H, Wang F *et al.* LDLRAD2 overexpression predicts poor prognosis and promotes metastasis by activating Wnt/beta-catenin/EMT signaling cascade in gastric cancer. Aging2019 Oct 24;11(20):8951-68.
19. Okada T, Sinha S, Esposito I, Schiavon G, Lopez-Lago MA, Su W *et al.* Author Correction: The Rho GTPase Rnd1 suppresses mammary tumorigenesis and EMT by restraining Ras-MAPK signalling. Nature cell biology2019 Apr;21(4):534.
20. Zhou X, Men X, Zhao R, Han J, Fan Z, Wang Y *et al.* Correction: miR-200c inhibits TGF-beta-induced-EMT to restore trastuzumab sensitivity by targeting ZEB1 and ZEB2 in gastric cancer. Cancer gene therapy2020 Dec;27(12):976-78.
21. Gwon MG, An HJ, Kim JY, Kim WH, Gu H, Kim HJ *et al.* Anti-fibrotic effects of synthetic TGF-beta1 and Smad oligodeoxynucleotide on kidney fibrosis in vivo and in vitro through inhibition of both epithelial dedifferentiation and endothelial-mesenchymal transitions. FASEB journal : official publication of the Federation of American Societies for Experimental Biology2020 Jan;34(1):333-49.
22. Wang B, Cao C, Liu X, He X, Zhuang H, Wang D *et al.* BRCA1-associated protein inhibits glioma cell proliferation and migration and glioma stem cell self-renewal via the TGF-beta/PI3K/AKT/mTOR signalling pathway. Cellular oncology2020 Apr;43(2):223-35.
23. Baek SH, Ko JH, Lee JH, Kim C, Lee H, Nam D *et al.* Ginkgolic Acid Inhibits Invasion and Migration and TGF-beta-Induced EMT of Lung Cancer Cells Through PI3K/Akt/mTOR Inactivation. Journal of cellular physiology2017 Feb;232(2):346-54.

24. You X, Liu Q, Wu J, Wang Y, Dai J, Chen D *et al.* Galectin-1 Promotes Vasculogenic Mimicry in Gastric Cancer by Upregulating EMT Signaling. Journal of Cancer2019;10(25):6286-97.
25. Echizen K, Oshima H, Nakayama M, Oshima M. The inflammatory microenvironment that promotes gastrointestinal cancer development and invasion. Advances in biological regulation2018 May;68:39-45.
26. Huang YJ, Shiau AL, Chen SY, Chen YL, Wang CR, Tsai CY *et al.* Multivalent structure of galectin-1-nanogold complex serves as potential therapeutics for rheumatoid arthritis by enhancing receptor clustering. European cells & materials2012 Mar 13;23:170-81; discussion 81.
27. Leffler H, Carlsson S, Hedlund M, Qian Y, Poirier F. Introduction to galectins. Glycoconjugate journal2002;19(7-9):433-40.
28. Camby I, Le Mercier M, Lefranc F, Kiss R. Galectin-1: a small protein with major functions. Glycobiology2006 Nov;16(11):137R-57R.
29. Cooper DN, Massa SM, Barondes SH. Endogenous muscle lectin inhibits myoblast adhesion to laminin. The Journal of cell biology1991 Dec;115(5):1437-48.
30. Tsai YT, Liang CH, Yu JH, Huang KC, Tung CH, Wu JE *et al.* A DNA Aptamer Targeting Galectin-1 as a Novel Immunotherapeutic Strategy for Lung Cancer. Molecular therapy Nucleic acids2019 Dec 6;18:991-98.
31. Qin H, Qin B, Yuan C, Chen Q, Xing D. Pancreatic Cancer detection via Galectin-1-targeted Thermoacoustic Imaging: validation in an in vivo heterozygosity model. Theranostics2020;10(20):9172-85.
32. Shen KH, Li CF, Chien LH, Huang CH, Su CC, Liao AC *et al.* Role of galectin-1 in urinary bladder urothelial carcinoma cell invasion through the JNK pathway. Cancer science2016 Oct;107(10):1390-98.
33. Fanfone D, Stanicki D, Nonclercq D, Port M, Vander Elst L, Laurent S *et al.* Molecular Imaging of Galectin-1 Expression as a Biomarker of Papillary Thyroid Cancer by Using Peptide-Functionalized Imaging Probes. Biology2020 Mar 14;9(3).
34. Chetry M, Song Y, Pan C, Li R, Zhang J, Zhu X. Effects of Galectin-1 on Biological Behavior in Cervical Cancer. Journal of Cancer2020;11(6):1584-95.
35. Wang W, Zhou Z, Xiang L, Lv M, Ni T, Deng J *et al.* CHIP-mediated ubiquitination of Galectin-1 predicts colorectal cancer prognosis. International journal of biological sciences2020;16(4):719-29.
36. Derynck R, Budi EH. Specificity, versatility, and control of TGF-beta family signaling. Science signaling2019 Feb 26;12(570).
37. Mullen AC, Wrana JL. TGF-beta Family Signaling in Embryonic and Somatic Stem-Cell Renewal and Differentiation. Cold Spring Harbor perspectives in biology2017 Jul 5;9(7).
38. Hao Y, Baker D, Ten Dijke P. TGF-beta-Mediated Epithelial-Mesenchymal Transition and Cancer Metastasis. International journal of molecular sciences2019 Jun 5;20(11).

39. Chiang AC, Massague J. Molecular basis of metastasis. The New England journal of medicine2008 Dec 25;359(26):2814-23.
40. Jung HY, Fattet L, Yang J. Molecular pathways: linking tumor microenvironment to epithelial-mesenchymal transition in metastasis. Clinical cancer research : an official journal of the American Association for Cancer Research2015 Mar 1;21(5):962-68.

Figures

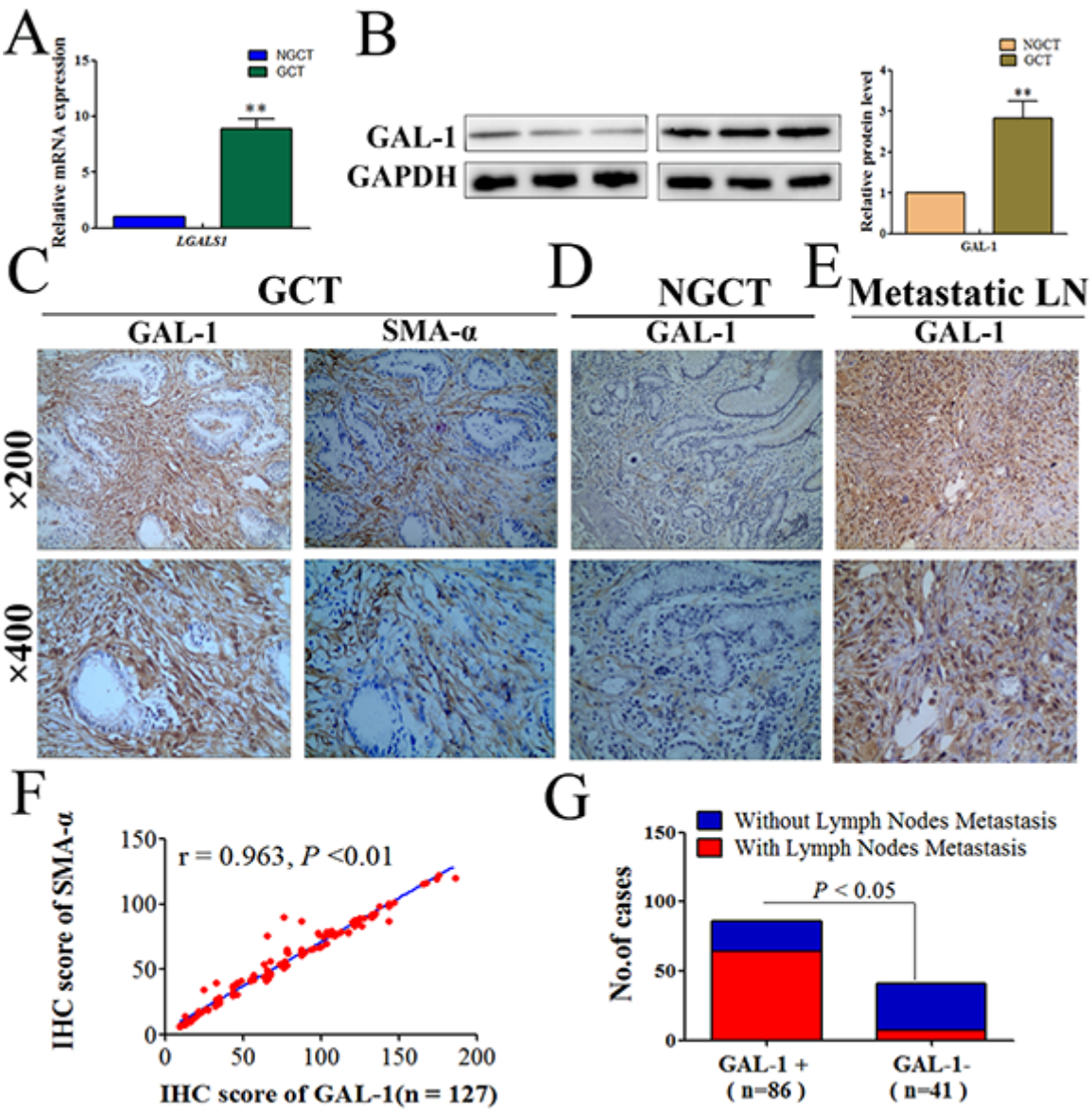

Figure 1

GAL-1/LGALS1 is overexpressed in GC tissues derived from CAFs and promotes lymph node metastasis. (A) GCT exhibited significantly higher levels of the LGALS1 mRNA than that in NGCT. (B) GAL-1 is overexpressed in GC tissues. (C–E) Representative images of IHC for GAL-1 and SMA-α protein levels in GCT, NGCT, and metastatic LN. (F) The IHC score of GAL-1 correlated positively with the IHC score of

SMA-α in GC tissues (r = 0.963; P < 0.01). (G) The lymph node metastasis rate of the GAL-1-positive group was significantly higher than that in the GAL 1 negative group (P < 0.01).

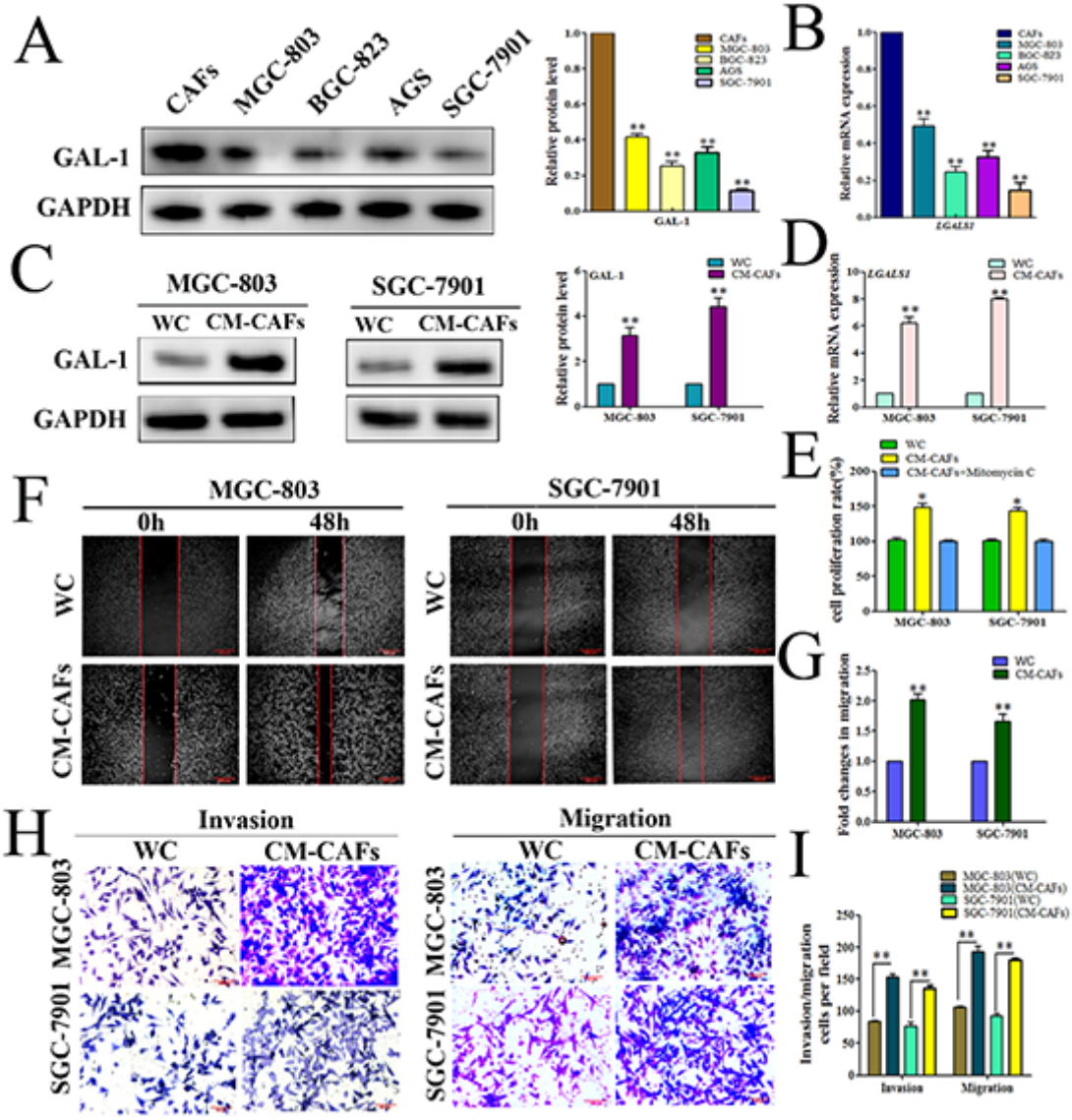

Figure 2

CAF-derived GAL-1/ LGALS1 promotes the invasion and metastasis ability of GC cell lines in vitro. (A and B) GAL-1 protein and LGALS1 mRNA levels in CAFs and GC cell lines, GAL-1/LGALS1 levels were high in CAFs. (C and D) Treatment of MGC-803 and SGC-7901 cells with CM-CAFs significantly increased GAL-1 protein levels and LGALS1 mRNA expression. (E) CM-CAFs increased the proliferation of MGC-803 and SGC-7901 cells, and the proliferation effect was abolished when the medium contained 10 μg/mL mitomycin C. (F and G) MGC-803 and SGC-7901 cells treated with CM-CAFs exhibited a significantly enhanced migration capacity compared with the wild-type control (P < 0.01). (H and I) Transwell assay showing that MGC-803 and SGC-7901 cells treated with CM-CAFs had increased cell invasion and migration abilities (P < 0.01).

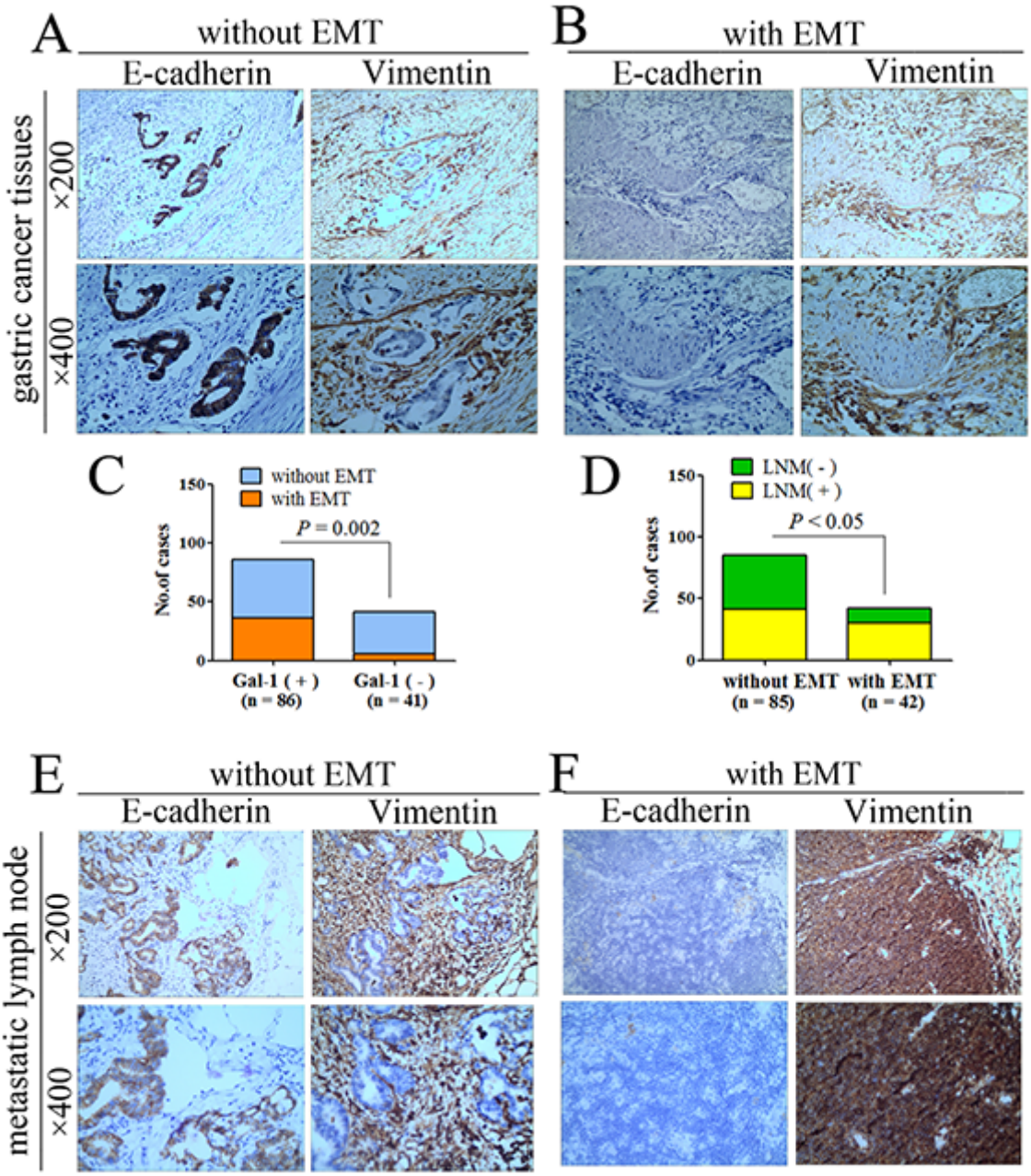

Figure 3

GAL-1/LGALS1 promotes EMT in GC, and EMT promotes lymph node metastasis of GC. (A) Representative images of GCT without EMT. (B) Representative images of GCT with EMT. (3) GAL-1/LGALS1 significantly promoted EMT in GC ($P < 0.05$). (D) GC with EMT was more prone to lymph node metastasis ($P < 0.05$). (E and F) Representative images of GC cells with or without EMT in metastatic lymph nodes.

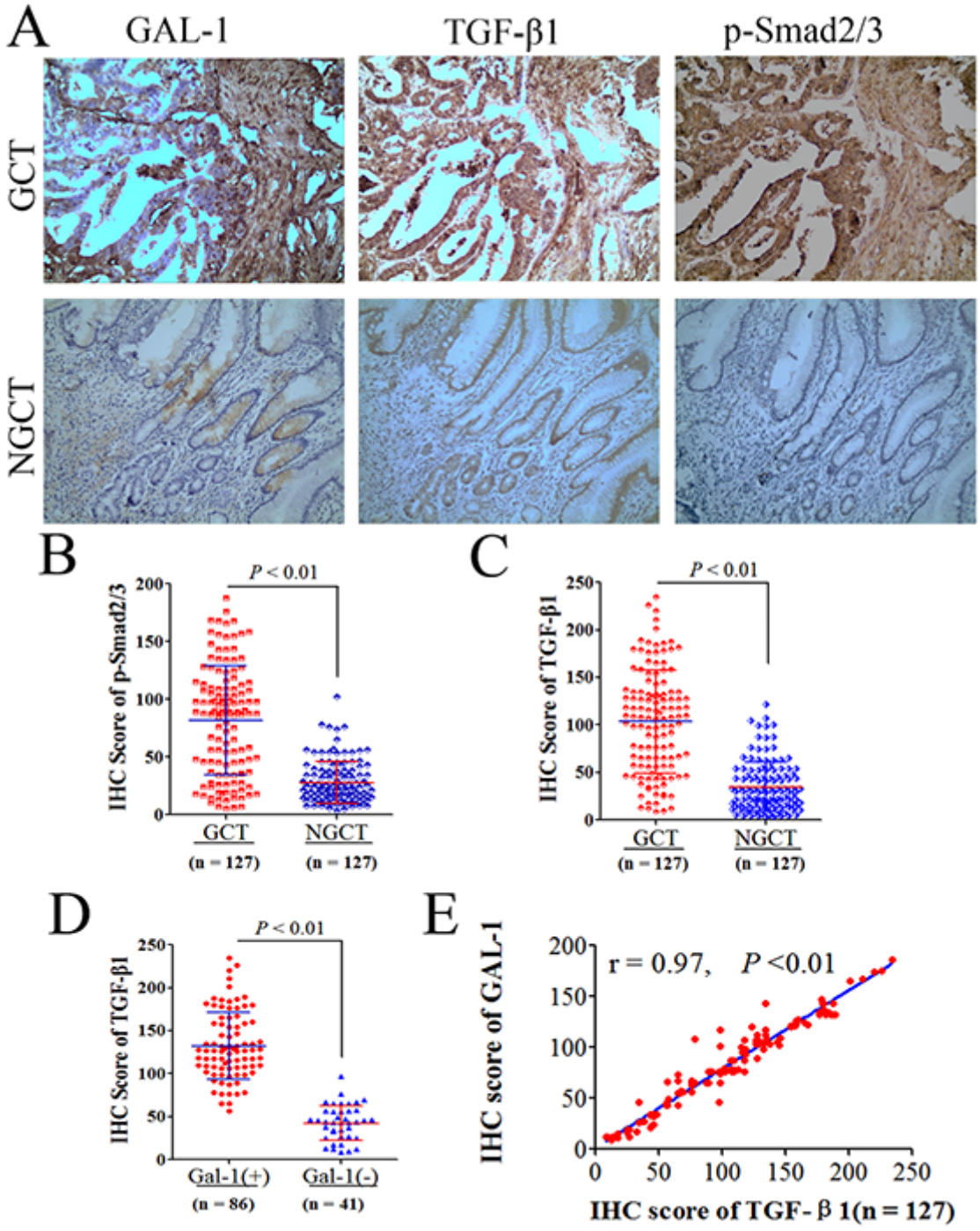

Figure 4

GAL-1/LGALS1 activates TGF-β/Smad signaling pathways in GC tissues. (A) Representative images of IHC for GAL-1, TGF-β1, and p-Smad2/3 protein levels in GCT and NGCT. (B and C) Significant differences in TGF-β1 and p-Smad2/3 levels were observed between GCT and NGCT (all P < 0.01). (D) The TGF-β1 IHC scores in GAL-1-positive GCT (P < 0.01). (E) The TGF-β1 IHC scores correlate positively with the GAL-1 IHC scores in GC tissues (r = 0.97; P < 0.01).

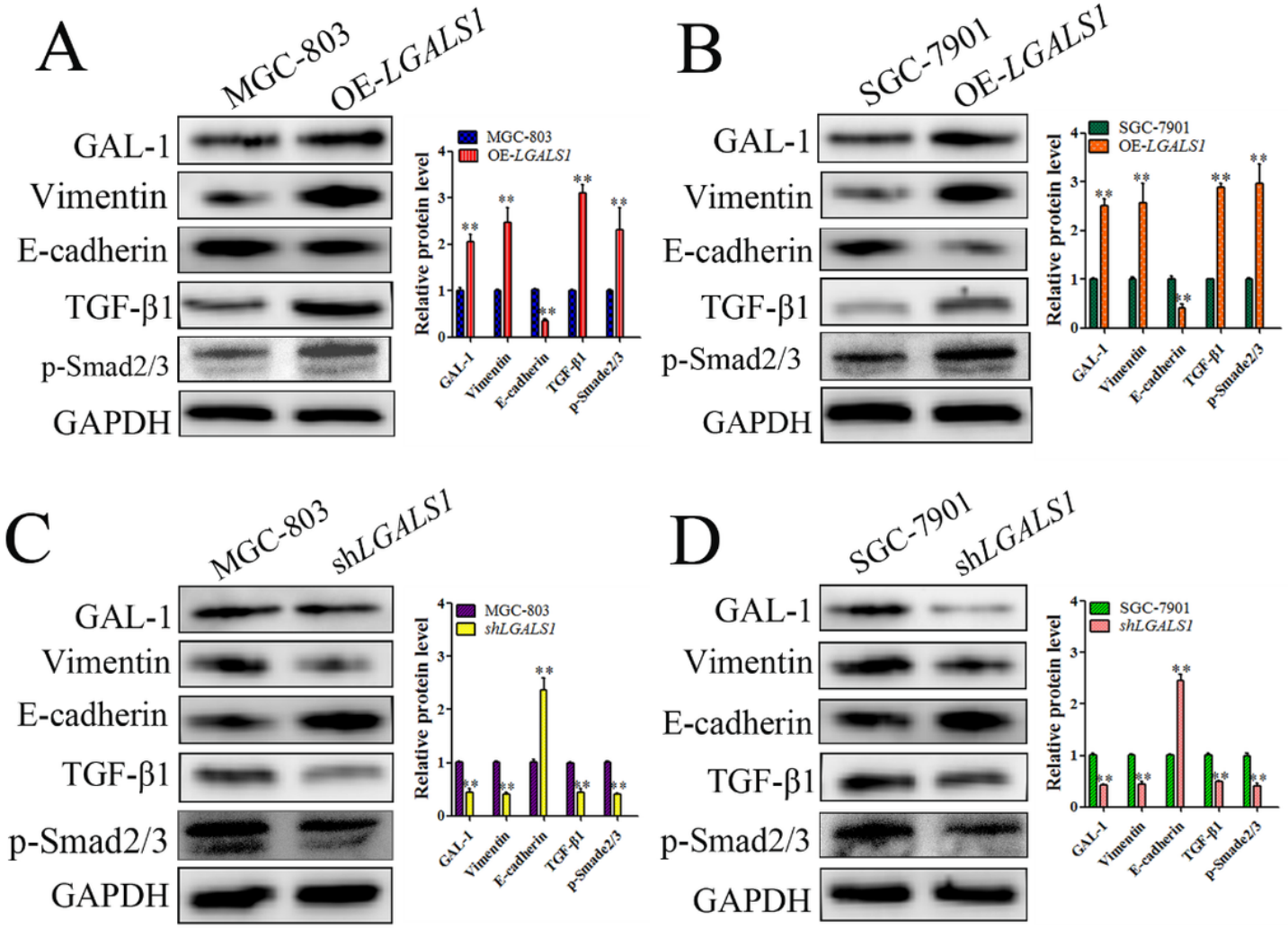

Figure 5

GAL-1/ LGALS1 induces EMT through TGF-β/Smad signaling pathways in vitro. (A and B)WB showing that OE-LGALS1 efficiently increased the expression of TGF-β1 and p-Smad2/3 and induced EMT in MGC-803 and SGC-7901 cells compared with the wild-type cells (all $P < 0.01$). (C and D) Silencing LGALS1 in MGC-803 and SGA-7901 cells efficiently decreased the levels of TGF-β1 and p Smad2/3 and inhibited EMT (all $P < 0.01$).

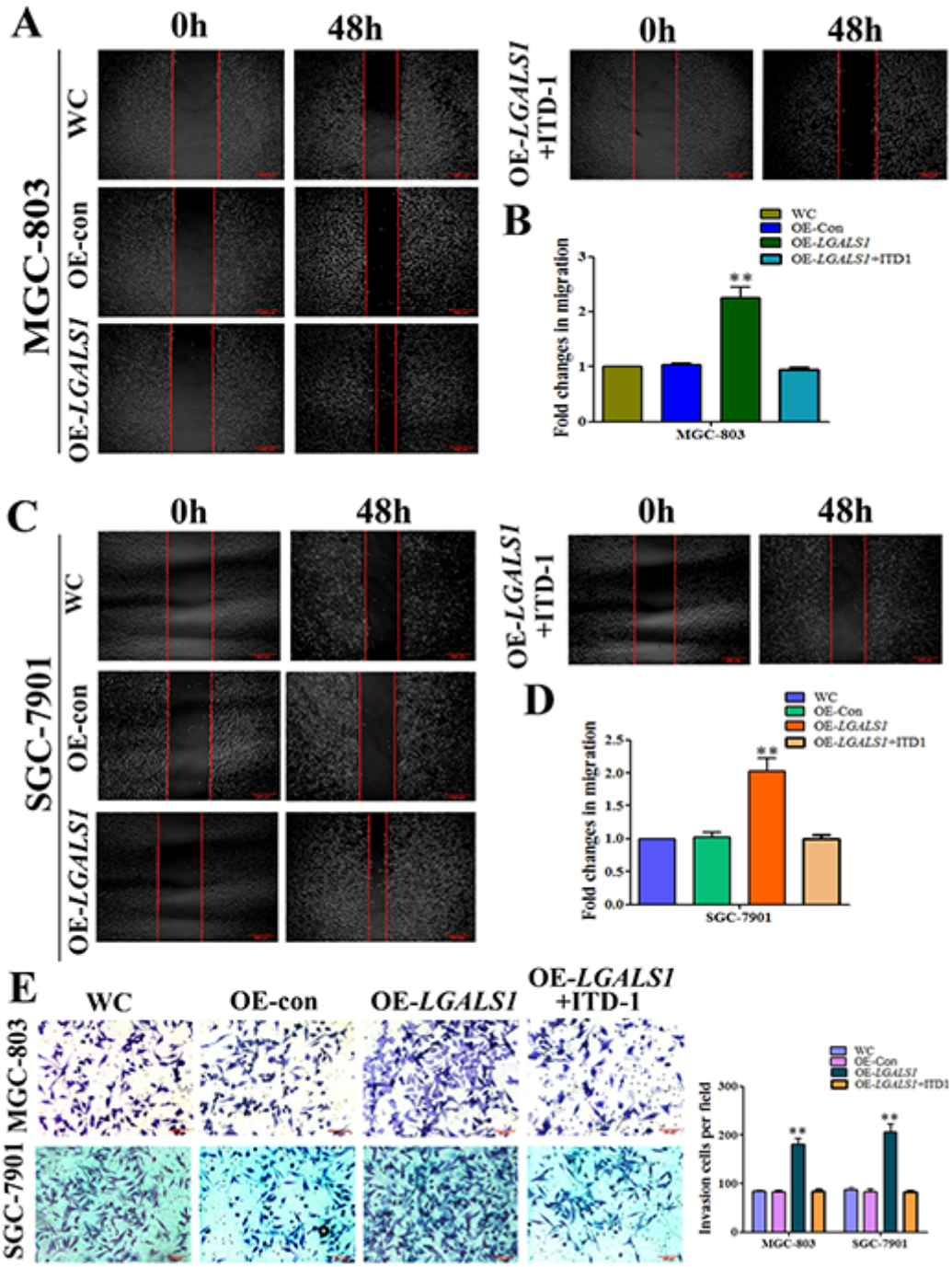

Figure 6

GAL-1/ LGALS1 promotes the migration and invasion of GC cells in vitro through TGF-β/Smad signaling pathways. (A-D). OE-LGALS1 significantly enhanced the migration capacity of MGC-803 and SGC-7901 cells compared with WC and OE-con. The migration capacity was abolished when the medium contained 10 μM ITD1 ($P < 0.01$). (E) Transwell assay showing that MGC-803 and SGC-7901 cells increased their invasive ability after transfection with LV-LGALS1-OE, and 10 μM ITD1 abolished this increase in invasive ability (n = 3)

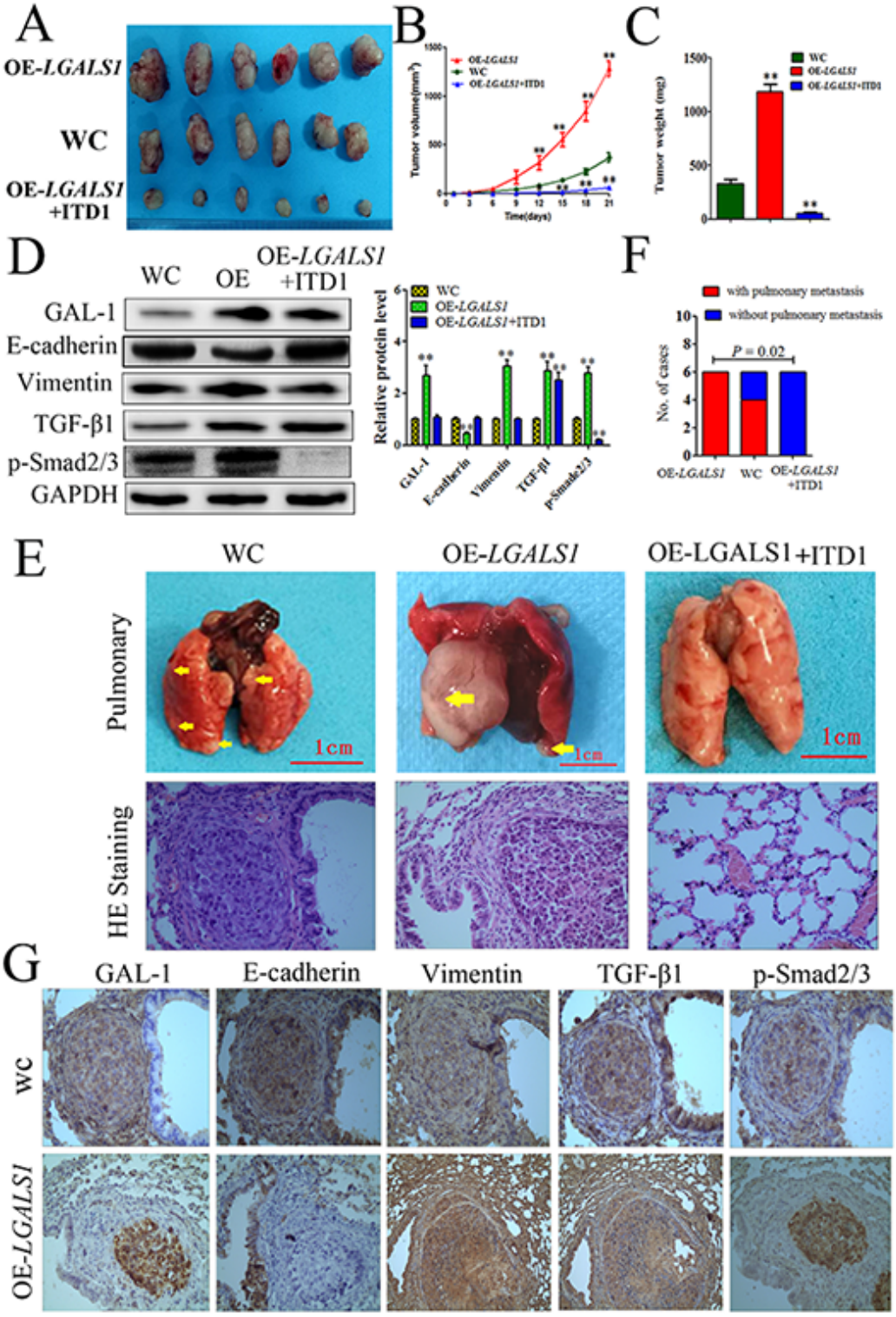

Figure 7

GAL-1/ LGALS1 promotes GC cell metastasis in vivo through the TGF β/Smad signaling pathway.(A) OE-LGALS1 induced MGC-803 to form subcutaneous xenograft tumors with larger volumes (B) and weighs (C) (expressed as the mean ± SE). * P <0.05, ** P< 0.01, n = 6. (D) OE-LGALS1 increased the levels of TGF-β1 and p-Smad2/3, and induced EMT in the subcutaneous xenograft tumor, ITD1 could inhibit this effect. (E) Representative images of metastasis (yellow arrows) in the lungs at 50 days after inoculation, and

representative images of H&E staining. Original magnification: ×400. Metastases were frequent in the (F). (G) Immunostaining showing GAL-1, E-cadherin, vimentin, TGF-β1 and p-Smad2/3 levels in pulmonary metastases. Magnification: ×400.