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Original Article

Galectin-1 Promotes Gastric Carcinoma Progression and Cisplatin Resistance Through the NRP-1/c-JUN/Wee1 Pathway

Zhengyang Pan [1,2,*], **Guoxi Xu** [3,*], **Yan Zhang** [2], **Meiling Wu** [4], **Jiahui Yu** [2], **Xujun He** [2], **Wei Zhang** [1], **Junfeng Hu** [4]

[1]Department of Gastrointestinal Surgery, Zhejiang Chinese Medical University, Hangzhou, China
[2]Cancer Center, Department of Genetic and Genomic Medicine, Zhejiang Provincial People's Hospital, Affiliated People's Hospital, Hangzhou Medical College, Hangzhou, China
[3]Department of Gastrointestinal Surgery, Jinjiang Hospital, Quanzhou, China
[34]General Surgery, Cancer Center, Department of Gastrointestinal and Pancreatic Surgery, Zhejiang Provincial People's Hospital, Affiliated People's Hospital, Hangzhou Medical College, Hangzhou, China

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Correspondence to
Junfeng Hu
General Surgery, Cancer Center, Department of Gastrointestinal and Pancreatic Surgery, Zhejiang Provincial People's Hospital, Affiliated People's Hospital, Hangzhou Medical College, 158 Shangtang Road, Gongshu District, Hangzhou, Zhejiang 310014, China.
Email: hjfeng234@126.com

Wei Zhang
Department of Gastrointestinal surgery, Zhejiang Chinese Medical University, 548 Binwen Road, Binjiang District, Hangzhou, Zhejiang 310053, China.
Email: zhangweils1968@163.com

Xujun He
Cancer Center, Department of Genetic and Genomic Medicine, Zhejiang Provincial People's Hospital, Affiliated People's Hospital, Hangzhou Medical College, 158 Shangtang Road, Gongshu District, Hangzhou, Zhejiang 310014, China.
Email: hxj0105099@163.com

*Zhengyang Pan, Guoxi Xu contributed equally to this work.

ABSTRACT

Purpose: Gastric cancer (GC) is among the deadliest malignancies and the third leading cause of cancer-related deaths worldwide. Galectin-1 (Gal-1) is a primary protein secreted by cancer-associated fibroblasts (CAFs); however, its role and mechanisms of action of Gal-1 in GC remain unclear. In this study, we stimulated GC cells with exogenous human recombinant galectin-1 protein (rhGal-1) to investigate its effects on the proliferation, migration, and resistance to cisplatin.
Materials and Methods: We used simulated rhGal-1 protein as a paracrine factor produced by CAFs to induce GC cells and investigated its promotional effects and mechanisms in GC progression and cisplatin resistance. Immunohistochemical (IHC) assay confirmed that Gal-1 expression was associated with clinicopathological parameters and correlated with the expression of neuropilin-1 (NRP-1), c-JUN, and Wee1.
Results: Our study reveals Gal-1 expression was significantly associated with poor outcomes. Gal-1 boosts the proliferation and metastasis of GC cells by activating the NRP-1/C-JUN/Wee1 pathway. Gal-1 notably increases GC cell resistance to cisplatin The NRP-1 inhibitor, EG00229, effectively counteracts these effects.
Conclusions: These findings revealed a potential mechanism by which Gal-1 promotes GC growth and contributes to chemoresistance, offering new therapeutic targets for the treatment of GC.

Keywords: Galectin-1; Gastric cancer; Cisplatin; Drug resistance

INTRODUCTION

Gastric cancer (GC) is among the deadliest malignancies and the third leading cause of cancer-related deaths worldwide [1]. Although advances in tumor treatment have led to positive outcomes in the management of GC, surgical treatment remains the primary and prevailing approach. However, most patients are typically diagnosed at advanced stages, missing the opportunity for surgery and resulting in a less than 5% 5-year survival rate [2].

ORCID iDs
Zhengyang Pan
https://orcid.org/0009-0004-4501-7049
Guoxi Xu
https://orcid.org/0000-0001-9755-1068
Yan Zhang
https://orcid.org/0009-0006-3023-1995
Meiling Wu
https://orcid.org/0009-0005-6973-5747
Jiahui Yu
https://orcid.org/0009-0005-4262-8052
Xujun He
https://orcid.org/0000-0002-6979-2072
Wei Zhang
https://orcid.org/0000-0001-8557-0800
Junfeng Hu
https://orcid.org/0009-0008-6875-5190

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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

Author Contributions
Conceptualization: H.X., Z.W., H.J.; Data curation: P.Z., X.G.; Formal analysis: P.Z., X.G., Z.Y., W.M., Y.J.; Investigation: P.Z., Z.Y., W.M., Y.J.; Supervision: H.X.; Writing - original draft: P.Z.; Writing - review & editing: H.J.

In recent years, the role of the tumor microenvironment (TME) in tumor proliferation and drug resistance has garnered increasing attention. As primary stromal cells in the TME, cancer-associated fibroblasts (CAFs) play a crucial role in tumor proliferation, invasion, migration, and drug resistance by producing soluble factors via paracrine mechanisms [2,3]. For example, the secretion of transforming growth factor (TGF)-β leads to cisplatin resistance in esophageal cancer, while paracrine exosomes affect the Wnt/β-catenin pathway in colorectal cancer, resulting in resistance to oxaliplatin and 5-fluorouracil [3,4]. Therefore, it is essential to further explore the paracrine components and their mechanisms in GC and CAFs.

Galectin-1 (Gal-1), encoded by the LGALS1 gene, is a 14-kDa lectin belonging to the galactoside family, with a specific affinity for β-galactosides. It facilitates cell-cell or cell-extracellular component communication by binding to glycol-conjugated proteins on the cell surface [5]. CAFs produce Gal-1 via secretory and paracrine pathways [2]. Gal-1 binds to tumor cell-glycogenated membrane receptors such as neuropilin-1 (NRP1), to regulate downstream signal transduction, which affects tumor cell adhesion, migration, invasion, tumor-induced angiogenesis, and apoptosis [6,7]. Furthermore, previous studies have shown that Gal-1 regulates signal transduction and gene transcription by interacting with tumor cell surface or nuclear proteins such as H-Ras, FOXP3, and Gemin4 [8-10]. However, the role of Gal-1 in GC remains unclear.

Currently, platinum-based chemotherapy remains one of the standard therapies for various cancers, including GC, colorectal cancer, and breast cancer [11,12]. Chemotherapy is effective in enhancing the survival and quality of life of patients with locally advanced, unresectable, or metastatic GC. Unfortunately, primary or secondary resistance remains a significant factor limiting the efficacy of chemotherapy [1]. The resistance mechanism in GC is complex and involves multiple factors, including inactivation of apoptosis signaling pathways, loss of cell cycle checkpoint control, accelerated cell proliferation, autophagy flux, enhanced DNA damage repair ability, reduced drug uptake, increased epithelial-mesenchymal transition (EMT) through upregulation of multiple drug resistance (MDR)-related proteins, and activation of cancer stem cells (CSCs) [13-16]. In addition, components of the tumor microenvironment, such as CAFs, play a significant role in chemotherapy resistance. However, the mechanism of cisplatin resistance in GC remains unclear, and further studies at the molecular level are needed to benefit a subset of patients with GC.

In the current study, we used simulated rhGal-1 protein as a paracrine factor produced by CAFs to induce GC cells and investigated its promotional effects and mechanisms in GC progression and cisplatin resistance. This study offers a new perspective and potential therapeutic target for GC by elucidating the molecular mechanisms of Gal-1 in GC proliferation, invasion, and cisplatin resistance.

MATERIALS AND METHODS

Cell lines and cell culture

The GC cell lines AGS and MKN-1 were obtained from the Chinese Academy of Science Cell Bank (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 μg/mL streptomycin at 37°C in a humidified incubator with 5% CO_2.

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The chemical inhibitors used were Galectin-1/LGALS1 (HY-P73072; MedChemExpress, Monmouth Junction, NJ, USA), EG00229 (HY-10799; MedChemExpress), and cisplatin (HY-17394; MedChemExpress).

Cell treatment

To determine whether Galectin-1 had an effect on gastric cells, the human Gal-1/LGALS1 (rhGal-1) group was treated with 25 or 50 ng/mL rhGal-1 for 48 hours. Then, the cells were treated with cisplatin (HY-17394, AGS, 40 μM; MKN-1, 20 μM; MedChemExpress) to assay chemotherapy-induced cytotoxicity. Subsequently, to investigate the role of NRP-1 in the process, special inhibitors of 3 μM EG00229 (HY-10799; MedChemExpress) were used 12 hours after rhGal-1 treatment for 48 hours.

We employed a Transwell noncontact co-culture system (6 well), with inserts with a 0.4 μm pore size polycarbonate membrane. A total of 1×10^5 of either human splenic fibroblast and GC cells were plated in the upper and lower chambers of the Transwells for 72 hours, respectively. Cells in the upper or lower chambers are unable to migrate through the 0.4 μm pores of the polycarbonate membrane, which creates a noncontact co-culture system.

Cell Counting Kit-8 (CCK-8) assay

The 5×10^3 AGS or MKN-1 cells were seeded in 96-well plates. After culturing for 24 hours at 37°C in 5% CO_2, allowed to attach overnight, and then the cells were divided into several groups with different treatments. Cell viability was determined using the CCK-8 assay (#C0037; Beyotime, Shanghai, China). CCK-8 reagent was added to each well, and the absorbance at 450 nm was measured using a microplate reader.

Bromodeoxyuridine (BrdU) staining assay

BrdU labeling and immunostaining were used to assess spatial and temporal changes in cellular proliferation. GC cells were seeded onto a cavity slide (CS-08-TC; LABSELECT, Hefei, China) at a density of 5×10^4 cells/well. After different treatments, the GC cells were treated with 10 μL/mL BrdU for 12 hours and then fixed with 4% paraformaldehyde. After blocking with 5% normal goat serum for 1 hour, the slides were incubated overnight with BrdU primary antibodies (1:50, sc-32323; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cell nucleus was stained with DNA binding reagent 4′,6-diamidino-2-phenylindole (DAPI). Immunofluorescent images of the cells were captured and photographed using a high-resolution microscope (Nikon, Tokyo, Japan).

Cell invasion and wound healing assays

For the cell invasion assay, 5×10^4 cells were suspended in 200 μL of DMEM without serum and were placed in the upchamper inserts which were precoated with different treatments. Culture medium containing 20% fetal bovine serum was added to each well. Then, the cells were incubated in the incubator for 48 hours at 37°C in 5% CO_2. The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), stained with 0.3% Crystal Violet Staining Solution (#C0121; Beyotime), and counted under a light microscope (Nikon) in 3 randomly selected areas.

For the wound healing assay, cells were seeded in 6-well plates and scratched with sterile 200 μL pipette tips to artificially create wounds. After 48 hours of treatment, wound healing was observed and photographed.

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Western blot

Briefly, the cells were homogenized in RIPA lysis buffer (#P0013B; Beyotime) with a protease inhibitor cocktail (#P1005; Beyotime) on ice. A BCA protein assay kit (Thermo Fisher Scientific, Boston, MA, USA) was used to measure protein concentration. Proteins were fractionated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis and electrotransferred onto polyvinylidene fluoride membranes using the semi-dry transfer method. The membranes were blocked with 5% bovine serum albumin for 2 hours, then incubated overnight with primary antibodies at 4°C. After extensive washing, sections were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000; Huaan Biotech, Hangzhou, China) for 2 hours at room temperature. Enhanced chemiluminescence (Generay, Shanghai, China) was added to the membranes and Bio-Rad ChemiDoc-XRS with Image Lab software (Bio-Rad, Richmond, CA, USA) was used to expose and acquire images. Relative protein expression was analyzed using the Image-Pro Plus and ImageJ software (MEDIA Systems, Bethesda, MD, USA).

The primary antibodies used were against: NRP-1 (1:2,000, ET1609-69; Huabio, Hangzhou, China); GADPH (1:1,000, #5174; Cell Signaling Technology, Danvers, MA, USA); β-Tubulin (1:1,000, #2144; Cell Signaling Technology); c-JUN (1:1,000, #9165; Cell Signaling Technology); Wee1 (1:1,000, #13084; Cell Signaling Technology); Capase3 (1:1,000, #14220; Cell Signaling Technology); Bcl-2 (1:1,000, #4223; Cell Signaling Technology); α-SMA (1:1,000, HA600032; Huabio); and Vimentin (1:1,000, ET1610-39; Huabio).

RNA isolation and quantitative real-time polymerase chain reaction (PCR) analysis

Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total of 500 ng of RNA was reverse transcribed into cDNA using an oligo (dT) primer and then amplified with a specified primer. Then cDNA amplified with SYBR Green Real-time PCR Master Mix (Yeasen, Shanghai, China). The PCR primers used are listed in **Supplementary Table 1**.

TUNEL assay

Cells were stained using the One Step TUNEL Apoptosis Assay Kit (#C1086; Beyotime) according to the manufacturer's protocol. Immunofluorescent images of the cells were captured and photographed using a high-resolution microscope (Nikon).

Dual-luciferase reporter assay

The dual-luciferase reporter assay was performed as previous report [17]. HEK293T cells were used for the dual-luciferase reporter assay, and all luciferase reporter vectors were constructed by Shanghai RiboBio Company (Shanghai, China). All luciferase assays were performed in at least 3 independent experiments, each consisting of 3 wells per transfection.

Immunohistochemical (IHC) staining

GC specimens were acquired from the Zhejiang Provincial People's Hospital (Hangzhou, China) between 2010 and 2019. None of the patients had received any radiotherapy or chemotherapy in advance of the operation. The project was approved by the Ethics Committee of the Zhejiang Provincial People's Hospital. IHC staining and quantification of staining intensity were performed at the Department of Pathology of Zhejiang Provincial People's Hospital according to standard protocols.

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The following primary antibodies were used: anti-Gal-1 (1:250, #6837-1; Epitomics, Burlingame, CA, USA), c-JUN (1:200, #9165; Cell Signaling Technology), Wee1 (1:150, #13084; Cell Signaling Technology), and NRP-1 (1:200, ET1609-69; Huabio).

Xenograft mouse model

For subcutaneous tumor xenografts, an estimated 5×10^6 MKN-1 cells in 0.1 mL PBS (control, Gal-1-treated alone, and the combination of Gal-1 and EG00229 treated for 72 hours) were subcutaneously injected into the right axillary region of a 4-week-old female nude mouse. Fourteen days later, the mice were intraperitoneally injected with 10 mg/kg cisplatin twice weekly for 2 weeks (twice a week). After 14 days of treatment, the tumors were collected, and the tumor diameters were measured and photographed.

Bioinformatics

GC RNA sequencing data were downloaded from The Cancer Genome Atlas (TCGA-STAD, 375 tumor samples; https://portal.gdc.cancer.gov/). The differentially expressed genes were identified by the "limma" package ($P<0.01$, and $|\text{fold-change}| \geq 2$).

Statistical analysis

All in vitro functional assays were analyzed by the Student's test for statistical significance using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) or SPSS 12.0 software (SPSS Inc., Chicago, IL, USA) Spearman's ρ-test was used to assess the staining correlation. Statistical analyses of patient and clinical data were performed using Fisher's exact tests. Data are expressed as the mean ± standard error of the mean. Statistical significance was set at $P<0.05$ significant.

RESULTS

Gal-1 promotes the proliferation, invasion, and migration of GC cell lines

To investigate Gal-1 as a positive regulator of cancer tumor growth and progression, we sought to experimentally confirm this possibility. As demonstrated by CCK-8, BrdU proliferation, and colony formation assays, the proliferation of AGS and MKN-1 cells treated with rhGal-1 was significantly higher than that in the untreated control group (**Fig. 1A-C**). Simultaneously, wound healing and invasion assays demonstrated that rhGal-1 significantly promoted the invasion and migration of AGS and MKN-1 cells compared to those in the untreated control group (**Fig. 1D-F**). Therefore, Gal-1 promotes the proliferation, invasion, and migration of GC cells.

Gal-1 activates NRP-1/c-JUN/Wee1 signaling in GC cells

To probe the molecular mechanism of Gal-1 regulation in GC cells, we divided patients with GC into high- and low-expression groups based on LGALS1 (Gal-1) expression data from TCGA. We conducted Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of the genes associated with differentially expressed genes (DEGs) between the high LGALS1 expression and low LGALS1 expression groups. KEGG enrichment analysis revealed that these DEGs were primarily associated with the "Cell cycle" (**Fig. 2A**). We also found a significant association between LGALS1 and c-JUN expression using Gene Expression Profiling Interactive Analysis (GEPIA) ($P=0.002$, $R=0.15$; **Fig. 2B**). Besides, c-JUN expression showed a significant association with Wee-1 expression in GEPIA ($P<0.0001$, $R=0.2$; **Fig. 2B**).

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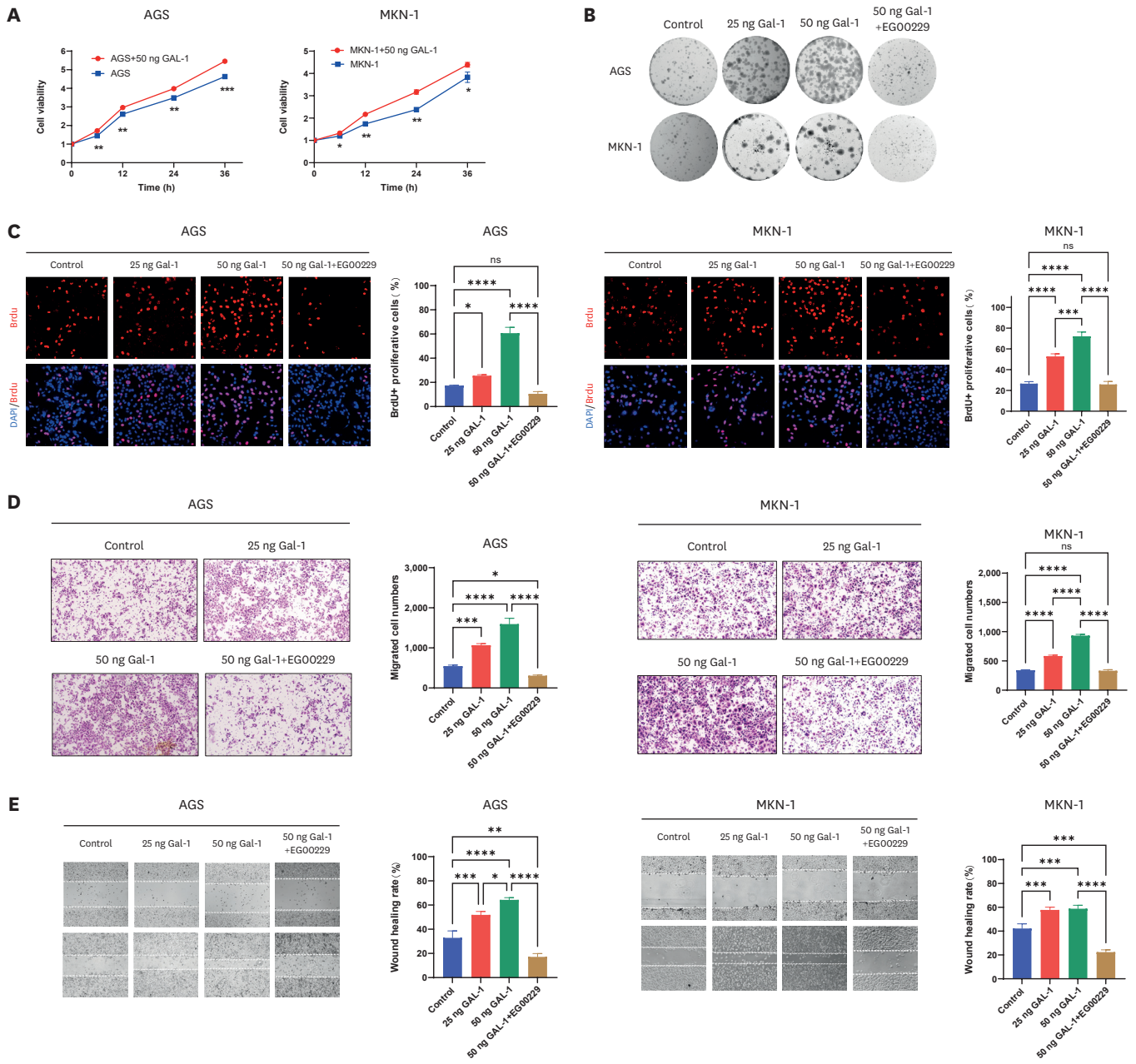

Fig. 1. Exogenous Gal-1 promotes the proliferation and invasion of GC cells and the capacity can be reversed by NRP-1 inhibitor (EG00229). (A) CCK8 assay showed Gal-1-treated AGS and MKN-1 (group was treated with 50 ng/mL rhGal-1 for 48 hours) had stronger cell growth ability than untreated cells (n = 3). (B) Gal-1-treated AGS and MKN-1 cells (group was treated with 25 or 50 ng/mL rhGal-1 for 48 hours) showed stronger colony formation ability than untreated cells. And this effect was inhibited by treating with EG00229 (3 μM) compared with the 50 ng/mL Gal-1-treated group (n=3). (C) BrdU staining assay showing AGS and MKN-1 cells treated with Gal-1 exhibited a significantly enhanced proliferation capacity compared with the wild-type control. The proliferation capacity was abolished when the medium contained 3 μM EG00229 (n=3). Magnification: ×200. (D, E) Transwell assay and Wound-healing assay showing that AGS and MKN-1 cells treated with Gal-1 had increased cell invasion and migration abilities, and 3 μM EG00229 abolished this increase in invasive ability (n=3). Magnification: ×40.
Gal-1 = galectin-1; GC = gastric cancer; NRP-1 = neuropilin-1; CCK-8 = Cell Counting Kit-8; BrdU = Bromodeoxyuridine.
Statistical significance was assessed by t-test; *P<0.05, **P<0.01, ***P<0.005, ****P<0.001.

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NRP-1, which serves as a receptor for the secreted Gal-1 protein, is upregulated in various types of tumors [18]. CAFs promoted the migration of GC cells, and this effect was significantly inhibited by the NRP-1 inhibitor (EG00229) (**Supplementary Fig. 1**). The mRNA levels of NRP-1, c-JUN, and Wee1 were upregulated after treatment with Gal-1 through quantitative real-time PCR (qRT-PCR).

In addition, the inhibition of NRP-1 (EG00229) effectively reversed this situation. The mRNA levels of other c-JUN/Wee1 downstream pathway genes, CDH2/Vim (EMT-related protein), were upregulated after treatment with Gal-1, and inhibition of NRP-1 (EG00229) effectively reversed this effect. The c-JUN/Wee1 downstream pathway genes CDK1/TP53/CCNB1/CDH1/MMP9/CCND1 showed no significant differences (**Supplementary Fig. 2**).

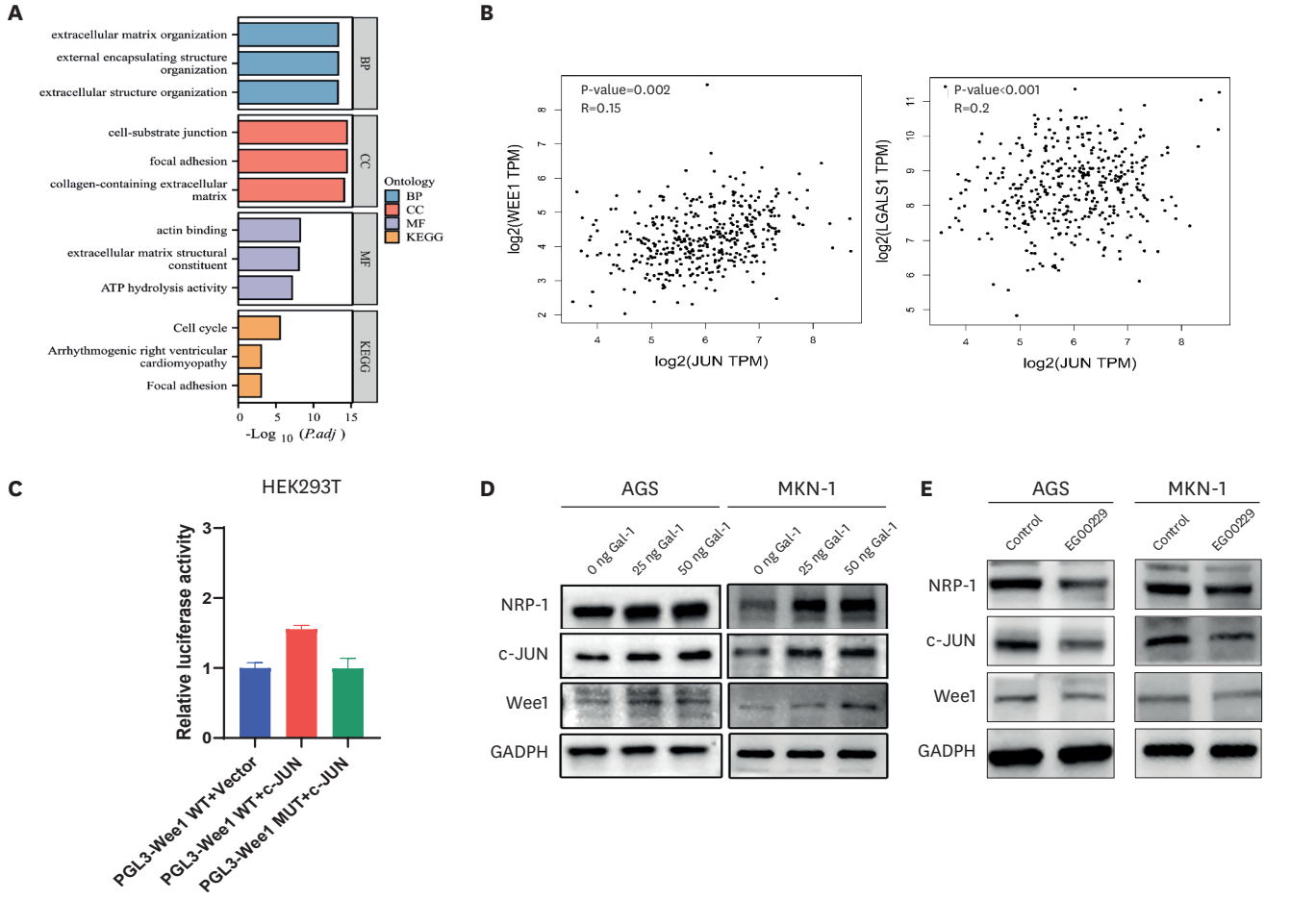

Fig. 2. Gal-1 promotes GC cell metastasis in vivo through the NRP-1/c-JUN/Wee1 signaling pathway. (A) The enrichment of KEGG pathways and GO terms among differentially expressed genes. The DEGs was determined by GC patients divided by LGALS1 expression into high and low group using expression data from TCGA (n=375). (B) The GC patients correlation between the mRNA expression of LGALS1 and c-JUN in GEPIA. The GC patients correlation between the mRNA expression of c-JUN and Wee1 in GEPIA. (C) Relative luciferase reporter assays in HEK293 cells co-transfected with plasmid constructs containing the Wee1 promoter with a c-JUN-overexpressing construct or control construct. The result confirmed the correlation between c-JUN and Wee1. (D) Western blot showing that Gal-1 efficiently increased the expression of NRP-1, c-JUN, and Wee1 in AGS and MKN-1 cells compared with untreated cells. (E) Western blot showing NRP-1 inhibitor (3 μM EG00229) efficiently decreased the levels of protein NRP-1, c-JUN, and Wee1 in AGS and MKN-1 cells. (F) Immunofluorescence staining showing Gal-1 efficiently increased the protein expression of NRP-1, c-JUN, and Wee1 in AGS and MKN-1 cells compared with untreated cells. And this effect was inhibited by treating with EG00229 (3 μM).
Data are presented as the means ± standard error of the mean.
Gal-1 = galectin-1; GC = gastric cancer; NRP-1 = neuropilin-1; KEGG = Kyoto Encyclopedia of Genes and Genomes; GO = Gene Ontology; DEGs = differentially expressed genes; TCGA = The Cancer Genome Atlas; GEPIA = Gene Expression Profiling Interactive Analysis.
*P<0.05, **P< 0.01, ***P<0.005, ****P<0.001.

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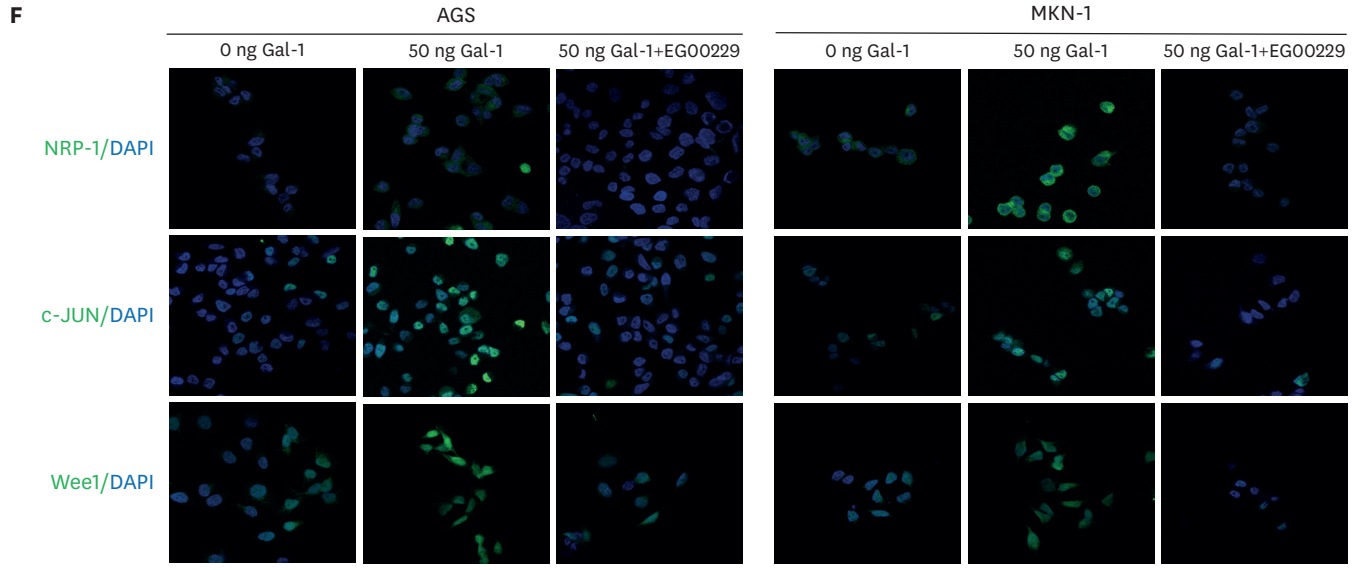

Fig. 2. (Continued) Gal-1 promotes GC cell metastasis in vivo through the NRP-1/c-JUN/Wee1 signaling pathway. (A) The enrichment of KEGG pathways and GO terms among differentially expressed genes. The DEGs was determined by GC patients divided by LGALS1 expression into high and low group using expression data from TCGA (n=375). (B) The GC patients correlation between the mRNA expression of LGALS1 and c-JUN in GEPIA. The GC patients correlation between the mRNA expression of c-JUN and Wee1 in GEPIA. (C) Relative luciferase reporter assays in HEK293 cells co-transfected with plasmid constructs containing the Wee1 promoter with a c-JUN-overexpressing construct or control construct. The result confirmed the correlation between c-JUN and Wee1. (D) Western blot showing that Gal-1 efficiently increased the expression of NRP-1, c-JUN, and Wee1 in AGS and MKN-1 cells compared with untreated cells. (E) Western blot showing NRP-1 inhibitor (3 μM EG00229) efficiently decreased the levels of protein NRP-1, c-JUN, and Wee1 in AGS and MKN-1 cells. (F) Immunofluorescence staining showing Gal-1 efficiently increased the protein expression of NRP-1, c-JUN, and Wee1 in AGS and MKN-1 cells compared with untreated cells. And this effect was inhibited by treating with EG00229 (3 μM).
Data are presented as the means ± standard error of the mean.
Gal-1 = galectin-1; GC = gastric cancer; NRP-1 = neuropilin-1; KEGG = Kyoto Encyclopedia of Genes and Genomes; GO = Gene Ontology; DEGs = differentially expressed genes; TCGA = The Cancer Genome Atlas; GEPIA = Gene Expression Profiling Interactive Analysis.
*P<0.05, **P< 0.01, ***P<0.005, ****P<0.001.

Hence, we hypothesized that the NRP-1/c-JUN/Wee1 pathway also plays a crucial role in GC. As depicted in **Fig. 2D and F**, following rhGal-1 stimulation, the expression of NRP-1 increased, and the expression of c-JUN and Wee1 was significantly upregulated in GC cells. Luciferase reporter assays confirmed the correlation between c-JUN and Wee1 (**Fig. 2C**). This finding agrees with that reported by Zhu et al. [19] and Li et al. [20]. To further confirm whether the activation of the NRP-1/c-JUN/Wee1 signaling pathway affects the proliferation, metastasis of GC. We utilized the NRP-1 inhibitor EG00229 to suppress the expression of NRP-1. Simultaneously, the levels of c-JUN and Wee1 protein expression significantly decreased in AGS and MKN-1 cells after the suppression of NRP-1 expression (**Fig. 2E and F**). In vitro cell experiments using CCK-8, BrdU, Transwell, scratch, and cloning assays demonstrated that EG00229 effectively inhibited the proliferation, invasion, and migration of GC cells stimulated by Gal-1 (**Fig. 1B-F**). These data suggest that Gal-1 promotes GC progression via the NRP-1/c-JUN/Wee1 signaling pathway in GC cells.

Gal-1 positive correlated with NRP-1/c-JUN/Wee1 expression in GC

We further confirmed the correlation between Gal-1, NRP-1, c-JUN, and Wee1 expression in GC tissues by IHC staining. Gal-1 was expressed in CAFs, whereas NRP-1, c-JUN, and Wee1 were expressed in tumor cells (**Fig. 3C and D**). Gal-1 expression was positive in 43 of 86 tumor samples (50.0%). Gal-1 expression was significantly associated with lymph node metastasis, vascular invasion, distant metastasis, lymphatic invasion, and TNM stage of the clinicopathologic parameters in GC (P<0.05; **Table 1**). Spearman's ρ-test shown a positive correlation between Gal-1, NRP-1, c-JUN and Wee1(R=0.279, P=0.009; R=0.424, P=0.000;

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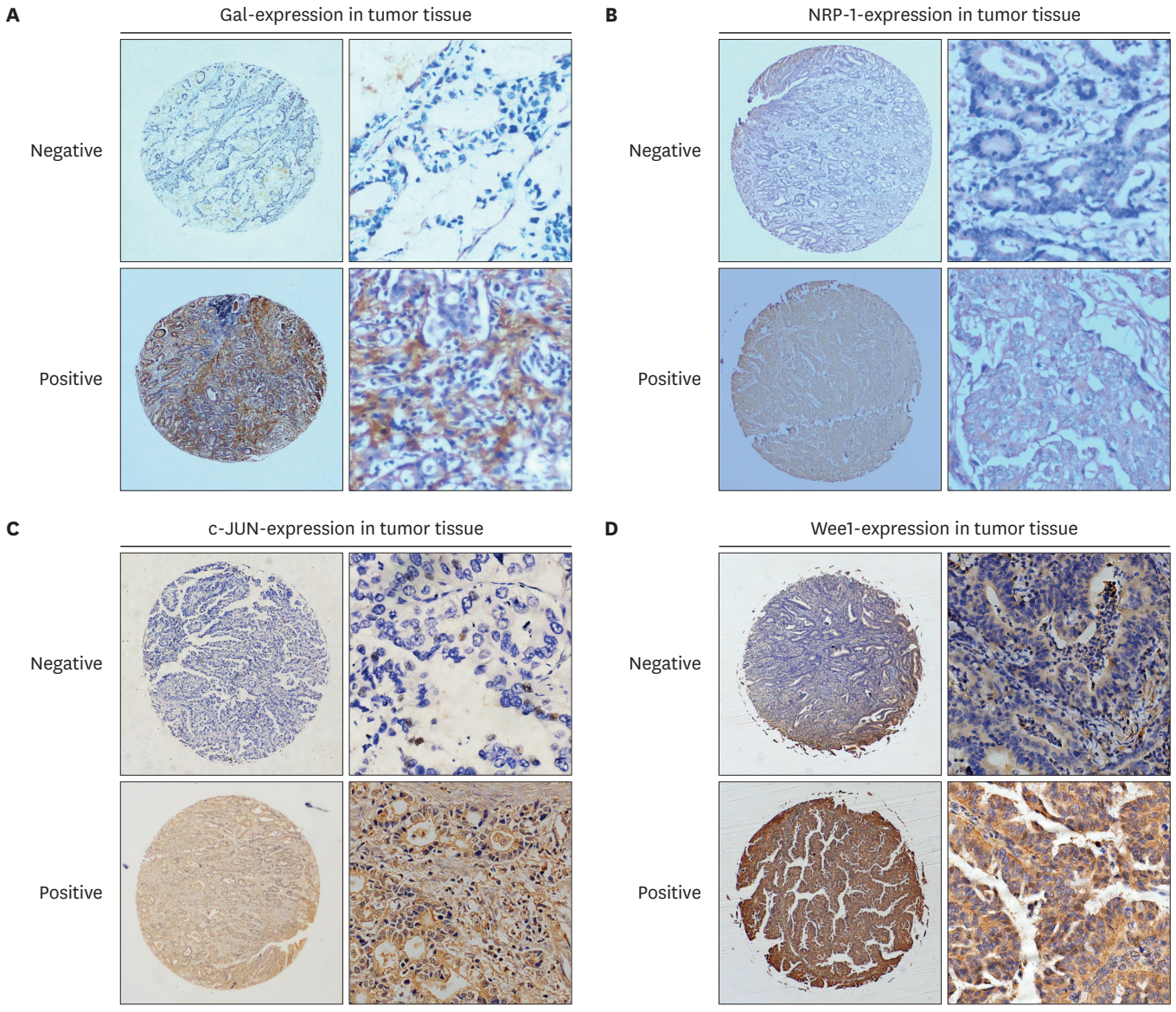

Fig. 3. Immunohistochemical staining for Gal-1, NRP-1, c-JUN, and Wee1 in GC tissues. (A) Negative and positive Gal-1 staining in tumor tissue. (B) Negative and positive NRP-1 staining in tumor tissue. (C) Negative and positive c-JUN staining in tumor tissue. (D) Negative and positive Wee1 staining in tumor tissue. Magnification: ×10, ×200.
Gal-1 = galectin-1; NRP-1 = neuropilin-1; GC = gastric cancer.

R=0.305, P=0.004; R=0.548, P=0.000, respectively; **Tables 2-5**). In conclusion, the findings above indicate that Gal-1 plays a pivotal role in the onset and progression of GC via its influence on the NRP-1/c-JUN/Wee1 pathway.

Gal-1 reduces sensitivity to cisplatin therapy in vitro GC and inhibition of NRP-1 (EG00229) can effectively reverse cisplatin resistance in GC

To demonstrate that Gal-1 stimulation enhances cisplatin resistance in GC cells, we treated GC cell lines stimulated with rhGal-1 and an untreated control group with varying doses of cisplatin. Compared with the control group, rhGal-1 stimulation significantly enhanced the proliferation of GC cells after cisplatin treatment, indicating that rhGal-1 promoted cisplatin resistance in GC cells (**Fig. 4A-E**). Transwell cell invasion assays and scratch healing

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Table 1. Relationship between Gal-1 and clinicopathologic features in 86 gastric cancer patients

Groups	No. of cases	Cases positive for Gal-1	P-value
Sex			0.118
Male	54	23 (42.6)	
Female	32	20 (62.5)	
Tumor diameter			0.380
<5 cm	51	23 (45.1)	
≥5 cm	35	20 (57.1)	
Differentiation			0.193
Well	2	2 (100.0)	
Moderate	38	16 (42.1)	
Poor	46	25 (54.3)	
Histological classification			0.951
Papillary adenocarcinoma	4	2 (50.0)	
Tubular adenocarcinoma	66	32 (48.5)	
Mucinous adenocarcinoma	2	1 (50.0)	
Signet-ring cell carcinoma	14	8 (57.1)	
Lauren classification			0.388
Diffuse	41	23 (56.1)	
Intestinal	45	20 (44.4)	
Lymph node metastasis			0.000
Yes	61	38 (62.3)	
No	25	5 (20.0)	
Distant metastasis			0.049
Yes	11	9 (81.8)	
No	75	34 (45.3)	
Vascular invasion			0.050
Yes	23	16 (69.6)	
No	63	27 (42.9)	
Lymphatic invasion			0.000
Yes	59	38 (64.4)	
No	27	5 (18.5)	
TNM stage			0.003
I	17	2 (11.8)	
II	16	8 (50.0)	
III	41	24 (58.5)	
IV	12	9 (75.0)	

Gal-1 = galectin-1; TNM = tumor, node, metastasis.

Table 2. Correlation between Gal-1 and NRP-1 expression in 86 gastric cancer patients

	NRP-1 expression		Spearman's ρ-test	
	NRP-1 (–)	NRP-1 (+)	R-value	P-value
Gal-1 expression				
Gal-1 (–)	27	16	0.279	0.009
Gal-1 (+)	15	28		

Gal-1 = galectin-1; NRP-1 = neuropilin-1.

Table 3. Correlation between Gal-1 and c-JUN expression in 86 gastric cancer patients

	c-JUN expression		Spearman's ρ-test	
	c-JUN (–)	c-JUN (+)	R-value	P-value
Gal-1 expression				
Gal-1 (–)	27	16	0.424	0.000
Gal-1 (+)	9	34		

Gal-1 = galectin-1

experiments demonstrated that rhGal-1 stimulation attenuated the inhibitory effects of cisplatin on the invasion and metastasis of GC cells (**Fig. 4F**). TUNEL staining also showed that rhGal-1 treatment weakened the effect of cisplatin on apoptosis (**Fig. 5A and B**), reflected by decreased expression of the apoptosis proteins Caspase-3 and increased expression of the anti-apoptosis

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Table 4. Correlation between Gal-1 and Wee1 expression in 86 gastric cancer patients

	Wee1 expression		Spearman's ρ-test	
	Wee1 (–)	Wee1 (+)	R-value	P-value
Gal-1 expression				
Gal-1 (–)	25	18	0.305	0.004
Gal-1 (+)	12	31		

Gal-1 = galectin-1

Table 5. Correlation between c-JUN and Wee1 expression in 86 gastric cancer patients

	Wee1 expression		Spearman's ρ-test	
	Wee1 (–)	Wee1 (+)	R-value	P-value
c-JUN expression				
c-JUN (–)	27	9	0.548	0.000
c-JUN (+)	10	40		

protein Bcl-2 compared with the untreated group in the presence of cisplatin (**Fig. 5C and D**). Moreover, EG00229 partially rendered GC cells sensitive to cisplatin (**Figs. 4A-4H**, **5A-5D**). In conclusion, Gal-1 stimulation effectively improved the resistance of GC cells to cisplatin, and drug resistance can be effectively reversed this drug EG00229.

Gal-1 reduces sensitivity to cisplatin therapy in vivo and the capacity can be reversed by the NRP-1 inhibitor (EG00229)

In vitro experiments demonstrated that Gal-1/LGALS1 reduced the sensitivity of GC cells to cisplatin therapy, as well as their invasion and migration, through the NRP-1/c-JUN/Wee1 signaling pathway, and this effect could be reversed by EG00229. Subcutaneous GC implantation models were established using athymic mice (n=6 per group). Fourteen days later, the mice were intraperitoneally injected with 10 mg/kg cisplatin twice weekly for 2 weeks (twice a week). After 21 days after the subcutaneous implantation of GC cells, the Gal-1 treatment group exhibited larger tumors than the untreated group. Significantly smaller tumors were formed by cells treated with a combination of Gal-1 and EG00229 than by those formed in the Gal-1-treated alone group (**Fig. 5E and F**). These results further confirmed the important role of Gal-1 in cisplatin resistance in GC and that EG00229 potentially reversed this process.

DISCUSSION

The TME encompasses a range of cell types, including fibroblasts/myofibroblasts, vascular endothelial cells, immune/inflammatory cells, and lymph endothelial cells, all of which are intricately associated with tumor proliferation, angiogenesis, and immunosuppression during tumor initiation and metastasis [21]. CAFs represent a significant component in numerous tumors, exerting influence on cancer progression and chemotherapy resistance through the secretion of various growth factors and soluble proteins, including IL-6, IL-8, TGF-β, and others [22]. For example, CAFs can secrete IL-6 and IL-8, thereby promoting the generation of tumor stem cells and consequently influencing both tumor initiation and resistance to chemotherapy [22]. The secretion of lysophosphatidylcholine targets pancreatic ductal adenocarcinoma, leading to the activation of AKT, thus facilitating tumor progression [23]. TGF-β-stimulated CAFs secreted IL-11 to activate GP130/STAT3 in colon cancer and promote tumor metastasis [24]. This suggests that CAFs employ various paracrine mechanisms to modify the signaling and behavior of cancer cells and that cisplatin serves as the cornerstone of first-line chemotherapy for the majority of tumors. Cisplatin resistance

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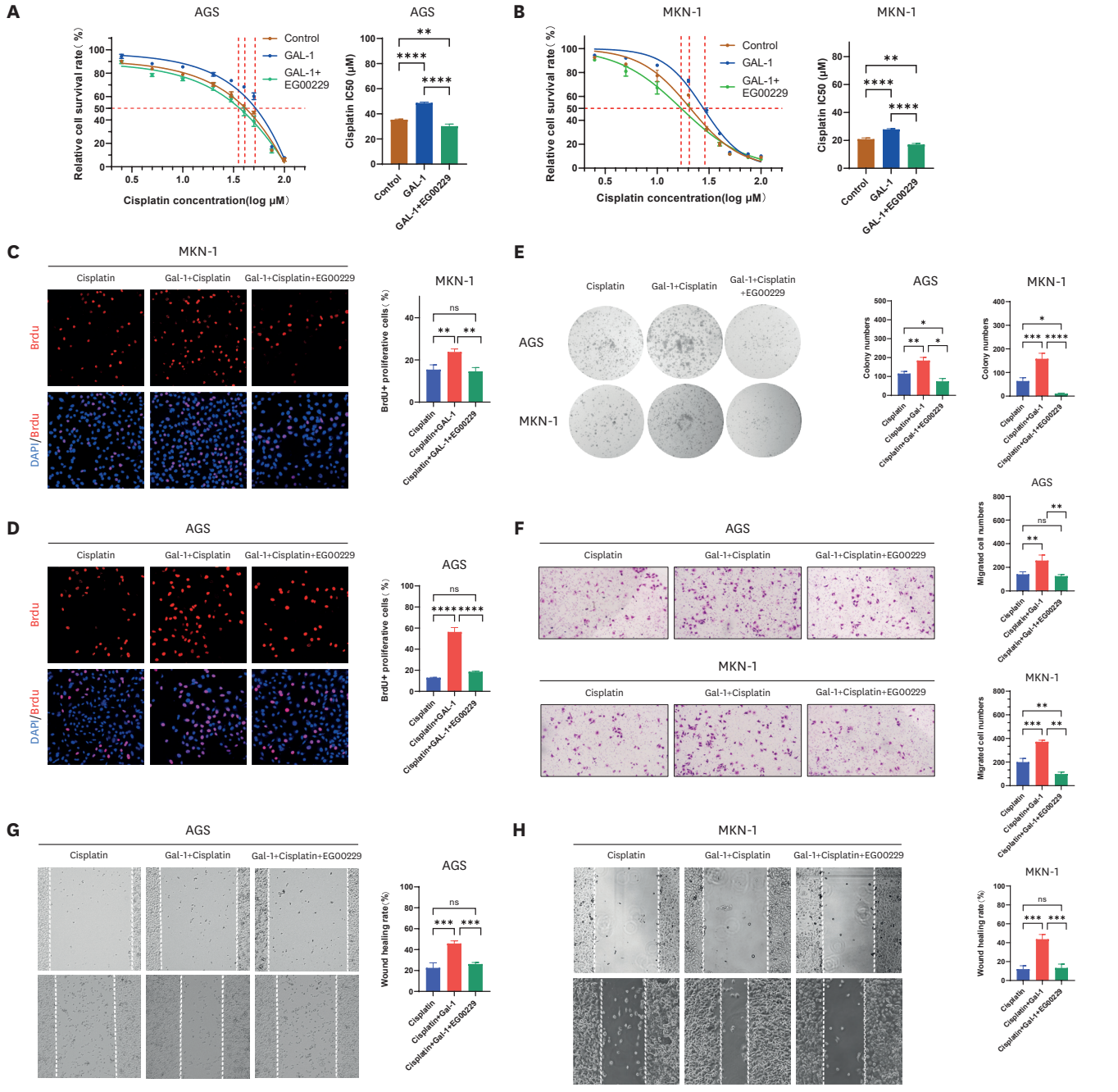

Fig. 4. Gal-1 reduced the sensitivity of GC cells to cisplatin and the capacity can be reversed by NRP-1 inhibitor (EG00229). (A, B) The IC50 value of control, Gal-1-treated alone (treated with 50 ng/mL rhGal-1 for 48 hours), and the combination of Gal-1 and EG00229 treated with 50 ng/mL rhGal-1 for 48 hours and 3 μM EG00229 for 12 hours) in AGS and MKN-1 cells were measured after treated with cisplatin for 48 hours. (C, D) BrdU staining was performed control, Gal-1-treated alone (treated with 50 ng/mL rhGal-1 for 48 hours), and the combination of Gal-1 and EG00229 (treated with 50 ng/mL rhGal-1 for 48 hours and 3 μM EG00229 for 12 hours) in AGS and MKN-1 cells were exposed to cisplatin (AGS, 40 μM; MKN-1, 20 μM) for 24 hours. Magnification: ×200. (E) Control, Gal-1-treated alone (treated with 50 ng/mL rhGal-1 for 48 hours, and the combination of Gal-1 and EG00229 treated with 50 ng/mL rhGal-1 for 48 hours and 3 μM EG00229 for 12 hours) in AGS and MKN-1 cells were exposed to cisplatin (AGS, 40 μM; MKN-1, 20 μM) for 24 hours. The cells were trypsinized and collected. One thousand cells from each treatment group were reseeded in 6-well plates. A clonogenic assay was performed. (F-H) The migration and invasion of AGS and MKN-1 cells after untreated, Gal-1-treated alone (treated with 50 ng/mL rhGal-1 for 48 hours, and the combination of Gal-1 and EG00229 treated with 50 ng/mL rhGal-1 for 48 hours and 3 μM EG00229 for 12 hours)were exposed to cisplatin (AGS, 40 μM; MKN-1, 20 μM) for 48 hours. Magnification: ×40.
Data are presented as the means ± standard error of the mean.
Gal-1 = galectin-1; GC = gastric cancer; NRP-1 = neuropilin-1; IC50 = half maximal inhibitory concentration; BrdU = Bromodeoxyuridine.
*P<0.05, **P<0.01, ***P<0.005, ****P<0.001.

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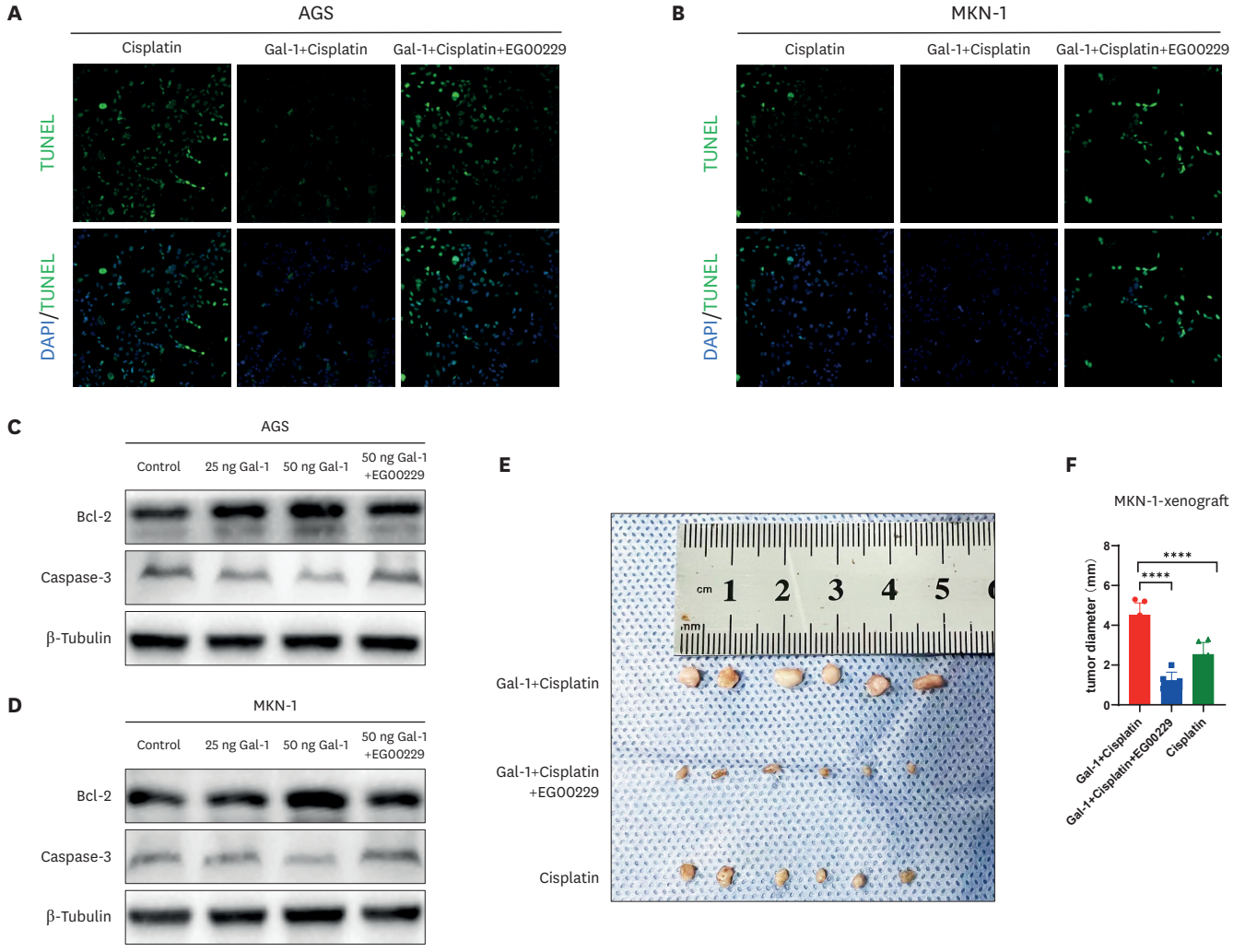

Fig. 5. Gal-1 attenuated the apoptotic effect of cisplatin on GC cells and the capacity can be reversed by NRP-1 inhibitor (EG00229). (A, B) TUNEL staining in AGS and MKN-1 cells after untreated, Gal-1-treated alone (treated with 50 ng/mL rhGal-1 for 48 hours), and the combination of Gal-1 and EG00229 treated with 50 ng/mL rhGal-1 for 48 hours and 3 μM EG00229 for 12 hours)were exposed to cisplatin for 48 hours. Magnification: ×200. (C, D) Western blot showing that Gal-1-treated AGS and MKN-1 cells efficiently increased the expression of anti-apoptosis proteins Bcl-2 compared with untreated group in the presence of cisplatin for 48 hours. Western blot showing that Gal-1-treated AGS and MKN-1 cells efficiently decreased the expression of apoptosis proteins Caspase-3 compared with untreated group in the presence of cisplatin for 48 hours. And the capacity can be reversed by EG00229. (E, F) Gal-1 induced MKN-1 to form subcutaneous xenograft tumors with larger volumes with cisplatin. EG00229 could inhibit this effect.
Gal-1 = galectin-1; GC = gastric cancer; NRP-1 = neuropilin-1.

often plays a crucial role in determining the prognosis of patients [11]. Among them, CAFs are pivotal in influencing drug resistance within the tumor microenvironment. However, the precise mechanisms by which CAFs affect tumor drug resistance via paracrine pathways remain unclear [25].

Gal-1 is a soluble protein secreted by CAFs and has been reported to play a significant regulatory role in cancer tumor growth and progression [26,27], but the mechanism underlying the effect of exogenous Gal-1 on GC cells remains unclear. In the present study, we found that exogenous Gal-1 stimulation enhanced the proliferation and metastasis of GC cells. GC cells stimulated with Gal-1 displayed reduced sensitivity to cisplatin, attenuating the apoptotic effect of cisplatin on GC. Moreover, patients with GC with elevated intermediate Gal-1 expression in CAFs have an increased incidence of lymph node metastasis, vascular

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invasion, distant metastasis, lymphatic invasion, and a more advanced TNM stage. These findings suggest that CAFs promote GC progression via paracrine Gal-1.

Previous studies have demonstrated that Gal-1 regulates cell proliferation and repair by interacting with the NRP-1 receptor [28]. And the c-JUN protein, a component of the activated protein-1 (AP-1) transcription complex and the most transcriptionally active factor within the AP-1 complex, has been reported to impact tumor proliferation, metastasis, differentiation, and apoptosis [29]. Wee1 belongs to the cyclin-dependent protein kinase family. It binds to terminal phosphorylation sites and inactivates cyclin B, leading to cell cycle arrest in G2 phase cells in response to DNA damage. This process is closely associated with drug resistance during tumor cell proliferation. Using a luciferase assay, we demonstrated that c-JUN could bind to the Wee1 promoter, thereby regulating the expression of Wee1. This finding agrees with that reported by Li J et al. [20]. In our in vitro experiment, we observed that treatment with rhGal-1 significantly upregulated the expression of NRP-1 receptor proteins c-JUN and Wee1 [30]. Notably, this effect was counteracted by the NRP-1 inhibitor (EG00229). Furthermore, immunohistochemistry confirmed a positive correlation between Gal-1 and NRP-1/C-JUN/Wee1 in GC. Simultaneously, NRP-1 inhibitors suppressed the proliferation and metastasis induced by Gal-1 in GC cells and restored the sensitivity of GC cells to cisplatin.

In summary, our study revealed that Gal-1 can boost the proliferation and metastasis of GC cells while increasing their resistance to cisplatin through the activation of the NRP-1/c-JUN/Wee1 pathway. Notably, the NRP-1 inhibitor EG00229 effectively counteracted these effects. This reveals a potential mechanism through which Gal-1 promotes the growth of GC cells and contributes to chemoresistance, offering new therapeutic targets for the treatment of GC.

SUPPLEMENTARY MATERIALS

Supplementary Table 1
Primers of genes used in the study

Supplementary Fig. 1
CAFs promotes GC cell invasion and the capacity can be reversed by NRP-1 inhibitor (EG00229). (A) Western blot showed that fibroblasts after co-culture with GC cells the expression of protein α-SMA and Vimentin were efficiently increased It revealed that fibroblasts were effectively induced to differentiate into CAFs. (B) and the Transwell assay showed that CAFs promote GC cell invasion and that GC cells after treatment with an NRP-1 inhibitor (EG00229) can reverse this phenomenon.

Supplementary Fig. 2
qRT-PCR analysis of indicated NRP-1/c-JUN/Wee1 genes and c-JUN downstream pathway gene in untreated group, 50 ng Gal-1-treated group, and 50 ng Gal-1+3 μM EG00229-treated group.

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