

## Original Article

# Galectin-1 mediates TGF- $\beta$ -induced transformation from normal fibroblasts into carcinoma-associated fibroblasts and promotes tumor progression in gastric cancer

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**Abstract:** Carcinoma-associated fibroblasts (CAFs) are a major constituent of the tumor microenvironment. Cancer cells can induce the transformation from normal fibroblasts (NFs) into CAFs, reciprocally, CAFs promote tumor invasion and proliferation. TGF- $\beta$  has been the mostly accepted factor to fuel NFs transformation into CAFs. Galectin-1 (Gal1) is highly upregulated in CAFs of multiple human cancers, and overexpression of Gal1 in CAFs promotes tumor progression. The effect of Gal1 on TGF- $\beta$ -induced CAFs activation has not yet been established in gastric cancer (GC). In this study, we show that Gal1 expression in stroma is positively related to TGF- $\beta$  in epithelial cells by retrospective analysis of GC patient samples. Meanwhile, conditioned media (CMs) from gastric cancer cells induce expression of both Gal1 and the CAFs marker alpha smooth muscle actin ( $\alpha$ -SMA) in NFs via TGF- $\beta$  secretion. Knockdown of Gal1 prevents TGF- $\beta$ -induced the conversion of NFs to CAFs. CMs from fibroblasts overexpressing Gal1 inhibits cancer cells apoptosis, promotes migration and invasion in vitro. Thus, Gal1 is significantly involved in the development of tumor-promoting microenvironment by enhancing TGF- $\beta$  signaling in a positive feedback loop. Targeting Gal1 in tumor stroma should be considered as a potential therapeutic target for GC.

**Keywords:** Gastric cancer, carcinoma-associated fibroblast, normal fibroblast, TGF- $\beta$ , transformation, galectin-1

## Introduction

Gastric cancer (GC) is one of the most common cancers worldwide and the leading causes of cancer-related death in China [1]. Despite many improvements in treatment of this disease during the past decade, the prognosis for GC remains poor, especially in advanced stages [2]. Therefore, there is a definite need to evaluate new biological markers that accurately predict the natural history of the disease to improve the outcome of patients with GC.

Cancer has long been considered a cell-autonomous process in which progressive genetic and epigenetic alterations transform cells independently of the external milieu. Emerging evidence suggests that the behavior of carcinomas, including cancer progression and drug susceptibility, is influenced by interaction between tumor cells and the tumor microenvironment, which comprises the extracellular matrix

(ECM), growth factors, cytokines and a variety of stromal cells, such as endothelial cells, inflammatory cells and fibroblasts [3, 4]. In this context, a sub-population of fibroblasts with a myofibroblastic phenotype in cancerous lesions is known as carcinoma-associated fibroblasts (CAFs), which are characterized by the overexpression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), fibroblast activation protein (FAP), fibroblast surface protein (FSP), and vimentin [5]. CAFs have recently been implicated in important aspects of cancer progression through remodeling of the ECM, induction of angiogenesis, and recruitment of inflammatory cells and directly stimulate cancer cell proliferation via the secretion of growth factors and mesenchymal-epithelial cell interactions [6, 7].

In the tumor environment, normal fibroblasts (NFs) undergo changes in protein expression that represent an 'activated' myofibroblastic phenotype, which typically involves the up-regu-

lation of markers such as  $\alpha$ -SMA [8, 9]. The conversion of NFs to CAFs is a general phenomenon operative in multiple cancers, giving rise to tumor progression. Although the mechanisms underlying the CAFs activation remains elusive, transforming growth factor beta (TGF- $\beta$ ) is a potent inducer [10, 11]. In a coculture model of human fibroblasts with cancer cells or treated with conditioned media from cancer cells, TGF- $\beta$  is the dominant factor to mediate CAFs activation [12, 13]. Furthermore, it was found that cancer cell-derived TGF- $\beta$  regulates ovarian cancer invasion by upregulating CAF-derived versican in the tumor microenvironment [10].

Galectin-1 (Gal1), a proto-type member of the galectin family of  $\beta$ -galactoside binding proteins, is a homodimer of 14-kDa subunits possessing two  $\beta$ -galactoside-binding sites. It participates in a variety of biological functions including cell-cell and cell-matrix interactions and cell growth [14]. Intracellularly, galectins interact with signaling pathways, whereas extracellularly, these soluble proteins function by cross-linking cell surface glycoconjugates, forming multimeric glycan arrays termed "lattices" and modulating intracellular signalling pathways [14]. Gal1 overexpression in CAFs promotes growth or invasion of adjacent cancer cells [15] and is correlated with poor survival in several types of cancer including breast, prostate, gastric cancer and laryngeal carcinoma [16-19]. Moreover, Gal1 has also been associated with dermal fibroblasts differentiation into myofibroblasts, and treatment with Gal1 was found to promote myofibroblast activation [20]. But the involvement of Gal1 and the roles of Gal1 in TGF- $\beta$ -induced CAFs activation in tumor-stroma interaction are uncertain.

In this study, we aimed to examine the correlation of Gal1 with TGF- $\beta$  in GC by a retrospective analysis of 134 GC patients, and to clarify the potential regulatory role of Gal1 in TGF- $\beta$ -induced CAFs activation in vitro experiments. Furthermore, the impact of Gal1 expression in CAFs on apoptosis and invasion of GC cells was evaluated.

## Materials and methods

### *Ethics statement*

The study protocol was approved by the ethics committee of the First Affiliated Hospital of

Zhejiang University. Written informed consent in the study has been obtained from all participants.

### *Gastric cancer tissue specimens*

From March 2008 to April 2010, 134 patients with all tumor stages GC who underwent gastrectomy at the Department of Surgical Oncology of the First Affiliated Hospital of Zhejiang University were enrolled in this retrospective study. There were 84 male and 50 female with a median age of 63 years (range, 32-76 years), none had received chemotherapy or radiotherapy before surgery. Patient clinicopathological parameters were collected, including age, gender, differentiation, tumor location, tumor size and TNM pathological classification according to the International Union against cancer (UICC).

### *Immunohistochemistry analysis*

Gastric cancer tissue samples were fixed by immersion in 4% paraformaldehyde overnight at 4°C and then embedded in regular paraffin wax and cut into 4- $\mu$ m sections. Immunohistochemical analyses of Gal1 and TGF- $\beta$  expression were performed on formalin-fixed paraffin-embedded sections of surgical specimens. For immunohistochemistry, tissue sections were deparaffinized and rehydrated in PBS. After antigen retrieval with target retrieval solution, endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide. Nonspecific binding was blocked by pre-incubation with 5% bovine serum albumin (BSA), and then the slides were incubated in a humidified chamber with primary antibodies against Gal1 (1:250, Abcam, UK) and TGF- $\beta$  (1:250, Abcam, UK) overnight at 37°C. After washing with PBS, sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Boster, Wuhan, China) at room temperature for 30 min. After washing twice with PBS, antibody complexes were colored with diaminobenzidine and then counterstained with hematoxylin. Slides were dehydrated and evaluated.

### *Evaluation of immunohistochemical staining*

The final effective immunohistochemical staining was evaluated by two independent pathologists without knowing the information of patients. The immunoreactivity score of Gal1 and TGF- $\beta$  was resulted from the multiplication of

**Table 1.** The sequences of small interfering RNA (siRNA) for the indicated gene

	The sequences of small interfering RNA (siRNA) for the indicated gene
Galectin-1	Sense: 5'-CUAAGAGCUUCGUGCUGAA-3' Antisense: 5'-ACGGUGACUUAAGAUCAA-3'
Smad2	Sense: 5'-GUCCCAUGAAAAGACUUAATT-3' Antisense: 5'-UUAAGUCUUUUAUGGGACTT-3'
TGF- $\beta$	Sense: 5'-GGACTATCCACCTGCAAGA-3' Antisense: 5'-GCAGAGTACACACAGCATA-3'

the positively stained cells and staining intensity. Briefly, the percentage of positive staining was scored as "0" (<5%, negative), "1" (5-25%), "2" (26-50%), "3" (51-75%) or "4" (>75%). Staining intensity was scored as "0" (no staining), "1" (weak staining), "2" (moderate staining), or "3" (strong staining). We defined Gal1 and TGF- $\beta$  expression levels according to median immunoreactivity score.

#### Cell lines and culture

The gastric cancer cells SGC-7901 and MKN-45 were obtained from Institute of Cellular Biology (Chinese Academy of Science, Shanghai, China) and cultured in RPMI-1640 medium (Gibco, Rockville, MD, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin (Life Technologies, Rockville, MD, USA) in 5% CO<sub>2</sub> at 37°C.

#### Isolation and culture of human gastric fibroblasts

CAFs and normal human gastric fibroblasts (NFs) were isolated from gastric tumor tissues and their normal counterparts at least 5 cm away from the outer tumor margin, as previously described [19]. Briefly, the tissue were cut into the smallest pieces in sterile PBS solution and were then digested with collagenase (0.1% collagenase type II, Sigma) at 37°C for 2 h. The suspension was filtered through a 20- $\mu$ m stainless steel wire mesh to collect a single-cell suspension. Then the filtrate was centrifuged at 1,000 rpm for 5 min and the supernatant was carefully discarded. The cell pellet was washed three times with serum-free DMEM and resuspended in DMEM supplemented with 20% fetal bovine serum (FBS) cultured at 37°C and 5% CO<sub>2</sub>/95% air atmosphere. After 30 min culture the nonadherent cells (mainly tumor cells) were

removed to obtain a population of pure fibroblasts because the adhesion time needed for fibroblasts is much shorter than that for tumor cells. After 3 passages, the percentage of purified NFs and CAFs was further examined by immunofluorescence and western blot targeting vimentin, cytokeratin and  $\alpha$ -SMA. As [Figure S1](#) showed, vimentin (a fibroblast marker) was expressed in NFs and CAFs, cytokeratin (a marker for epithelial) was not expressed, and  $\alpha$ -SMA (a CAFs marker) was strongly expressed in CAFs but not in NFs. All NFs and CAFs were used less than 10 passages.

#### Immunofluorescence

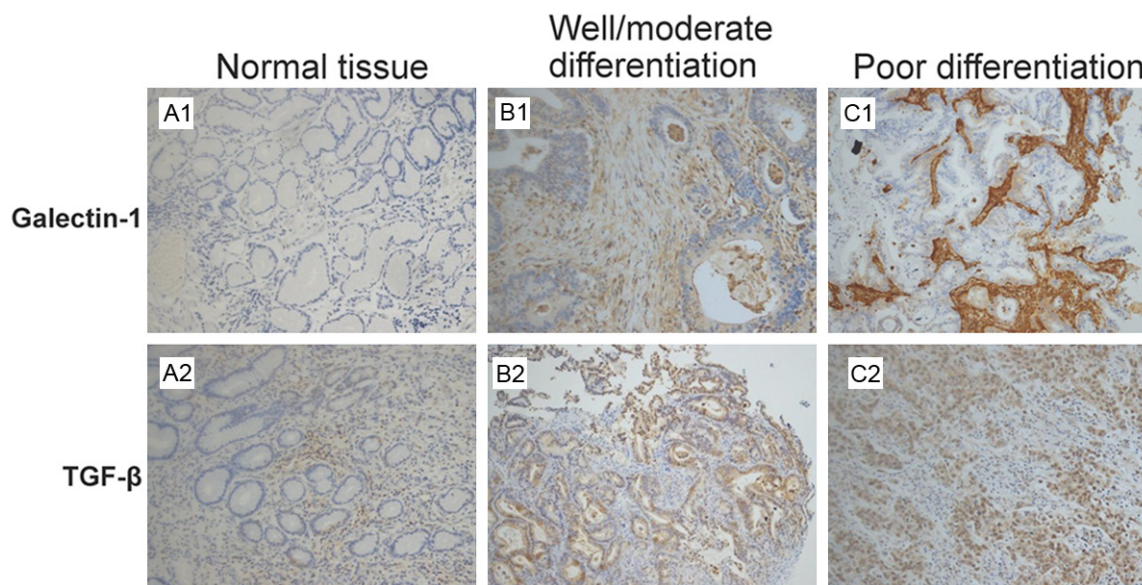
The isolated NFs and CAFs were further identified using immunofluorescence. Cells were seeded at  $1 \times 10^5$  on coverslips in 6-well plates cultured for 24 h and then fixed in 4% paraformaldehyde at room temperature (RT) for 20 min. Cells were subsequently incubated with 5% FBS in PBS for 30 minutes at RT to block nonspecific interactions and then were incubated with vimentin (Santa Cruz, USA; 1:100), cytokeratin (abcam, UK; 1:100), and  $\alpha$ -SMA (R&D Systems, USA; 1:100) primary antibodies at 4°C overnight. After several washes in PBS, the cells were incubated with FITC-labeled secondary antibody (Beyotime Biotechnology, China) at room temperature for 1 h. Finally, the cells were washed with PBS and stained with DAPI before imaging by fluorescence microscopy (OLYMPUS IX81, Japan).

#### Preparation of the conditioned media (CMs)

Gastric cancer cells (MKN7 and MKN45) and fibroblasts (CAFs and NFs) were seeded into T75 culture flasks and grown in normal growth media for 48 h until the cells were approximately 80% confluent. Then normal growth media was refreshed with serum-free media. After 24 h, the culture supernatants were collected, centrifuged at 4°C, 4000 rcf for 10 min, and stored at -70°C for subsequent use.

#### Recombinant lentivirus vector construction and transfection of targeted cells

The Gal-1 sequences were amplified by PCR, confirmed by sequencing. We assembled a pIRES2-eGFP-Gal1 construct by inserting Gal1 cDNA into pIRES2-eGFP vector. We next trans-



**Figure 1.** Increased galectin-1 and TGF- $\beta$  expression in surgical specimens of primary gastric cancer detected by immunohistochemistry. (A1-C1) Representative photographs of immunohistochemical staining of Gal1 in normal gastric tissue (A1), GC samples from well/moderately (B1) and poorly differentiated (C1). (A2-C2) Immunohistochemical staining of TGF- $\beta$  in normal gastric tissue (A2), GC samples from well/moderately (B2) and poorly differentiated (C2). Original magnification  $\times 200$ . Gal1 was mainly expressed in tumor stromal cells and weakly or negatively in cancer cells, whereas cancer TGF- $\beta$  was found in cancer cells.

ected pIRES2-eGFP-Gal1 into NFs using LipofectamineRNAiMAX (Invitrogen, Carlsbad, USA) in accordance with the manufacturer's protocol. The transfection of pIRES2-eGFP vector as a negative control.

#### Small interfering RNA (siRNAs) transfection

To inhibit the indicated gene expression, the transfection of synthetic siRNAs (GenePharma, Shanghai, China) were performed by Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. We also used a scrambled siRNA as a negative control. Knockdown efficiency was confirmed by qRT-PCR and western blot. The sequence of siRNA used was described in **Table 1**.

#### Real-time PCR analysis

We performed Quantitative real-time PCR analysis using GAPDH as an internal control. Total RNA was extracted with the TRIzol Kit (Invitrogen, USA) according to the manufacturer's instructions. qRT-PCR was performed using a SYBR Green PCR kit (TaKaRa, Japan) on the ABI 7500 Real-Time PCR System (Applied Biosystems, USA) according to the manufacturer's instructions. The primer sequences used were

as follows: Gal-1 forward primer 5'-CATGGCTTGTGGTCTGG-3', reverse primer 5'-TGGGCTGGCTGATTTCA-3'; TGF- $\beta$  forward primer 5'-TGGCGATACCTCAGCAACC-3', reverse primer 5'-CTCGTGGATCCACTTCCAG-3';  $\alpha$ -SMA forward primer 5'-TAGCACCCAGCACCATGAAGAT-3', reverse primer 5'-GAAGCATTGCGGTGGACAATG-3'; MMP-9 forward primer 5'-CAACATCACCTATTGGATCC-3', reverse primer 5'-CGGGTGTAGAGTCTCTCGCT-3', MMP-2 forward primer 5'-GTGCTGAAGGACACACTAAAGAAGA-3', reverse primer 5'-TTGCCATCCTTCTCAAAGTTGTAGG-3'. GAPDH as an internal control forward primer 5'-CCTGGCACCAGCACAAT-3', reverse primer 5'-GGGCCGACTCGTCATCG-3'. Each sample was run in triplicate for the target gene and the internal control gene.

#### Western blot analysis

Western blotting of all samples was performed using the following primary antibodies: anti- $\alpha$ -SMA, anti-cytokeratin, anti-vimentin, anti-Smad2, anti-p-Samd2, anti-p38, anti-p-p38, anti-ERK, anti-pERK1/2, anti-phospho-Akt-Ser<sup>473</sup>, anti-Akt (Cell Signal Technology), anti-galectin-1 (Abcam). GAPDH was used as a loading control. Cells were washed twice with cold PBS and

**Table 2.** Galectin-1 expression and clinicopathologic features in 134 patients with gastric cancer

Clinicopathological parameters	N	Galectin-1 expression		P value
		High	Low	
All cases	134	81	53	
Age (years)				0.288
<60	62	34	28	
$\geq$ 60	72	37	35	
Gender				0.716
Male	84	52	32	
Female	50	29	21	
Tumor size				0.710
<5 cm	43	25	18	
$\geq$ 5 cm	91	56	35	
Tumor location				0.469
Upper	31	21	11	
Middle	38	26	12	
Lower	53	28	25	
Entire	12	7	5	
Histological grade				0.018 <sup>a</sup>
Well to moderate	49	23	26	
Poor or others	85	58	27	
Invasion depth				0.011 <sup>a</sup>
T1-T2	50	23	27	
T3-T4	84	58	26	
Lymph node metastasis				0.013 <sup>a</sup>
Positive	93	63	30	
Negative	41	18	23	
TNM stage				<0.001 <sup>a</sup>
I-II	55	23	32	
III-IV	79	58	21	
TGF- $\beta$ expression				0.031 <sup>a</sup>
High	71	49	22	
Low	63	32	31	

Note: <sup>a</sup>P<0.05.

lysed in cold lysisbuffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF. Equal amounts of extracted proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% skim milk, each membrane was incubated with the indicated primary antibody, followed by the appropriate HRP-conjugated secondary antibody. The results were detected using an enhanced chemiluminescence (ECL) detection system.

#### Enzyme-linked immunosorbent assay (ELISA)

The conditioned media from CAFs and NFs were harvested after incubation of 48 hours. Concentration of secreted galectin-1 was evaluated by ELISA according to the manufacturer's protocol (R&D Systems).

#### Apoptosis assay

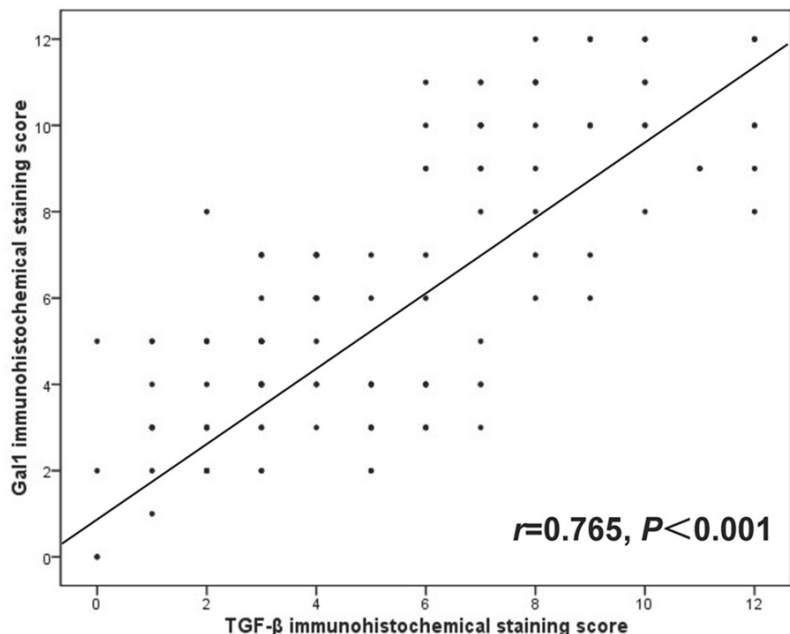
Gastric cancer cells were collected after 24 h treated with various CMs from NFs, CAFs and CAFs-siGal1, and subjected to apoptosis measurement using the annexin V/propidium iodide (PI) detection kit (BD Bioscience, San Jose, CA, USA) according to the manufacturer's protocol. The numbers of stained cells were analyzed by a flow cytometer (BD Bioscience, San Jose, CA, USA). Each experiment was conducted in triplicate.

#### Transwell assay for cell migration and invasion

Cell migration and invasion assays were done in 24-well Transwell polycarbonate filters (8  $\mu$ m pore size; Corning Costar) coated with or without Matrigel (BD Biosciences). Gastric cancer cells of pre-starved in serum-free medium for 12 h were plated in the upper chamber, and the conditioned media of NFs, CAFs-siCtr, CAF-siGal1 cells were added to the lower chamber. After 24 h incubation at 37°C with 5% CO<sub>2</sub>, non-penetrating cells on the upper surface of the filter were wiped off with a cotton swab. Penetrating cells were fixed with 95% ethanol, stained with 0.1% crystal violet, washed with PBS. For quantification, all of the stained cells representative of invading or migrating into the lower surface were counted under a light microscope. All of the experiments were performed in triplicate. Results show the mean  $\pm$  SD of 3 independent assays.

#### Statistics

Results were expressed as means  $\pm$  SD. All statistical analyses were carried out using SPSS version 16.0 (SPSS, Chicago, IL, USA). For in vitro experiments, unpaired Students' t-test or one-way ANOVA were used to analyze the significance of differences among groups. Categorical data were assessed using Chi-square or Fisher's exact tests. The relationship between Gal1 and TGF- $\beta$  was determined by the Pearson correlation test. The Kaplan-Meier test was employed to evaluate the survival rate, and the



**Figure 2.** Gal1 expression was positively associated with TGF- $\beta$  expression in gastric cancer. Relationship between Gal1 expression and TGF- $\beta$  expression was examined by Pearson correlation test according to the immunohistochemical staining score ( $r=0.765, P<0.001$ ).

survival rate curves were compared using the log-rank test. Cox's proportional hazards model was used to identify factors that had a significant influence on survival. Two-tailed levels of significance were used and  $P<0.05$  was considered statistically significant.

## Results

*Gal1 expression is positively associated with TGF- $\beta$  expression in patients with gastric cancer*

To clarify the correlation between Gal1 and TGF- $\beta$  expression, immunohistochemistry was carried out in 134 gastric cancer samples. As shown in **Figure 1**, Gal1 was highly expressed in tumor stromal cells and weakly or negatively in cancer cells, whereas cancer cell staining of TGF- $\beta$  was found in the evaluated tissue samples. Gal1 and TGF- $\beta$  were negative expressed in normal gastric tissue, and more strongly in GC with poorly differentiated than those with well or moderately differentiated. The relationship between Gal1 and the clinicopathological parameters of 134 GC were summarized in **Table 2**. As shown in **Table 2**, high Gal1 expression in GC was significantly related to histological grade ( $P=0.018$ ), invasion depth ( $P=0.011$ ), lymph node metastasis ( $P=0.013$ ) and

TNM stage ( $P<0.001$ ), whereas no significant correlation was found between Gal1 expression in GC tissues and age ( $P=0.288$ ), gender ( $P=0.716$ ), tumor size ( $P=0.710$ ) and tumor location ( $P=0.469$ ). In addition, Pe- arson correlation analysis of immunohistochemical staining score of the indicated markers uncovered remarkable positive correlation between Gal1 expression and TGF- $\beta$  expression ( $r=0.765, P<0.001$ , **Figure 2**).

*Gal1 expression is an independent prognostic predictor for patients with GC*

Post-surgical follow-up was completed on 30th June 2015. All patients underwent follow-up until cancer-related death or more than

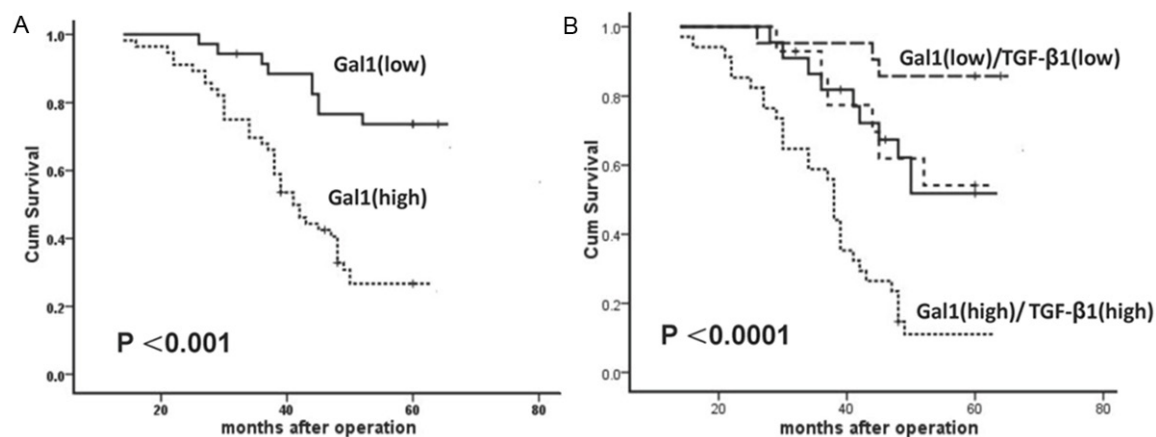
5 years after tumor resection. High Gal1 expression was significantly associated with a poor prognosis. As **Figure 3A** showed, overall survival was markedly lower in the group with high Gal1 expression than those with low Gal1 expression ( $P<0.001$ ). Taken together with TGF- $\beta$  expression in patients with GC, those with co-expression of Gal1 and TGF- $\beta$  showed the shortest overall survival time (**Figure 3B**,  $P<0.0001$ ).

Further multivariate Cox proportional hazard regression analysis was employed to identify the independent value of each variable for predicting overall survivaltime in GC (**Table 3**), including Gal1 expression, TGF- $\beta$  expression and clinicopathologic characteristics. As shown in **Table 3**, overall survival is related with Gal1 expression (HR=2.057, 95% CI: 1.034 to 4.091,  $P=0.039$ ), histological grade (HR=2.752, 95% CI: 1.261 to 6.010,  $P=0.011$ ) and TNM stage (HR= 3.260, 95% CI: 2.203 to 4.825,  $P<0.001$ ). Thus, Gal1 may be a useful maker for predicting the overall survival of patients with GC.

*Galectin-1 is associated with transformation from NFs to CAFs*

Firstly, five pairs of primary CAFs and NFs were used to examine Gal1 expression by qRT-PCR

## Regulation of TGF- $\beta$ -induced carcinoma-associated fibroblasts activation by galectin-1



**Figure 3.** Kaplan-Meier survival curves for cumulative survival rate of 134 patients with GC. A. Gal1 overexpression is correlated with poor survival of patients with GC ( $P < 0.001$ ). B. Overall survival analysis was carried out based on the coexpression of Gal1 and TGF- $\beta$ . The patients with both Gal1 and TGF- $\beta$  overexpression showed the significantly shortest survival time ( $P < 0.0001$ ).

**Table 3.** Multivariate analysis of overall survival in gastric cancer

Variables	Multivariate analyses		
	HR	95% CI	P value
Age			NS
Gender			NS
Tumor size			NS
Tumor location			NS
Histological grade	2.752	1.261-6.010	0.011 <sup>a</sup>
Lymph node metastasis			NS
TNM stage	3.260	2.203-4.825	<0.001 <sup>a</sup>
TGF- $\beta$ expression			NS
Galectin-1 expression	2.057	1.034-4.091	0.039 <sup>a</sup>

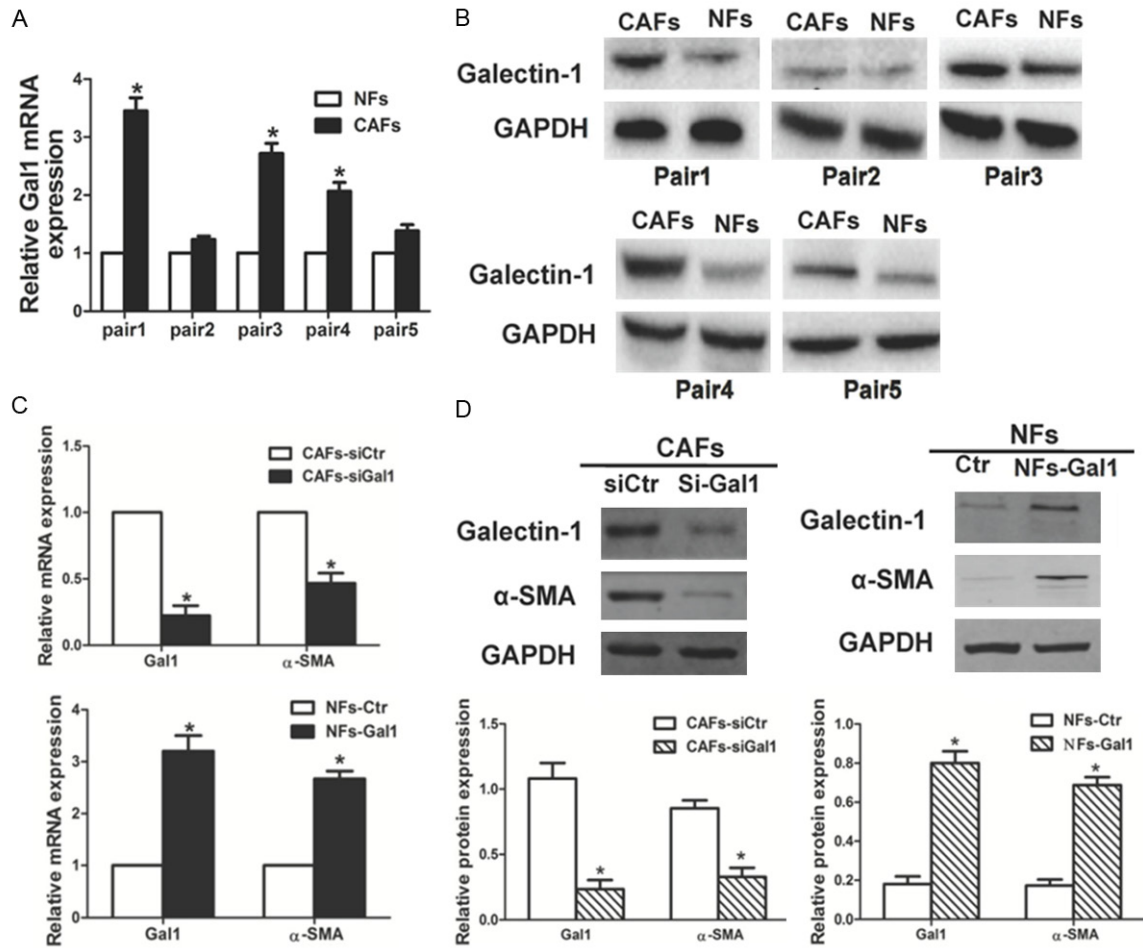
Note: <sup>a</sup> $P < 0.05$ ; NS: No significance.

and Western blot analysis. As shown in **Figure 4A & 4B**, in three pairs of fibroblasts, a significantly increased Gal1 expression was observed in CAFs compared with the corresponding NFs. Because this tendency was consistent with previously reported studies, we further evaluated whether Gal-1 expression is associated with CAFs activation in GC. Inhibition of Gal-1 expression through infection CAFs with siRNA targeting Gal1 significantly repressed  $\alpha$ -SMA expression. Meanwhile, up-regulation of Gal1 expression in NFs via transfection with recombinant Gal1 expression lentivirus vector significantly resulted in higher expression of  $\alpha$ -SMA compared with the negative control lentivirus vector (**Figure 4C & 4D**). In addition, to explore whether exogenous Gal1 contributed to CAFs activa-

tion, NFs were treated with human recombinant Gal1 at various concentration. As **Figure S2A** showed, addition of exogenous Gal1 could stimulated  $\alpha$ -SMA expression, but the concentration of 0.5  $\mu$ g/ml and 1  $\mu$ g/ml had the little effect on  $\alpha$ -SMA expression. Moreover, the conditioned supernatant obtained from fibroblasts culture contained detectable but quite low level of Gal that is not sufficient for induction of CAFs activation (**Figure S2B & S2C**). Thus, transformation from NFs into CAFs possibly mediated by intracellular Gal1 in our study.

### *Gal1-positive CAFs promotes GC cell lines anti-apoptosis*

In the next step, we evaluated the regulatory function of CAFs on GC cell lines in terms of apoptosis. GC cells apoptosis was determined with double staining of Annexin V and PI by flow cytometry 24 h after treatment with various CMs. The result showed that the ratio of apoptotic SGC7901 cells exposure to CMs from CAFs was significantly lower than those treated without or with CMs from NFs, whereas the anti-apoptotic activity of SGC7901 was abolished upon treated with CMs from CAFs with Gal1 knockdown. The similarity was also observed in MKN45 cells ( $P < 0.05$ , **Figure 5A & 5B**). These results suggest that Gal1 in CAFs may play an important anti-apoptotic role in GC development. Thus, to confirm the mechanism of Gal1 on anti-apoptosis, we detected the expression of certain pro-apoptotic proteins and anti-apoptotic proteins by western blot



**Figure 4.** Gal1 expression is positively with CAFs activation. A. Gal1 mRNA expression was analyzed by qRT-PCR of paired NFs and CAFs in five GC patients. The relative expression levels of Gal1 were calculated by comparing the  $2^{-\Delta\Delta CT}$  values of CAFs to NFs and shown in fold changes. GAPDH was used as an internal control. B. Gal1 expression in paired NFs and CAFs from five GC patients was analyzed by western blotting. C and D. Gal1 promotes NFs transformation into CAFs. Up-regulation of Gal1 expression in NFs transfected with Gal1 expression lentivirus vector (NFs-Gal1) contributed to  $\alpha$ -SMA expression compared with negative control lentivirus vector (NFs-Ctr), knockdown of Gal1 expression in CAFs by siRNA (siGal1) inhibited  $\alpha$ -SMA expression, but alteration of  $\alpha$ -SMA expression was not observed in CAFs treated with negative control siRNA (siCtrl). C. Relative changes of Gal1 and  $\alpha$ -SMA mRNA expression in fibroblasts. GAPDH was used as an internal control. D. Top: Western blotting of Gal1 and  $\alpha$ -SMA expression in fibroblasts. Bottom: Quantification of results shown in top using ImageJ software. GAPDH was used as the loading control. The results are shown as mean values  $\pm$  SD of three independent experiments and analyzed by unpaired Student's t-tests (\* $P < 0.05$ ).

analysis. As shown in **Figure 5C**, Gal1 knockdown in CAFs significantly suppressed the expression of the anti-apoptotic protein Bcl-2, but upregulated Bax expression both in SGC7901 and MKN45 cells.

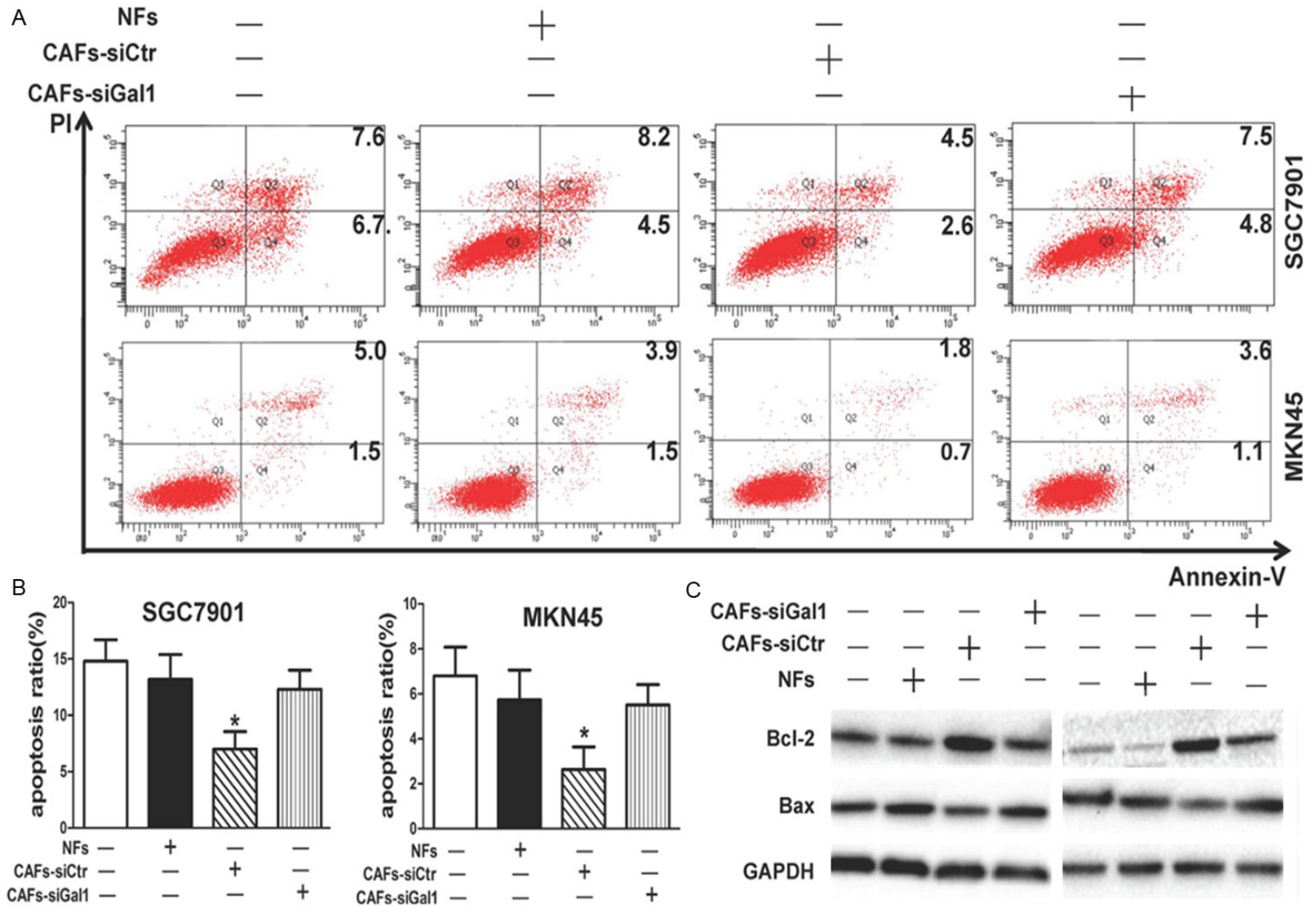
*Gal1-positive CAFs promotes gastric cancer cell migration and invasion*

Gal1 expression positively correlated with lymph node metastasis status and TNM stage. We further explored whether Gal1 in CAFs facili-

tates migration and invasion of GC cells. As shown in **Figure 6A & 6B**, CMs from CAFs markedly promoted GC cells migration and invasion, compared with in absence or presence of that from NFs ( $P < 0.01$ ). But inhibition of Gal1 expression in CAFs inactivated this effect. These findings indicate that Gal1 in CAFs contributes to GC cells migration and invasion. A change in the proteolytic degradation of adjacent tissue is required during tumor invasion. Matrix metalloproteinase (MMPs) such as MMP-2 and

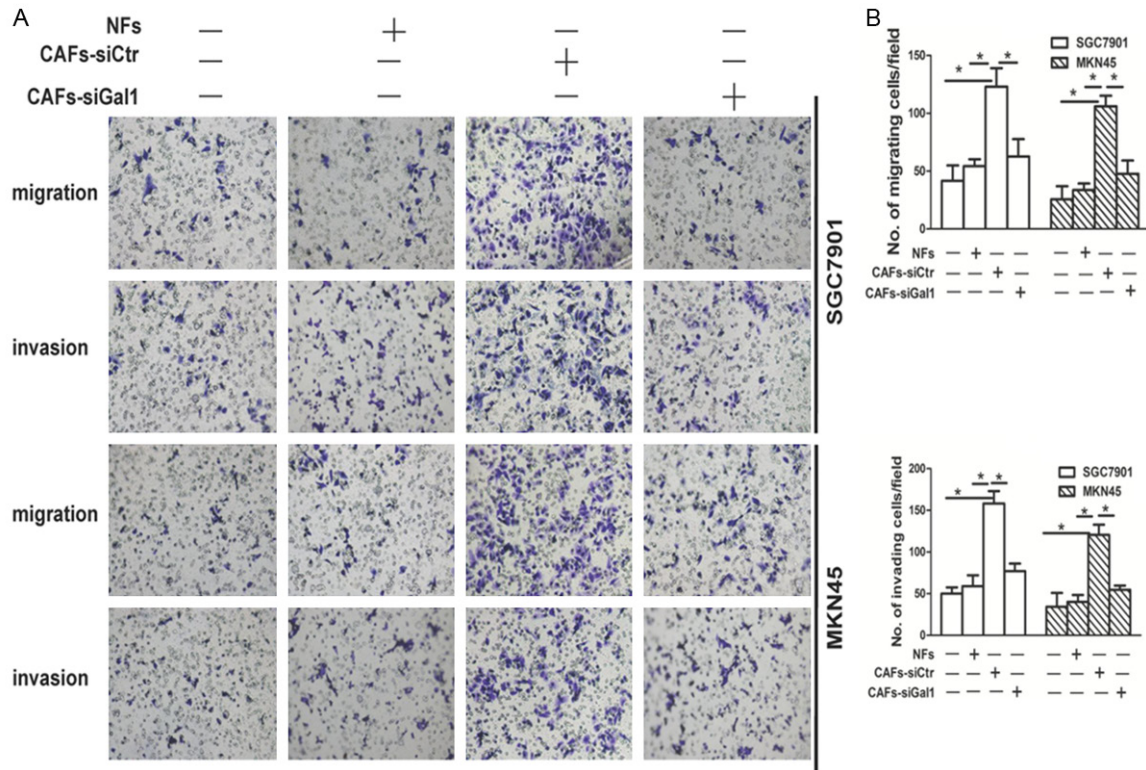


Regulation of TGF- $\beta$ -induced carcinoma-associated fibroblasts activation by galectin-1



**Figure 5.** Gal1-positive CAFs promote GC cells anti-apoptosis. **A.** Representative graphs of apoptosis assay by FACS analysis using Annexin V-FITC/PI staining. **B.** Changes in the number of apoptotic SGC7901 and MKN45 cells in absence or presence of CMs from NFs, CAFs-siCtr or CAFs-siGal1. **C.** Western blotting analysis of pro-apoptotic and anti-apoptotic proteins expressed by GC cells absence or in presence of CMs from NFs, CAFs-siCtr or CAFs-siGal1. The results are shown as mean values  $\pm$  SD of three independent experiments and analyzed by one-way ANOVA (\* $P < 0.05$ ).

Regulation of TGF- $\beta$ -induced carcinoma-associated fibroblasts activation by galectin-1



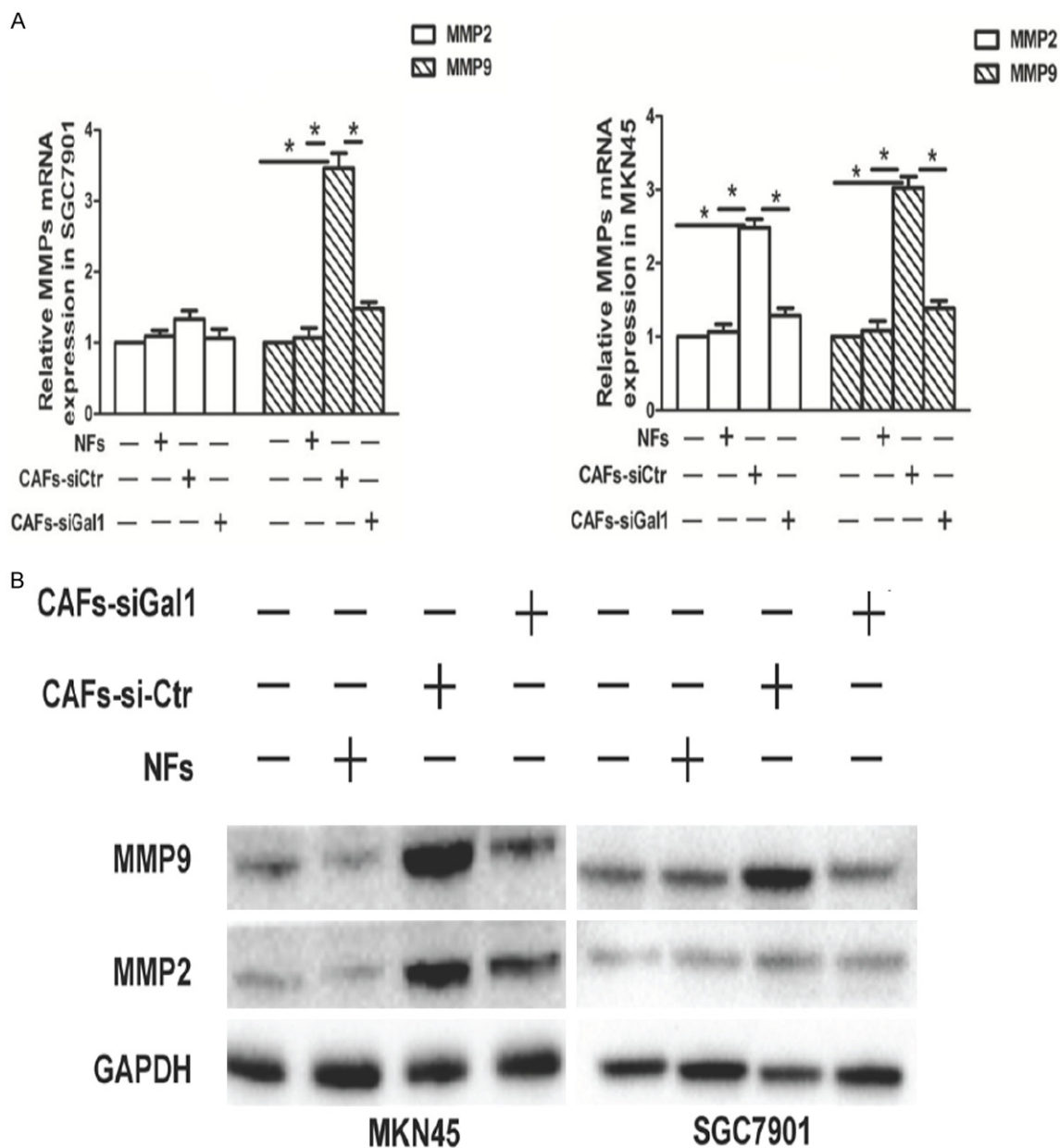
**Figure 6.** Gal1-positive CAFs promote GC cells migration and invasion. A. Representative graphs of GC cells migration and invasion assay in absence or presence of CMs from NFs, CAFs-siCtr or CAFs-siGal1 (magnification  $\times 200$ ). B. Changes in the number of migration and invasion GC cells in absence or presence of CMs from NFs, CAFs-siCtr or CAFs-siGal1. The results are shown as mean values  $\pm$  SD of three independent experiments and analyzed by one-way ANOVA ( $*P < 0.05$ ).

MMP-9 are major contributors to the degradation of extracellular matrix. Gal-1 expression was involved in regulating the production and activities of MMP-2 and MMP-9 in OSCC cells, which previously reported [21]. In our study, we found that MM9 was upregulated both in SGC7901 and MKN45 treated with CMs from CAFs compared with absence or presence of NFs, silencing of Gal1 in CAFs led to MMP9 expression inhibition (Figure 7A & 7B,  $P < 0.05$ ). These data indicate that Gal1 is responsible for CAFs-amplified migration and invasion of GC cells possibly through increasing MM9 expression.

*Gal1 is required for TGF- $\beta$  dependent transformation of NFs into CAFs*

TGF- $\beta$  is secreted by a range of tumor cells [22] and mediates the interaction of cancer cells with stromal fibroblasts [7]. Fibroblasts respond to TGF- $\beta$  and acquire a myfibroblastic phenotype with a distinctive pattern of gene expres-

sion, such as  $\alpha$ -SMA [23, 24]. Studies have shown that fibroblasts differentiation into myfibroblasts driven by TGF- $\beta$  treatment, accompanied by upregulation of Gal1 expression [25]. In light of our observation that Gal1 stimulated NFs transformation into CAFs, we next investigated the regulatory role of Gal1 on CAFs activation driven by TGF- $\beta$  in GC. As shown in Figure 8A-C, similar to the effect of recombinant TGF- $\beta$ , CMs from MKN7 which strongly expressed TGF- $\beta$  significantly induced Gal1 and  $\alpha$ -SMA expression in NFs, but not that from MKN45 negatively expressed TGF- $\beta$ . Meanwhile, the increased expression levels of Gal1 and  $\alpha$ -SMA stimulated by CMs from MKN7 was suppressed via TGF- $\beta$  knockdown. Furthermore, knockdown of Gal1 by siRNA targeting Gal1 gene in NFs abolished the TGF- $\beta$ -stimulating effects of  $\alpha$ -SMA expression (Figure 8D, 8E). These findings suggest that Gal1 mediates the process of NFs transformation into CAFs driven by TGF- $\beta$  derived from cancer cells.

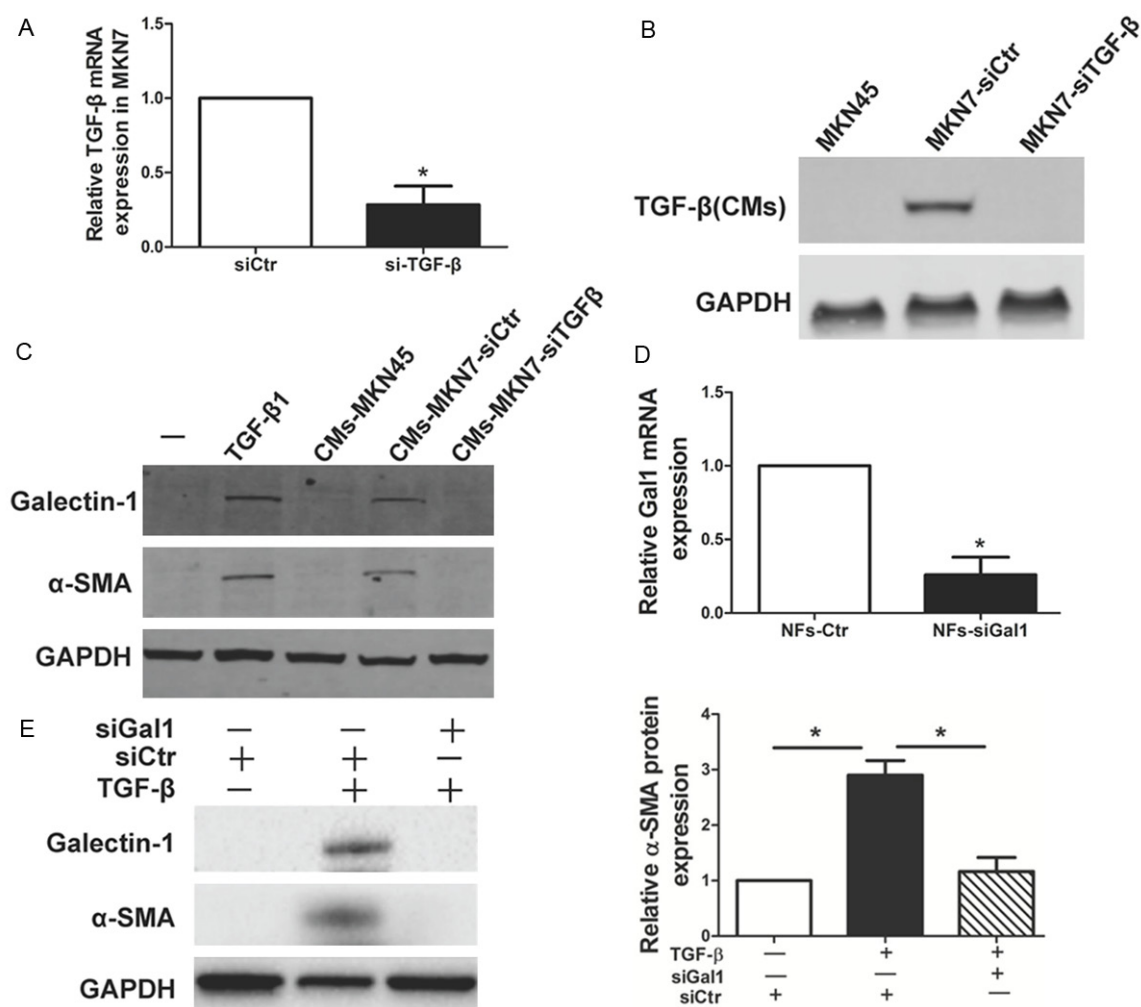


**Figure 7.** Gal1-positive CAFs regulate MMPs expression in GC cells. **A.** qRT-PCR analysis of MMPs mRNA expression in GC cells without or with CMs from NFs, CAFs-siCtr and CAFs-siGal1 treatment. **B.** Western blotting analysis of MMPs expression in GC cells without or with CMs from NFs, CAFs-siCtr or CAFs-siGal1. The results are shown as mean values  $\pm$  SD of three independent experiments and analyzed by one-way ANOVA (\* $P < 0.05$ ).

*Gal1 regulates TGF- $\beta$ -induced NFs differentiation into CAFs through Smad2 activation*

The major signaling pathway of TGF- $\beta$  acts through transmembrane receptor serine/threonine kinases and activates the cytoplasmic Smad proteins [26]. In addition, a non-Smad pathway that includes extracellular signal-regulated kinase (ERK), mitogen-activated protein

kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathway has also been well established to impact on TGF- $\beta$ -mediated signaling [27, 28]. As expected, we found that enhanced phosphorylation levels of Smad2 and Akt in a time-dependent manner in NFs treated with recombinant TGF- $\beta$ , whereas no difference in Smad2, Akt expression and p38 MAPK activation (**Figure 9A**). To investigate

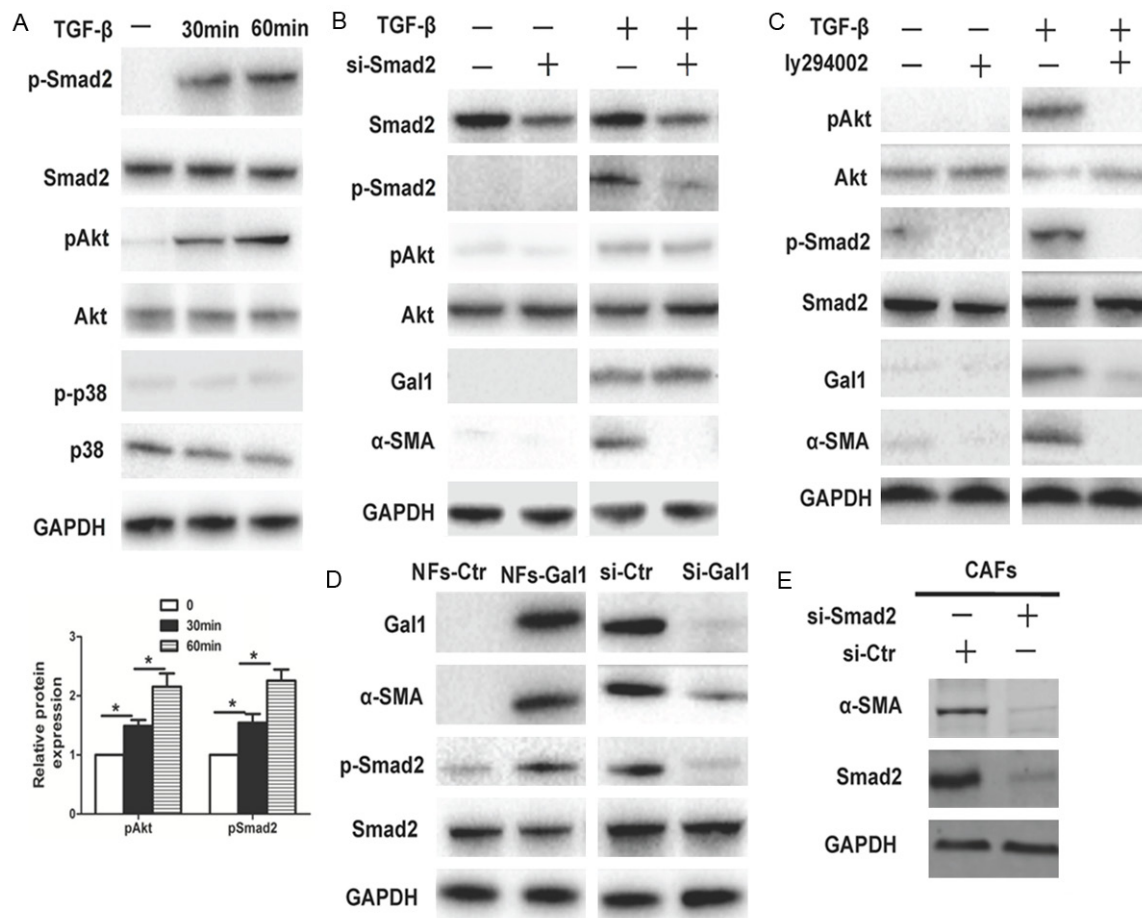


**Figure 8.** Gal1 is crucial for NFs transformation into CAFs induced by TGF- $\beta$  in GC. A. The efficacy of TGF- $\beta$  knock-down by siRNA (siTGF- $\beta$ ) in MKN7 cells which strongly express TGF- $\beta$  was determined by qRT-PCR. Infection with scramble siRNA was used as negative control (MKN7-siCtr). B. Western blotting analysis of TGF- $\beta$  expression in CMs from MKN45, MKN7-siCtr and MKN7-siTGF- $\beta$ . C. The effect of CMs from GC cell lines on Gal1 and  $\alpha$ -SMA expression was analyzed by western blotting, recombinant TGF- $\beta$  (10 ng/ml) used as a positive control and without CMs as a negative control. D. The efficiency of knockdown of Gal1 expression in NFs by infected with siRNA targeting Gal1 (NFs-siGal1) was evaluated by qRT-PCR. Infection with scramble siRNA was used as negative control (NFs-siCtr). GAPDH was used as an internal control. Data were calculated using the  $2^{-\Delta\Delta CT}$ . E. Gal1 regulates TGF- $\beta$ -induced  $\alpha$ -SMA expression. Left:  $\alpha$ -SMA expression levels in NFs-siCtr or NFs-siGal1 without or with TGF- $\beta$  treatment (10 ng/ml) for 24 h were examined by western blotting. Right: Quantification of  $\alpha$ -SMA expression using ImageJ. The results are shown as mean values  $\pm$  SD of three independent experiments and analyzed by one-way ANOVA or unpaired Student's t-tests (\* $P$ <0.05).

whether the observed intracellular signaling contribute to TGF- $\beta$ -stimulating Gal1 and  $\alpha$ -SMA expression, Smad2 expression was suppressed in NFs by infection with small interfering RNA (siRNA) (Figure S3A). The Smad2 siRNA significantly decreased the  $\alpha$ -SMA expression level of NFs although in presence with TGF- $\beta$  treatment, whereas difference of Gal1 expression and Akt activation levels between Smad2 siRNA and negative control siRNA was not

obtained (Figure 9B). As shown in Figure 9C, LY49002, a PI3K inhibitor, suppressed  $\alpha$ -SMA, Gal1 expression and Smad2 activation levels in NFs although treated with TGF- $\beta$ . Previous studies have indicated that Gal1 accelerated fibroblasts differentiation into myofibroblasts by regulating Smad2/3 pathway, such as enhancing nuclear retention of Smad2 [25] and Smad2/3 phosphorylation [29]. To further elucidate the mechanism of the regulatory role of

## Regulation of TGF- $\beta$ -induced carcinoma-associated fibroblasts activation by galectin-1



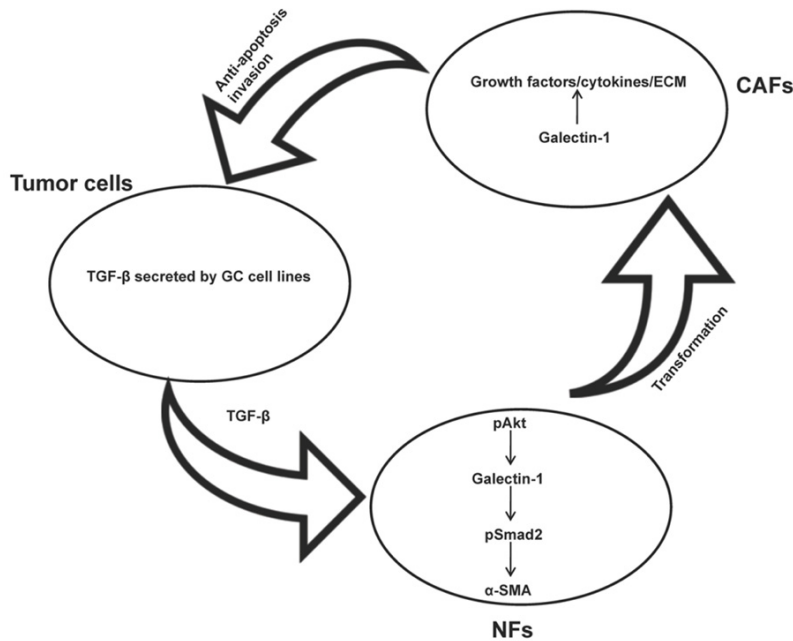
**Figure 9.** Gal1 mediates TGF- $\beta$ -induced NFs differentiation to CAFs by Smad2 activation. **A.** Akt and Smad2 phosphorylation levels were increased in presence of TGF- $\beta$  treatment in a time-dependent manner. Top: Western blotting analysis of intracellular signaling upon treatment of TGF- $\beta$  (10 ng/ml) at various time courses. Bottom: Quantification of pAkt and pSmad expression shown in **A.** using ImageJ. **B.** Western blotting analysis of Gal1, pAkt and  $\alpha$ -SMA expression levels in NFs with negative control siRNA or Smad2 siRNA upon TGF- $\beta$  treatment.  $\alpha$ -SMA expression was suppressed in NFs infected with siRNA targeting Smad2 although treated with TGF- $\beta$ , but not Gal1 and pAkt expression. **C.** Western blotting analysis of Gal1, pSmad2 and  $\alpha$ -SMA expression levels in NFs without or with LY294002 (20 nM) upon TGF- $\beta$  treatment. **D.** Western blotting analysis of Gal1, pSmad2 and  $\alpha$ -SMA expression levels in NFs without or with transfection with Gal1 expression lentivirus vector, and those in CAFs infection with negative control siRNA (siCtr) or Gal1 siRNA (siGal1). **E.** Western blotting analysis of  $\alpha$ -SMA expression in CAFs infection with negative control siRNA (siCtr) or Smad2 siRNA (siSmad2). The results are shown as mean values  $\pm$  SD of three independent experiments and analyzed by one-way ANOVA or unpaired Student's t-tests (\* $P$ <0.05).

Gal1 on TGF- $\beta$ -driven CAFs activation, we focused on the relationship between Gal1 and Smad2/3. As shown in **Figure 9D**, Smad2 phosphorylation level was markedly upregulated along with concomitant increased  $\alpha$ -SMA expression in NFs transfected with a Gal1 expression lentivirus vector, compared with that transfected with control lentivirus vector. Likewise, Smad2 activation in CAFs was inhibited by knockdown of Gal1 expression. Besides,  $\alpha$ -SMA expression was affected in Smad2-depleted CAFs (**Figure S3B** and **Figure 9E**). On the basis of these findings, we propose that TGF- $\beta$  stimu-

lates Gal1 expression in a non-canonical Akt-dependent pathway, which could be inhibited by LY294002. Gal1 facilitates TGF- $\beta$ -driven NFs transformation into CAFs through enhancing Smad2 activation (**Figure 10**).

### Discussion

Gal1 is well known to be involved in cancer cell proliferation, invasion, immuno-suppression and tumor angiogenesis [21, 30, 31]. Consistent with previous results that Gal1 was positively associated with CAFs activation [31-33], we



**Figure 10.** Schematic diagram depicting a working hypothesis of the crosstalk between stromal fibroblasts and cancer cells. TGF- $\beta$  derived from gastric cancer cells upregulates Gal1 expression in NFs in a PI3K/Akt dependent manner, which could be inhibited by LY294002, a PI3K/Akt inhibitor. Gal1 mediates TGF- $\beta$ -driven transformation from NFs to CAFs through Smad2 activation, and therefore promotes anti-apoptosis, migration and invasion of gastric cancer cells.

observed that knockdown of Gal1 expression in activated CAFs was accompanied by decreased expression of  $\alpha$ -SMA. Gal-1 has also been induced with hepatic and pancreatic stellate cell activation in fibrosis tissues [34, 35], which indicates that the level of Gal-1 expression in fibroblasts controls the progression of fibrosis and cancer. Although Gal1 is an important mediator of CAFs activation, the mechanism and regulation of Gal1-mediated CAFs activation are still unclear. Reactive oxygen species (ROS) have pivotal roles in physiological and pathological processes, including the progression of myofibroblast activation [36]. Several studies have indicated that the NADPH oxidase (NOX) family, especially NOX4 has an important role in ROS production in various cell types, including fibroblasts [37, 38]. Gal1 has been reported to stimulate NOX4 expression during myofibroblast activation, more importantly knockdown of NOX4 expression suppressed Gal1-induced myofibroblast activation [29], which indicates that Gal1 mediates myofibroblast activation dependent of NOX4 expression.

It has widely reported that TGF- $\beta$  produced by tumour cells may contribute to maintaining the myofibroblastic phenotype, and therefore might play an important role in the malignant phenotype in the cancer microenvironment [10, 24]. There have been a few reports on upregulation of Gal1 expression upon TGF- $\beta$  treatment, but with some conflicting results. Wu MH, et al. did not observe alteration of Gal1 expression in reaction to TGF- $\beta$  treatment, even more Okano et al reported decreasing levels of Gal1 in response to TGF- $\beta$  in HKC cells [32, 39]. In our study, we demonstrate for the first time that Gal1 accumulation in tumor stroma cells induced by cancer cell-derived TGF- $\beta$  in GC. Furthermore, a positive correlation between Gal1 and

TGF- $\beta$  expression is also observed in patient tissue samples with GC. The diversity of Gal1 expression in fibroblasts respond to TGF- $\beta$  treatment is possibly ascribed to the different tissue origin and attendant circumstance. Gal1 could also be regulated by other factors, for instance, PDGF-induced myofibroblasts activation is associated with upregulation of Gal1 [34]. Although compelling evidence indicates a critical role for Gal-1 in the modulation of tumor growth and metastasis [40]. There is still scarce information about molecular pathways involved in the regulation of Gal-1 gene expression within the tumor microenvironment. To date, transcription factors, such as NF- $\kappa$ B and HIF-1 $\alpha$  have been reported to be involved in Gal1 expression [41, 42]. The downstream mediators involved in TGF- $\beta$  signaling include p38 MAPK, NF- $\kappa$ B, ERK1/2, c-jun-NH2-kinase (JNK), and Akt. Our results are in agreement with a recent literature [25], LY294002, an inhibitor of PI3K suppressed Gal1 expression stimulated by TGF- $\beta$ , suggesting that PI3K/Akt axis may involved in TGF- $\beta$ -mediated upregulation of Gal1 expression in GC.

Smad acts as a major signaling molecule in TGF- $\beta$  action, a variety of signaling molecules are reported to regulate TGF- $\beta$ /Smad signaling. Intracellular Gal1 interacted with Smad2 and phosphorylated Smad2 to accelerate fibroblasts differentiation to myofibroblasts [25]. Extracellular Gal1 interacted with Neuropilin-1 (NRP1), a co-receptor for several growth factors, and subsequently enhanced Smad3 activation, which triggered NOX4 expression [29]. Additionally, Gal1 negatively regulated TGF- $\beta$ /Smad signaling by decreasing Smad3-complex from binding to the Smad binding element (SBE) to inhibit target gene transcription [39]. In our study, we demonstrated that Gal1 participated in the process of TGF- $\beta$ -driven CAFs activation through up-regulating Smad2 phosphorylation. In this regard, simultaneous blockade of the TGF- $\beta$  and Gal1 signaling pathways *in vivo* might have critical implications for cancer therapy.

Galectin-1 has attracted attention as a potential cancer target because of its contribution to a variety of events including immune suppression [43], tumor angiogenesis [42] and metastasis [18]. Galectin-1 may act both extracellularly after secretion and intracellularly [44]. Endogenous Gal1 expression in cancer cell interacts with H-Ras for membrane anchorage of H-Ras, and subsequent activation of a kinase cascade comprising Raf and ERK to induce Bcl2 and MMPs expression [28, 45]. Secreted Gal1 has been shown to interact with the extracellular glycans of cell-surface proteins including fibronectin, integrins, laminin and VEGFR2 that determines proliferation, adhesion, migration and angiogenesis [46, 47]. In our study, we employed the CMs from Gal1-positive CAFs, ruling out the intracellular pathway through which Gal1 promoted anti-apoptosis and invasion of cancer cells. Interestingly, we found quite a low amount of Gal1 in CMs from both Gal1-positive CAFs and NFs. Thus, we hypothesize that the indirect functions of Gal1 expression in cancer cell migration and invasion mainly due to CAFs activation and stimulation of growth factor, cytokines, MMPs and extracellular matrix (ECM) secretion from activated CAFs. An increasing body of evidence suggests that CAFs can secrete and deposit certain ECM components and further fuel the directional migration and invasion of carcinoma cells [48]. Gal1 knockdown in fibroblasts attenuates TGF- $\beta$ -induced fibronectin,

$\alpha$ -SMA and collagen expression [25]. Gal1 stimulates motility of human umbilical cord blood-derived mesenchymal stem cells by upregulation of NF- $\kappa$ B-dependent fibronectin/laminin5 expression [49]. ECM such as fibronectin induces adhesion, proliferation, and invasion of tumor cells through binding to integrins, which promotes integrin clustering and subsequently triggers integrin-mediated intracellular signal transduction [50]. Gal1 has been reported to modulate monocyte chemoattractant protein-1 (MCP-1) production from fibroblast, targeting MCP-1 reduces cancer cell migration and invasion induced by CMs from Gal1-positive CAFs [32]. Apart from MMPs secreted by CAFs itself, MMPs production and activity in epithelial ovarian cancer cells are affected by Gal1-positive fibroblast as well [51]. In our study, we observed MMP9 production in cancer cells in presence of CMs from Gal1-positive CAFs, and the decreased ability of cancer cells to migrate and invade coexisted with reduction of MMP9 in cancer cells treated with CMs from Gal1 knockdown CAFs. MMP9 probably participates in cancer cells migration and invasion accelerated by Gal1-positive CAFs according to such observation in the present study. However, some reports show Gal1 inhibits the viability, proliferation and Th1 cytokine production of non-malignant T cells in patients with leukemic cutaneous T-cell lymphoma, and Gal1 silencing favors colorectal cancer with the ability to proliferate and escape apoptosis [52, 53]. Knockdown of endogenous Gal-1 in epithelial ovarian cancer (EOC) cells resulted in the reduction of cell growth. In the current study, cancer cells escaped from apoptosis when supplemented with CMs from Gal1-positive CAFs. These observations together raise a possibility that Gal1 inhibits cell proliferation and induces apoptosis in susceptible cells, and plays vital roles in tumor progression within the tumor microenvironment [54]. Elucidation of the roles and mechanism of Gal1 in CAFs will facilitate better understanding and rational approaches for the treatment of gastric cancer.

In summary, we have shown that Gal1 in fibroblast was sensitive to the stimulation of paracrine TGF- $\beta$  from cancer cells, and participated in the process of TGF- $\beta$ -driven NFs transformation into CAFs via Smad2 activation. Consequently, activated CAFs contribute to tumor progression such as anti-apoptosis and invasion,

which means TGF- $\beta$ /Gal1 axis may mediate cross-talk between CAFs and cancer cells in GC (Figure 10). More importantly, Gal1 expression in CAFs is associated tumor progression and poor prognosis. Take together, our data suggest that Gal1 might serve as a prognostic factor for GC and have an important role in the diagnosis and treatment of GC.

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#### Disclosure of conflict of interest

None.

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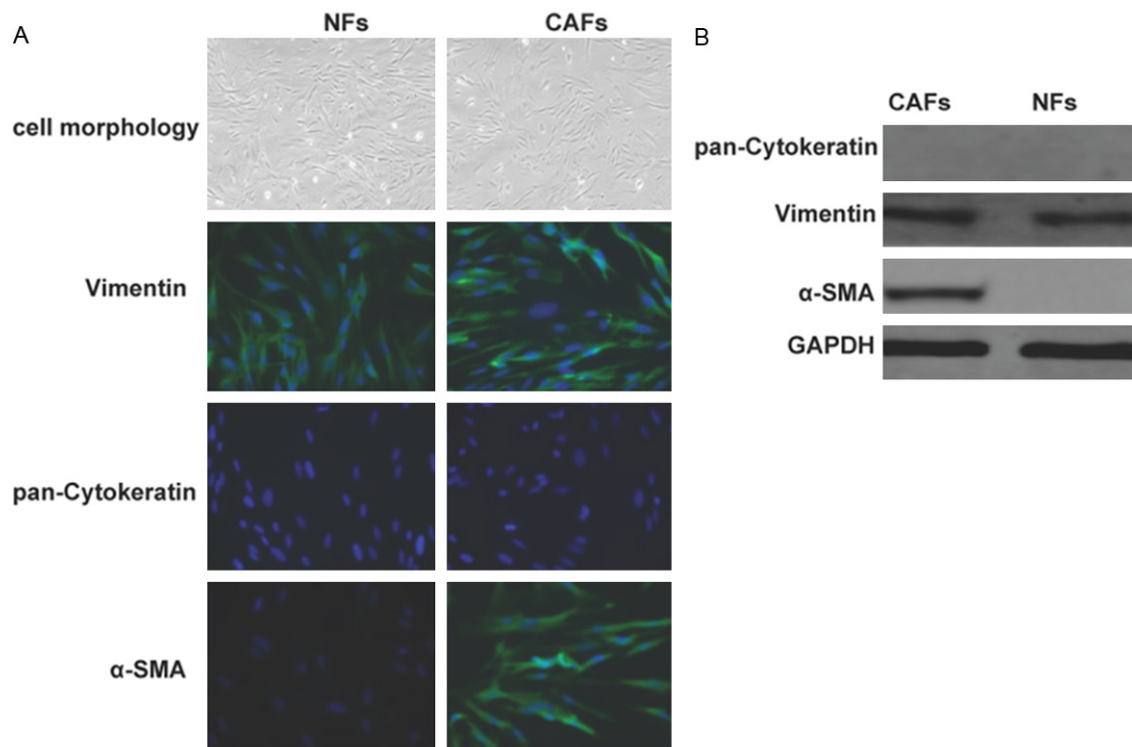


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## Regulation of TGF- $\beta$ -induced carcinoma-associated fibroblasts activation by galectin-1

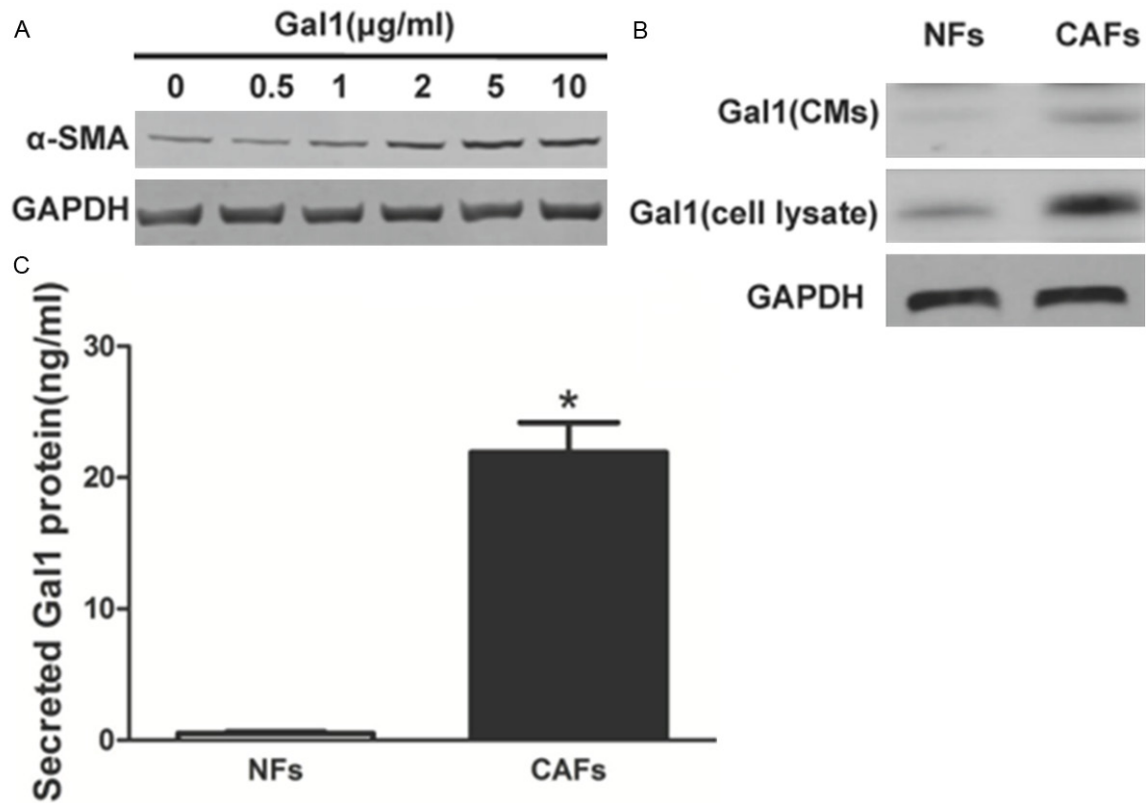
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# Regulation of TGF- $\beta$ -induced carcinoma-associated fibroblasts activation by galectin-1



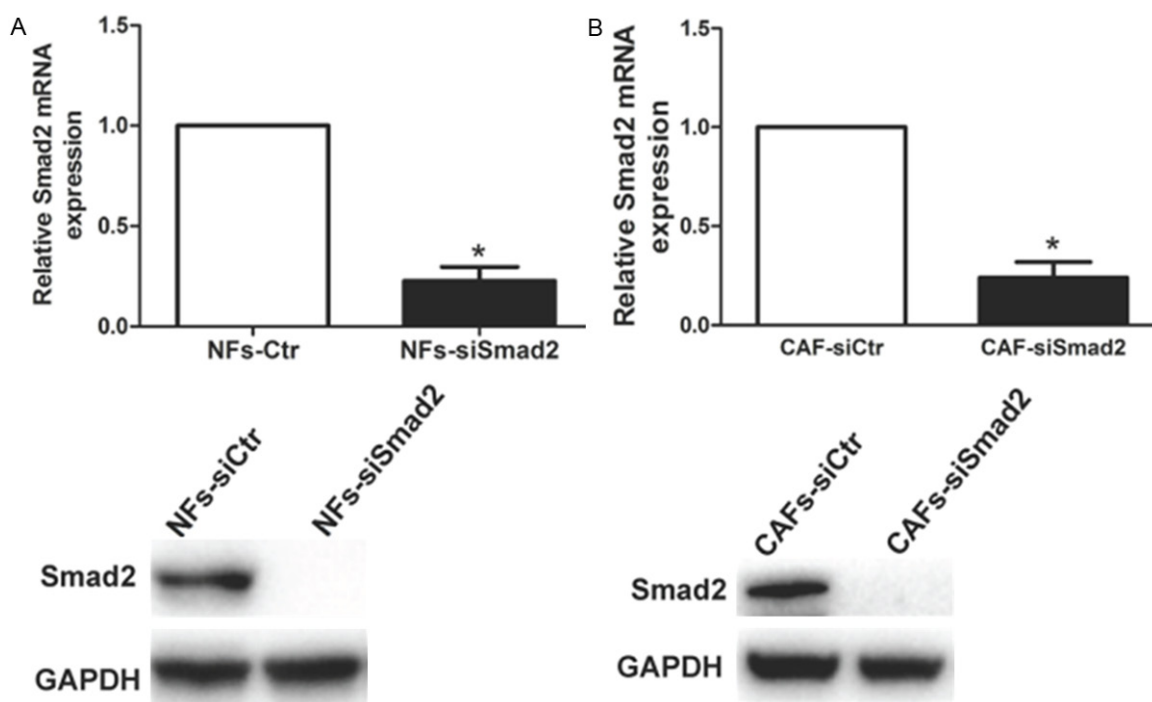
**Figure S1.** Identification of carcinoma-associated fibroblasts (CAFs) and normal fibroblasts (NFs) by immunocytochemistry and western blot analysis. A. Representative photographs of cell morphology and immunocytochemistry of CAFs and NFs (Magnification,  $\times 200$ ). B. Western blot analysis was used to analyze vimentin, pan-cytokeratin and  $\alpha$ -SMA expression in NFs and CAFs. Equal loading of protein was determined by GAPDH. Both NFs and CAFs positively expressed vimentin but negatively pan-cytokeratin. CAFs highly expressed  $\alpha$ -SMA, which differed from NFs.

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**Figure S2.** Exogenous Gal1 stimulates NFs transformation into CAFs in a dose-dependent manner. A. Western blot analysis of  $\alpha$ -SMA expression in NFs treated with recombinant Gal1 from 0.5  $\mu\text{g/ml}$  to 10  $\mu\text{g/ml}$ . B. Western blot analysis of Gal1 expression in cell lysate and CMs of NFs and CAFs. C. Secretion levels of Gal1 in CAFs and NFs were determined by ELISA. The results are shown as mean values  $\pm$  SD of three independent experiments and analyzed by unpaired Student's t-tests ( $*P < 0.05$ ).

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**Figure S3.** The efficiency of Smad2 knockdown by siRNA in fibroblasts was determined by qRT-PCR and western blotting. A. Top: Relative expression of Smad2 mRNA in NFs infected with either negative control siRNA (siCtrl) or Smad2 siRNA (siSmad2) using qRT-PCR. Bottom: Western blotting analysis of Smad2 expression in NFs of negative control siRNA and Smad2 siRNA. B. Top: Relative expression of Smad2 mRNA in CAFs of negative control siRNA and Smad2 siRNA using qRT-PCR. Bottom: Western blotting analysis of Smad2 expression in CAFs of negative control siRNA and Smad2 siRNA. The results are shown as mean values  $\pm$  SD of three independent experiments and analyzed by unpaired Student's t-tests (\* $P$ <0.05).