Chondromodulin-1 functions as a tumor suppressor in gastric adenocarcinoma

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Abstract. Chondromodulin-1 (ChM1) is a cartilage-specific glycoprotein that stimulates the growth of chondrocytes and inhibits the tube formation of endothelial cells. Endogenously, ChM1 is expressed in the cartilage and is an anti-angiogenic factor. ChM1 has been reported to suppress the proliferation of multiple human tumor cells in an anchorage-independent manner. However, the role of ChM1 in carcinogenesis of gastric cancer remains unknown. By quantitative RT-PCR and western blotting we examined the expression of ChM1 in gastric cancer tissue and normal gastric tissue. *In vitro* we investigated the functional and mechanistic roles of ChM1 in the inhibition of gastric cancer cell aggressiveness. We observed that ChM1 expression was remarkably downregulated in gastric cancer cell lines compared with the immortal normal gastric epithelial cell line GES-1. Importantly, ChM1 was frequently downregulated in gastric cancer tissue compared with normal gastric tissue. Low ChM1 mRNA expression was associated with higher clinical stages, higher lymph node metastasis, and poorer prognosis of patients. Functional assays *in vitro* showed that ectopic expression of ChM1 was able to inhibit gastric tumor cell proliferation by arresting the cell cycle. Overall, our findings indicate that ChM1 is a potential tumor suppressor in gastric cancer, suggesting that it may be useful as a biomarker for the treatment and prognosis of gastric cancer.

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Introduction

Gastric cancer is one of the leading causes of cancer-related death worldwide owing to its frequency, poor prognosis, and limited treatment options (1,2). According to a study released in 2011, a total of 98,9600 new gastric cancer cases and 73,8000 deaths were estimated to have occurred in 2008, accounting for 8% of the total cases and 10% of total deaths (3). The molecular mechanisms of gastric carcinogenesis is an area of active investigation (4,5), and multiple genes have been identified, including many tumor suppressor genes that contribute to the genesis of gastric cancer in a loss-of-function manner, such as SEMA3A (6), microRNA-34b/c (7), microRNA-30b (8), and LZTFL1 (9).

Chondromodulin-1 (ChM1) is a cartilage-specific glycoprotein that stimulates the growth of chondrocytes (10) and inhibits the tube formation of endothelial cells (11). Its expression is restricted to the cartilage, and is an endogenous anti-angiogenic factor. ChM1 has been shown to suppress the proliferation of multiple human tumor cells, such as human umbilical vein endothelial cells (12), human hepatocellular carcinoma HepG2 cells (13), and human osterogenic sarcoma U-2 OS cells (14), in an anchorage-independent manner. Previous preclinical studies have demonstrated that ChM1 has anti-angiogenic and antitumor properties *in vitro* and *in vivo* that involve several complicated mechanisms (12,15). In addition, ChM1 expression has been shown to be downregulated in certain pathologies, such as intervertebral disc (IVD) degeneration. Specifically, after administration of basic fibroblast growth factor (bFGF) in IVD cells, ChM1 was found to be downregulated and its expression correlated with the degree of IVD degeneration (16). However, the role of ChM1 in carcinogenesis of gastric cancer remains unknown.

Herein, we observed that ChM1 expression was remarkably downregulated in gastric cancer cell lines compared with the immortal normal gastric epithelial cell line, GES-1. ChM1 was frequently downregulated in gastric cancer tissue compared with normal gastric tissue. Low ChM1 mRNA expression was associated with higher clinical stages, higher lymph node metastasis, and poorer prognosis of patients. Functional assays *in vitro* showed that ectopic expression of ChM1 inhibited gastric tumor cell proliferation by inducing cell cycle arrest. Overall, our findings indicate that ChM1 is a potential tumor

suppressor, which could serve as a biomarker for therapeutic and prognostic use in gastric cancer patients.

Materials and methods

Ethics statement. For tissue samples, written informed consent was obtained from patients. The procedures used in this study were approved by the Institutional Review Board of the First Military Medical University and was conformed to the Helsinki Declaration, and to local legislation.

Tissue samples. Eighty-seven pairs of snap-frozen gastric tumor and matched normal tissues from adjacent regions were provided by the Xi'jing Digestive Hospital, the Fourth Military Medical University from February 2009 to December 2011. The samples were from patients treated surgically for clinical stage I-III gastric cancer (aged 31-84 years), with informed consent from each patient. No patient received preoperative chemotherapy, radiotherapy, or hormone therapy.

RNA purification, cDNA synthesis and quantitative real-time PCR. Total RNA of cultured cells was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and RNA was stored at -80˚C before qRT-PCR analysis. ChM1 expression was detected with primers: F, 5'-AGGGAAGCAAATGGAACTACTCT-3'; R, 5'-GGTGG GTCAGCAGTGTCAAA-3' (product length, 113 bp; Tm, 60˚C; GC F-43.48%, R-55%; start-end, 1,176-1,288 bp) and GAPDH was used as an internal control and the primers for it were: F, 5'-ACCACAGTCCATGCCATCAC-3'; R, 5'-TCCAC CACCCTGTTGCTGTA-3'. PCR products were separated on an ethidium bromide-stained 1.5% agarose gel and visualized with UV.

Cell lines and culture conditions. Gastric cancer cell lines SGC-7901, MKN-28, and the immortalized normal gastric epithelial cell line GES-1 were kindly bestowed by Professor Daiming Fan. All the cell lines were maintained in our institute according to recommended protocols. Cells were cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) at 37˚C in a 5% CO_2 incubator.

Immunohistochemistry. Tissue paraffin sections were deparaffinized, antigen retrieval was performed using citrate sodium buffer (pH 7.2) at 95˚C for 15 min, and the endogenous peroxidase was blocked using 3% hydrogen peroxide for 15 min. Then, the sections were treated with normal goat serum for 30 min to reduce non-specific binding followed by rabbit polyclonal anti-ChM1 (1:200, SC-33563, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) incubating for 1 h at 37˚C. Finally, sections were incubated with secondary antibody for 30 min at room temperature. Diaminobenzidine was used for color reactions (17).

DNA synthesis assay (BrdU incorporation). To assess the proliferation of cells, BrdU incorporation assay was used. Cells were harvested with trypsin/EDTA and suspended in RPMI-1640, as appropriate. The cells were seeded at $2x10^4$ cells/ml into a 96-well multi-titer plate (100 μl/well) and

cultured for 24 h. The cells were then starved in 0.5% FBS containing Opti-MEM for 12 h and stimulated with 10 ng/ml fibroblast growth factor-2 (FGF-2) (Yope Biotech Co., Ltd., Shanghai, China) in either the presence or absence of 25 μg/ml recombinant human ChM1 (rhChM1) for another 24 h. Cells were labeled with BrdU during the last 3 h of this incubation. The medium was then replaced with one containing either 10 or 25 μg/ml rhChM1, BrdU was added, and the cells were cultured for 6, 12 or 24 h. BrdU incorporation by the cells was measured at least in triplicate at each time-point using a cell proliferation ELISA BrdU colorimetric kit according to the manufacturer's instructions (Laizee Biotech Co., Ltd., Shanghai, China). The BrdU colorimetric kit was read for absorbance at 450 nm, and referenced at 655 nm, using a Model 680 Microplate Reader (Bio-Rad, Hercules, CA, USA).

Small interference RNA (siRNA) transfection. To knock down the ChM1 mRNA expression, siRNA transfection was performed. For transfections, Lipofectamine 2000 (Invitrogen) and 100 nM siRNA (Gene Pharma Co., Shanghai, China) were used according to the manufacturer's recommendations as described previously (18). Seventy-two hours after transfection, cells were used for examination, western blotting, and CCK-8 assay. The silenced cell line was named as SGC7901-siChM1 or MKN28-siChM1, while the matched control cell lines were named as SGC7901-siCtrl or MKN28-siCtrl, respectively. The siRNA sequences used are: 5'-UGGAUUUA UCCUACAGAUGCA-3'; 5'-CAUCUGUAGGAUAAAUCC AUA-3'.

Construction of pcDNA3.1(+)-ChM1 plasmid. To overexpress ChM1, pcDNA3.1(+)-ChM1 plasmid was constructed. The human ChM1 cDNA expression vector (pcDNA3.1(+)-ChM1) was constructed by CW Biotech Co., Ltd., Beijing, China. Briefly, the plasmid pcDNA3.1(+)-ChM1 was generated according to the cDNA sequence from GenBank. The ChM1 gene was generated by PCR amplification. The plasmid pcDNA3.1(+) was extracted through a Maxi Preparation kit (Omega, GA, USA). The PCR product was subcloned into the *Bam*HI (Takara, Mountain View, CA, USA) and *Hin*dIII (Takara) sites of pcDNA3.1 plasmid by T4 ligase (Takara). The pcDNA3.1(+)-ChM1 construct was verified by DNA sequencing (Invitrogen, Grand Island, NY, USA) (data not shown).

Generation of ChM1 stable cell lines. To empirically determine the proper concentration of G418 antibiotic to use for selection of ChM1 stable-expressing clones, SGC-7901, MKN-28 cells were cultured in 12-well plates with $1.0x10^5$ cells in each well, in an incubator with constant supply of 5% CO_2 at 37˚C. The medium was changed 24 h later with different concentrations of antibiotic G418 (0, 50, 100, 200, 400, 600, 800 and 1,000 μg/ml) and replaced every 3 days. Medium with 800 μg/ml G418 was used for further experiments as it is the minimum concentration to induce total cell death 14 days after cell culture. Having determined the proper G418 concentration for selection, parental cells were transfected with the pcDNA3.1(+)-ChM1 plasmids using Lipofectamine 2000 according to the manufacturer's instructions. The density of cells was $2x10^5$ cells per well in 6-well plates. Monoclonal cell

colony with G418 resistance was generated using the limiting dilution method by culturing single cell in 100 μl medium in 96-well plates for 24 h. Monoclonal cell colonies were digested 15 days later for further amplification to culture cells with stable ChM1 expression in 24-well plates. Cells were transferred to cell culture flask until ~90% confluent. The ChM1 overexpressed cell lines transfected by pcDNA3.1(+)-ChM1 were named as SGC7901-ChM1 or MKN28-ChM1, while the matched control cell lines were named as SGC7901-NC or MKN28-NC, respectively.

Cell counting kit (CCK-8) assay. Cell viability was performed using the Cell Counting kit (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assay, as described previously (19). Cells were seeded in 200 μl/well of medium at a concentration of 1×10^4 cells/well into 96-well plates and incubated overnight for attachment. Then, culture medium was removed and fresh medium (100 μl/well) and 10 μl CCK-8 solution were added and cells were incubated for 1 h at 37°C. The optical density (OD) value (absorbance) was measured at 450 nm by a microplate spectrophotometer (Multiskan, MK3, Thermo, USA). All experiments were performed in quadruple on three separate occasions.

Colony-formation assay. To assess the anchorage-dependent proliferation of cells, a colony-formation assay was performed. The log-phase cells were harvested, plated into 6-well plates (500 cells/well), and chemotherapeutic drugs were added into the culture medium on the second day. The resulting colonies were stained with Coomassie Brilliant Blue (Sigma, Inc., St. Louis, MO, USA), and the visible colonies were counted after 2 weeks.

Cell invasion and migration assays. Cell invasion and migration capacity was assessed by Transwell permeable supports with 8-μm pore size (Costar, Cambridge, MA, USA). As instructed by the manufacturer, cells suspended in serum-free medium were seeded into Transwell inserts either uncoated (for migration assay) or coated (for invasion assay) with growth factor-reduced Matrigel (BD Biosciences, Bedford, MA, USA) (20). Bottom wells were incubated with complete medium, and 24 h later the invaded cells were fixed with methanol and stained with a crystal violet solution. The number of cells that penetrated the membrane was determined by counting the mean cell number in five randomly selected high-power fields.

Western blotting. Total protein from cultured cells were lysed using lysis buffer supplemented with phenylmethylsulfonyl fluoride (1 mM) on ice. Protein was electrophoresed through 12% SDS polyacrylamide gels and then transferred to a PVDF membrane (Millipore, MA, USA). Membranes were blocked with 5% non-fat milk powder at room temperature for 1 h and incubated overnight with primary antibodies. Membranes were incubated with secondary antibodies labeled with HRP for 1 h at room temperature after three 10-min washes in triethanolamine buffered saline solution with Tween (TBS-T). Finally, the signals were detected using an ECL kit (Pierce Biotech., Rockford, IL, USA) and the membranes were scanned and analyzed using a ChemiDoc XRS+ (Bio-Rad, CA, USA) imaging system with imaging software (Version 1.0). The

protein expression was normalized to an endogenous reference GAPDH and relative to the control. The Spectra multicolor broad-range protein ladder (Beyotime, Jiangsu, China) was used as a molecular marker. The antibodies used in the western blot assay are as follows: ChM1, sc-33563, 1:200, 25 kDa; Akt, sc-1618, 1:200, 62 kDa; GSK-3β, sc-377213, 1:100, 47 kDa; GAPDH, sc-365062, 1:5,000, 37 kDa (Santa Cruz Biotechnology, Inc. TX, USA)

Luciferase reporter assay. The nucleotide sequence of the STAT response elements was 5'-gatccagttcccgtcaatcg-3'. These constructs express Renilla luciferase. A reference construct was prepared by digesting the HSV-TK promoter between the *Bam*H1 site and *Hin*dIII sites from the pRL-TK vector (Promega Corp., Madison, WI, USA) that expresses Renilla luciferase, and cloning this fragment into the pGL4.18 [luc2p/Neo] vector (Promega) that expresses Firefly luciferase. The cells were infected with virus and cultured for 12 h then washed twice with culture medium and then transfected with various luciferase expression vectors by the lipofection method. The cells were harvested 24 h after transfection, and a Dual-Luciferase™ reporter assay system (Promega) was performed for sequential measurement of Firefly and Renilla luciferase activities using the specific substrates beetle luciferin and coelenterazine, respectively. Quantification of luciferase activities and calculation of relative ratios were carried out using a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA, USA). In these experiments, at least three independent transfections were performed.

Statistical analysis. The data were analyzed using SPSS 12.0 software (SPSS Inc., Chicago, IL, USA). All experiments in this study were repeated in triplicate. The Student's t-test was used to analyze the statistical significance of the differences between groups. χ^2 test and Fisher's exact test were used to assess the correlation between ChM1 and clinical pathologic parameters. For all the tests, P-values <0.05 was considered statistically significant.

Results

ChM1 was downregulated in human gastric cancer cells. ChM1 expression in four gastric cancer cell lines and one immortal normal gastric epithelial cell line were quantified by qRT-PCR (Fig. 1A) and western blotting (Fig. 1B). Among the five cell lines analyzed, ChM1 was found to be expressed at lower levels in gastric cancer cells, compared with normal mammary gastric epithelial GES-1 cells. Among the gastric cancer cells, SGC7901, MKN28, and GC9811 cells expressed relatively higher levels of ChM1, compared with the BGC-823 cell line, which had low expression or barely detectable ChM1 levels.

Expression of ChM1 is downregulated in human gastric cancer tissue. We found that ChM1 was significantly downregulated in 61 (70%) gastric cancer clinical tissues, compared with non-cancerous tissues (Fig. 2A). ChM1 expression of human gastric cancer clinical tissues was examined by immunohistochemistry and western blotting, which indicated that 59 and 63 patients had significantly lower ChM1 expression,
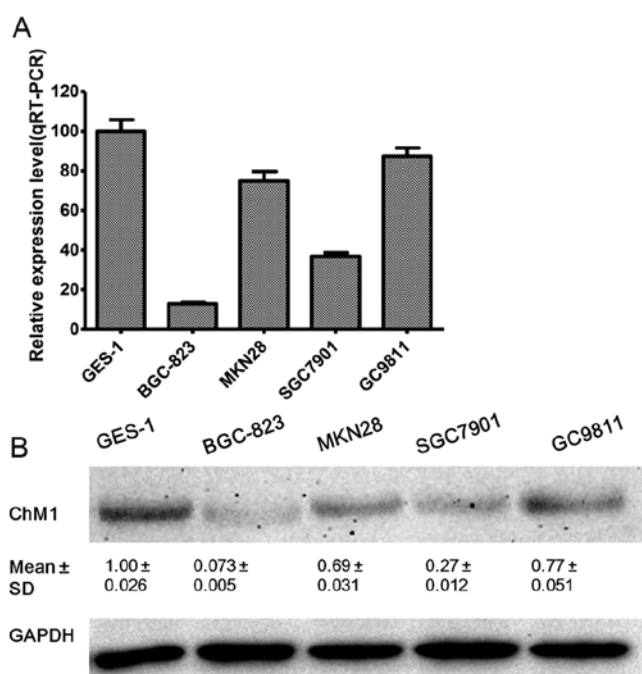
Figure 1. ChM1 expression in gastric cell lines. (A) ChM1 expression in four gastric cancer cell lines and GES-1 were quantified by qRT-PCR (one-way ANOVA analysis, F=689.4, ***P<0.0001). (B) ChM1 expression in four gastric cancer cell lines and GES-1 were quantified by western blotting (one-way ANOVA analysis, F=146.6, ***P<0.0001). Data were means of three separate experiments (mean ± SD).

Table I. The relationship between clinical parameters and ChM1 (mean ± SD) mRNA expression in primary gastric adenocarcinoma.

Clinical parameters	N (%)	Relative expression	P-value
Age (years)			
≥60	38 (43.7)	0.4317±0.02569	0.44
<60	49 (56.3)	0.4168±0.01972	
Gender			
Male	64 (73.6)	0.4095±0.01903	0.59
Female	23 (26.4)	0.4257±0.01655	
Size (cm)			
≥5	52 (59.8)	0.3921±0.02215	0.31
<5	35 (40.2)	0.4292±0.02734	
Histologic differentiation			
Well (W)	26 (29.9)	0.4325±0.01833	0.37
Moderately (M)	32 (41.4)	0.4196±0.02360	
Poorly (P)	29 (28.7)	0.4078±0.02074	
Lymphatic metastasis			
No	29 (29.9)	0.5702±0.05269	0.0017[a]
Yes	58 (70.1)	0.3615±0.02173	
TNM stage			
Stage I	22 (25.3)	0.5481±0.04722	0.0025[a]
Stage II/III	65 (74.7)	0.3969±0.03341	

as detected by immunohistochemistry (Fig. 2B) and western blotting (Fig. 2C) analysis, respectively. To gain further insight into this observation, we examined the relationship between ChM1 expression and the patients' clinical parameters. Analysis showed that ChM1 expression negatively correlated with lymph node metastasis and tumor-node-metastasis (TNM) stage (Tables I and II), but was irrelevant with age, sex, tumor differentiation, and tumor size.

ChM1 inhibits proliferation and growth in human gastric cancer cells in vitro. To investigate the role of ChM1 in the proliferation and growth of human gastric cancer cells, we generated gastric cancer cell lines to overexpress ChM1. Cells were transfected with pcDNA3.1(+)-ChM1, and after antibiotic selection the stable clones were named as SGC7901-ChM1 or MKN28-ChM1, while the matched control cell lines were named as SGC7901-NC or MKN28-NC, respectively. In addition, we also knocked down ChM1 using siRNA. The silenced cell line was named as SGC7901-siChM1 or MKN28-siChM1, while the matched control cell lines were named as SGC7901-siCtrl or MKN28-siCtrl, respectively. The expression levels were determined using both western blot (Fig. 3A and B) and qRT-PCR (Fig. 3C and D) analyses. As shown in Fig. 3E and F, ChM1 overexpression led to a significant decrease in cell proliferation, while ChM1 knockdown led to a significant increase in cell proliferation. To further demonstrate the mechanism by which ChM1 overexpression or knockdown affected proliferation, cell cycle progression was analyzed using flow cytometry. SGC7901-ChM1 cells showed a delayed G1 phase compared with SGC7901-NC cells, while MKN28-ChM1 cells also showed a delayed G1 phase

Table II. The relationship between clinical parameters and ChM1 protein expression in primary gastric adenocarcinoma.

| Clinical parameters	N (%)	ChM1 low expression	ChM1 high expression	P-value
Age (years)				
≥60	38 (43.7)	27	11	0.82
<60	49 (56.3)	36	13	
Gender				
Male	64 (73.6)	47	17	1.00
Female	23 (26.4)	17	6	
Size (cm)				
≥5	52 (59.8)	39	13	0.62
<5	35 (40.2)	24	11	
Histologic differentiation				
Well	26 (29.9)	18	8	0.99
Moderately	32 (41.4)	22	10	
Poorly	29 (28.7)	20	9	
Lymph node/ venous metastasis				
No	21 (24.1)	7	14	<0.01[a]
Yes	66 (75.9)	49	17	
TNM stage				
Stage I	22 (25.3)	9	13	0.016[a]
Stage II	30 (34.5)	21	9	
Stage III	35 (40.2)	29	6	
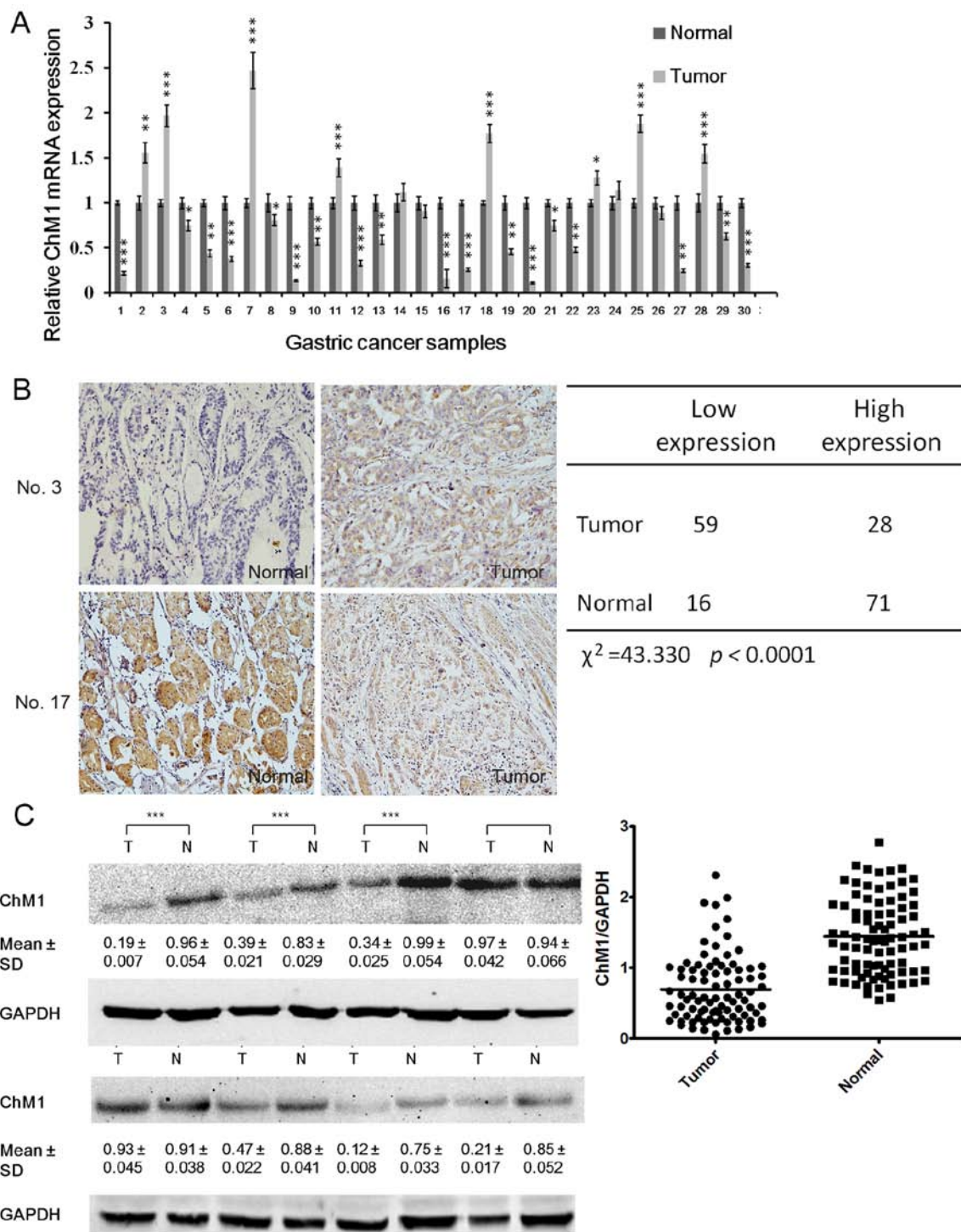
Figure 2. ChM1 expression in gastric cancer tissue. (A-C) ChM1 expression in gastric cancer tissues examined by qRT-PCR (A), immunohistochemistry (B) and western blotting (the gray value ratio of GES-1 cell line was taken as 1.00) (C). Data were means of three separate experiments (mean ± SD), one-way ANOVA analysis, ***$P<0.0001$, **$P<0.001$, *$P<0.05$.

compared with MKN28-NC cells (Fig. 3G). The ability of SGC7901 or MKN28 cells to form colonies was inhibited when ChM1 was overexpressed. Conversely, the ability of SGC7901 or MKN28 cells to form colonies was enhanced when ChM1 was knocked down (Fig. 3H).

ChM1 suppresses migratory and invasive potential. In addition to regulating cell proliferation, ChM1 was also found to regulate SGC7901 cell migration and invasion. As shown

in Fig. 4A and C, the overexpression of ChM1 decreased cell migration in a gap wound assay after 24 h by 45 μm (Fig. 4A), while ChM1 knockdown increased migration by 89 μm (Fig. 4C), compared with the control cells. In addition, a three-dimensional cell migration assay was performed using transwell chambers and an invasion assay was performed with Matrigel-precoated transwell chambers. It was found that ChM1 overexpression exhibited a significant reduction in the migration and invasion capabilities (Fig. 4B). Conversely,
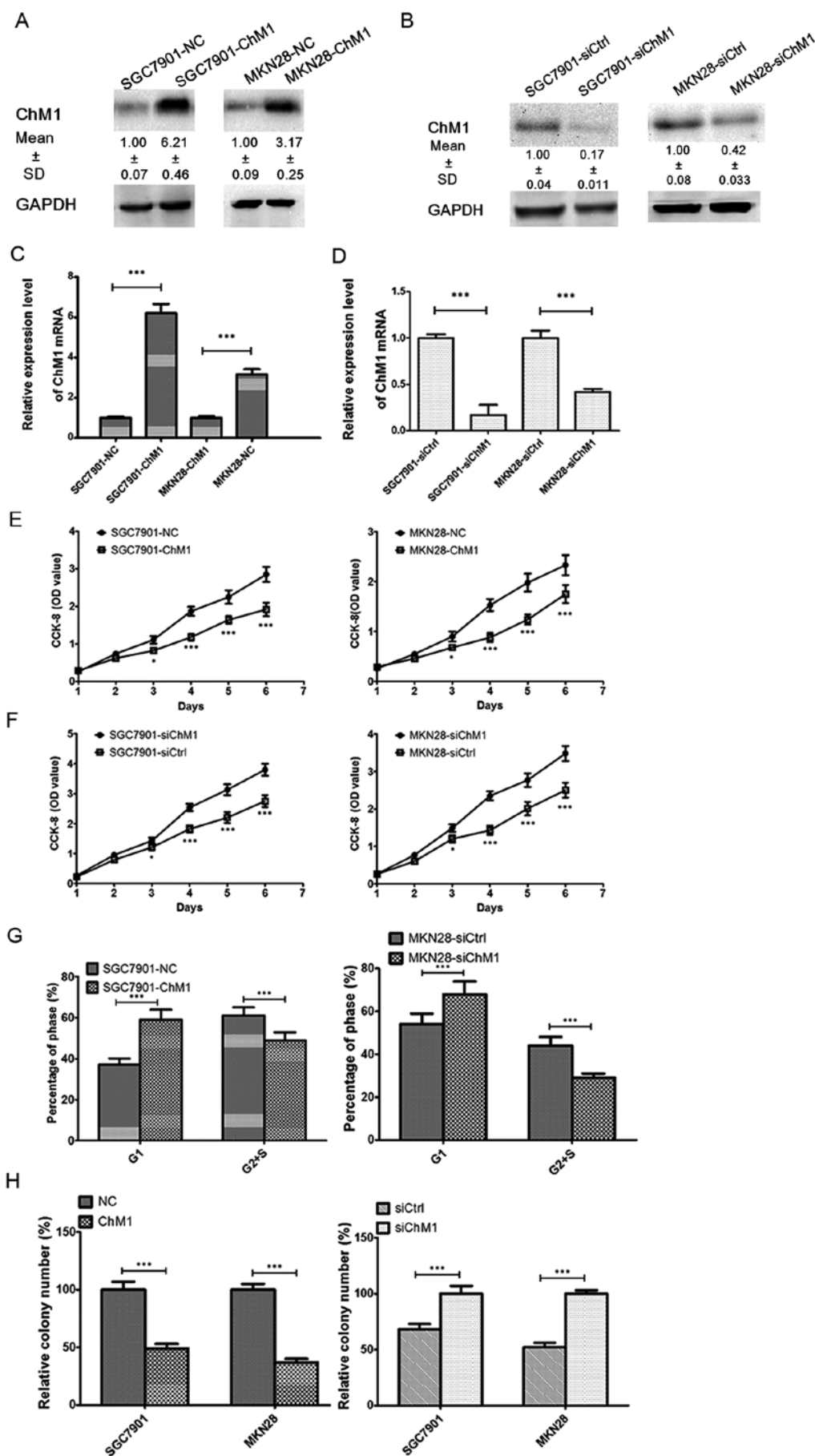
Figure 3. Effect of ChM1 on proliferation and growth of gastric cancer cell lines SGC7901 and MKN28. (A and B) Western blotting and (C and D) qRT-PCR methods were used to examine ChM1 expression. (E and F) The growth of cells over 6 days was examined using cell counting kit (CCK-8) assays. (G) Percentage of phase of cell cycle progression measured by flow cytometry. (H) Colony number of SGC7901 and MKN28 cells in soft agar. Data were means of three separate experiments (mean ± SD), *P<0.05, **P<0.001, ***P<0.0001.
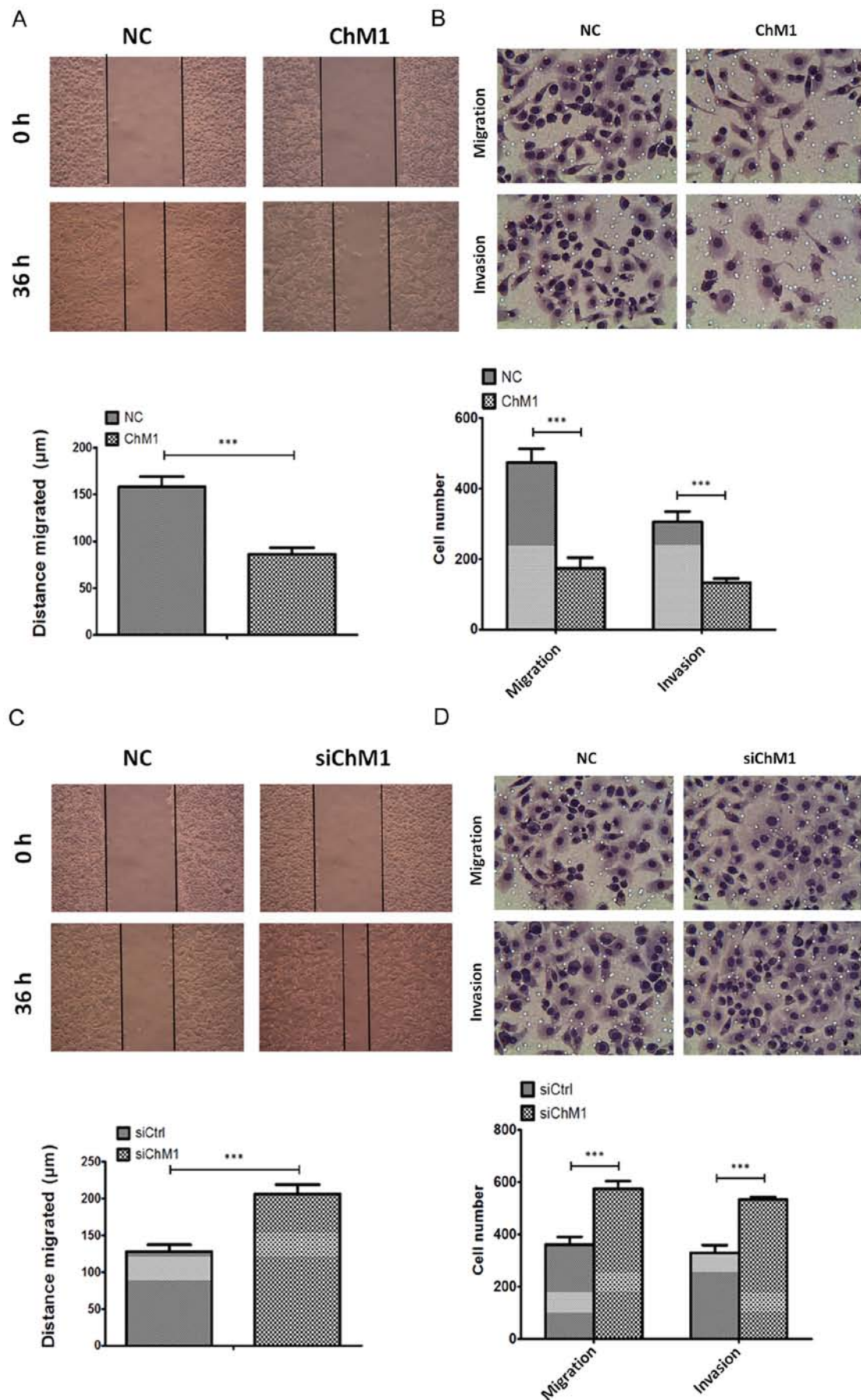
Figure 4. ChM1 significantly suppressed migratory and invasive potential of the gastric cancer cell line SGC7901. (A and C) Wound healing assay. Images of wound repair were taken at 0 and 36 h after wounding. The distance of wound closure is shown by area at 36 h. Representative images (upper) and quantification (lower) are shown, original magnification, x200. (B and D) Transwell migration assay and Matrigel invasion assay. Representative images (upper) and quantification (lower) are shown. Columns, average of three independent experiments, ***P<0.0001, original magnification, x200.
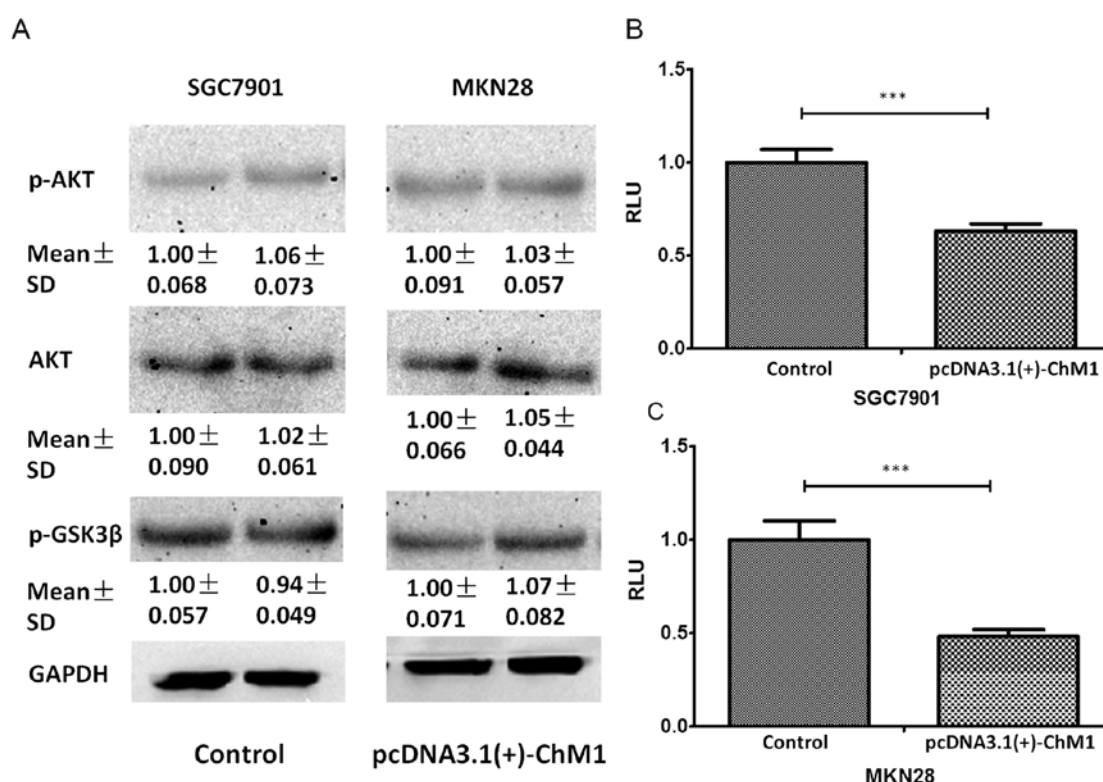
Figure 5. The effect of ChM1 on the downstream pathway of extracellular matrix-integrin signaling and STAT pathway. Western blotting (A) showing phosphorylation levels of Akt and GSK3β, the downstream molecules of the extracellular matrix-integrin signaling pathway. (B and C) pcDNA3.1(+)-ChM1 inhibited the promoter activity of STAT-luc in SGC7901 and MKN28. ***P<0.0001.
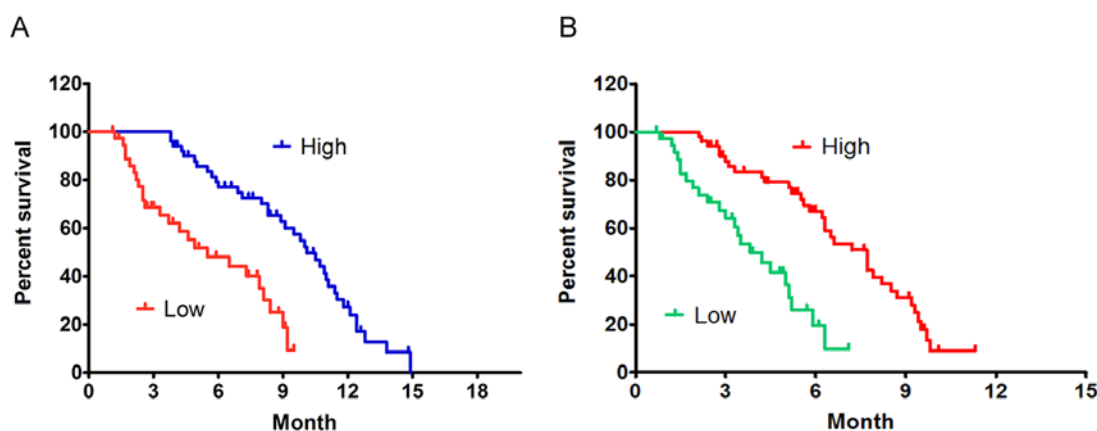

Figure 6. Low ChM1 expression correlates with poor survival in gastric cancer. Kaplan-Meier survival curves of overall (A) and event-free (B) survival. Low ChM1 expression correlates with favorable overall (P<0.0001) and event-free (P<0.0001) survival, whereas low ChM1 expression correlates with poor outcome. Cut-point analysis for overall and event-free survival was done for ChM1 using maximally selected rank statistics. The optimal cut-point was relative expression level at 0.56 for overall survival as well as event-free survival. Relative expression level above this cut-point were considered as high ChM1 expression, whereas lower values were considered as low ChM1 expression.

ChM1 knockdown exhibited a significant increase in migration and invasion capabilities (Fig. 4D).

Effect of ChM1 on downstream molecules of the extracellular matrix-integrin signaling and STAT pathways. Given that ChM1 has a direct antitumor effect by inhibiting the STAT signaling pathway, we verified this pathway to establish the potential pathway via which ChM1 exerted its tumor suppressor role (21). The results from our soft-

agar assay demonstrated that ChM1 directly suppressed anchorage-independent tumor cell growth. Therefore, to further illustrate the mechanism of this function, the anchorage-dependent signaling including integrins and their downstream signaling pathway, which includes Akt and glycogen synthase kinase 3-β (GSK3β) (22-24) were examined. It was found that phosphorylation of Akt and GSK3β was unaffected 24 h post-pcDNA3.1(+)-ChM1 transfection (Fig. 5A). Furthermore, the luciferase reporter assay showed

that pcDNA3.1(+)-ChM1 inhibited the promoter activity of STAT-luc in SGC7901 and MKN28 cultured on plates (Fig. 5B and C).

Low ChM1 expression levels indicates poorer clinical outcome of GC patients. Low ChM1 transcript level indicates poorer clinical outcome of GC patients. In this study, Kaplan-Meier estimates for overall survival and event-free survival were calculated to determine whether ChM1 expression levels are related to differences in clinical outcome. It showed that ChM1 expression was negatively correlated with patients outcome. In GCs with low ChM1 expression, median survival time was 5.5 months versus 10.1 months in tumors with high ChM1 expression (ratio=1.836, 95% confidence interval of ratio, 1.240-2.433) (Fig. 6A). Event-free survival was 3.8 months versus 7.7 months (ratio=2.026, 95% confidence interval of ratio, 1.437-2.615), respectively (Fig. 6B).

Discussion

In this study, we discovered three lines of evidence supporting a critical role for ChM1 in gastric cancer progression. First, we found that ChM1 expression was downregulated in gastric cancer, which was significantly associated with both lymph node metastasis and TNM stage of gastric cancer patients. Second, exogenous expression of ChM1 led to decreased cell growth and invasive properties *in vitro*, whereas knockdown of ChM1 resulted in greater cell growth and invasiveness. Third, ChM1 suppressed the expression of STAT. Therefore, we propose a new role for ChM1 as a novel suppressor of tumor invasion and metastasis in gastric cancer.

Invasion and metastasis have been shown to be important hallmarks of cancer. It has been found that local administration of recombinant human ChM1 almost completely blocked vascular invasion and tumor growth *in vivo*. Moreover, ChM1 also inhibited the growth of HT-29 colon adenocarcinoma cells *in vivo*, implying its therapeutic potential for solid tumors (25). Our study showed that ChM1 expression decreased the invasive potential and suppressed the metastasis potential of gastric cancer cells, suggesting ChM1 might serve as a suppressor of metastasis.

A previous study demonstrated that ChM1 knockout directly interfered with *in vivo* ectopic cartilage regeneration when chondrocytes were subcutaneously injected into nude mice with Matrigel (26,27). Moreover, ChM1 knockout compromised ectopic stability of *in vitro* regenerated cartilage after subcutaneous implantation (28). Furthermore, ChM1 removal from the inner meniscus-derived medium and functional blocking of ChM1 significantly increased endothelial cell proliferation, suggesting that ChM1 may be a key anti-angiogenic factor for maintaining the avascularity of the inner meniscus (29-31). Intriguingly, we found that ChM1 had a greater effect on gastric cancer cell invasion than cell growth, which prompted us to focus our studies on the role of ChM1 in gastric cancer invasion.

Signal transducer and activator of transcription 3 (STAT3) exerts an essential role in a variety of physiological functions, including development (32,33), proliferation (34,35), and immune defense (36). Increasing evidence indicates that STAT3 promotes tumorigenesis of a variety of cancers

(37,38), causing it to be recognized as an oncogene (39,40). In fact, strategies aimed at the co-targeting of STAT3/NF-κB activation and the interaction between them has garnered attention in other cancers, such as colorectal cancer, and might be an attractive and novel approach to combat gastric cancer (41).

In general, we have found that ChM1 acts as a tumor suppressor by inhibiting the growth of gastric cancer cells, and the mechanism of the induced growth arrest appears to involve the anchorage-independent Jak/STAT pathway.

Acknowledgements

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