

Bcl-2 associated athanogene 4 promotes proliferation, migration and invasion of gastric cancer cells

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Abstract. Currently, with the increase of morbidity and mortality rate, gastric cancer (GC) is attracting increasing attention in China. Bcl-2-associated athanogene 4 (BAG4) has been identified as a tumor promoter in several tumors, but its role in GC remains unknown. The present study aimed to detect the expression of BAG4 and determine its function in the progression of GC. The results from reverse transcription-quantitative polymerase chain reaction and western blotting revealed that BAG4 was markedly upregulated in highly metastatic cell lines (SGC7901 and MGC803), compared with the lower-metastatic cell lines (AGS and BGC823). Through Cell Counting Kit-8, cell cycle, apoptosis, Transwell and colony formation assays, BAG4 was demonstrated to promote the proliferation, migration and invasion of GC cells *in vitro*. Additionally, *in vivo* assays further certified that BAG4 can increase the proliferation and invasion of GC cells. In conclusion, these findings implicate BAG4 as a potential therapeutic target for GC.

Introduction

Currently, gastric cancer (GC) is the fourth most common malignancy in the world (1). The development of GC is a multistep process, involving progressive disruption of epithelial-cell proliferation, apoptosis, differentiation and survival mechanisms (2,3). With advancing modern medical therapies,

the rate of GC mortality has been decreased (4). It is necessary to identify the biomarkers that can distinguish between GC patients with poor or good prognosis.

Bcl-2 associated athanogene 4 [BAG4, also known as Silencer of Death Domains (SODD)] is a member of the BAG1-related protein family (5). The BAG proteins are located in both cytoplasmic and nuclear in cells (6), although the functional importance of this remains unclear. BAG proteins have a conserved BAG domain (BD) that binds to the ATPase domain of Hsp70/Hsc70, and regulates the activity of these molecular chaperones (7-9). Previous studies have reported that BAG protein family members are associated with aggressiveness of breast, gastric, and pancreatic cancer (10-13). Furthermore, loss of BAG4 is associated with the resistance of platinum chemotherapy (6,14). However, the role of BAG4 in gastric cancer remains unclear.

The present study detected the expression of BAG4 in GC cell lines and examined the effect of BAG4 on GC cell proliferation, migration and invasion *in vitro* and *in vivo*.

Materials and methods

Cell lines and animals. The SGC7901, BGC823, AGS, MGC803 and MKN45 human GC cell lines were purchased from the Chinese Academy of Sciences (Beijing, China) and cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.). All cell lines were cultured in 5% CO₂ at 37°C. A total of 24 male athymic BALB/c mice (age, 4-6 weeks old, 15.2-16.3 g) were purchased from the Central Laboratory of Animal Science at North Sichuan Medical College (Nanchong, China). They were housed at 24°C, with a humidity of 60-70%, a 12-h light/dark cycle and free access to food and water. All protocols for animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of North Sichuan Medical College in accordance with the NIH Guide for the Care and Use of Laboratory Animals (15) and the Animal Welfare Act.

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Construction of plasmids and transfection. For overexpression or depletion of BAG4, AGS cell lines were selected for stable transfection with a BAG4-expressing vector, and SGC7901 cell lines were selected for transient transfection with short hairpin

(sh)RNA lentivirus vectors (Shanghai GeneChem Co., Ltd., Shanghai, China) for BAG4. The lentivirus vectors carrying the fragment of coding sequence (CDS) or short hairpin (sh)RNA were transfected into 293T lentiviral packaging cell lines. The primers were as follows: Forward, 5'-ATGTCG GCCCTGAGGCGCT-3' and reverse, 5'-TCATAATCCTTT TTTTCTAATTTT-3' for BAG4; and 5'-CCAACAATCAAG ATCAAAGTA-3' for shRNA. Following this, 1 ml viral supernatant containing 4 μ g polybrene was added into GC cell lines for stable transduction. After 14 days, puromycin-resistant cell pools were established. After 72 h, the protein expression level of BAG4 was detected by western blot analysis, and transfected cells were subjected to Transwell assay.

Transwell assay. The invasion Boyden Chambers (BD Biosciences, Franklin Lakes, NJ, USA) were rehydrated with RPMI-1640 (serum-free) for 2 h at 37°C. RPMI 1640 supplemented with 100 ml/l fetal bovine serum was added to the lower compartment as the chemotactic factor. Following this, 1.5×10^5 tumor cells in serum-free RPMI 1640 were added to the upper compartment of the chamber. Each cell group was plated into three duplicate wells. After incubation for 48 h, the noninvasive cells were removed with a cotton swab. Cells that had migrated through the membrane and stuck to the lower surface of the membrane were fixed with methanol and stained with hematoxylin. Finally, the cells in lower compartment of the chamber that had migrated to the lower sides of the membrane were counted under a light microscope in five random visual fields (magnification, x200).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted from the five cell lines using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and cDNA was synthesized using an access reverse transcription system (Promega Corporation, Madison, WI, USA). The temp/duration of the RT reaction was as follows: 37°C for 15 min and 85°C for 5 sec. qPCR was performed using 7500 Fast Real-time PCR System (Thermo Fisher Scientific, Inc.), using the following thermal cycling profile: 95°C for 10 min, followed by 35 cycles of amplification (95°C for 10 sec, 60°C for 30 sec and 72°C for 34 sec). The method of quantification was adapted from Livak and Schmittgen (16). The PCR primers were as follows: forward, 5'-AATGGAGCGTATGGT CCAACA-3' and reverse, 5'-GGTGCATAATAAGCCCCT GAGT-3' for BAG4; and 5'-AGGTCGGTGTGAACGGAT TTG-3' and reverse, 5'-GGGGTCGTTGATGGCAACA-3' for GAPDH. First-strand cDNA was synthesized using a SYBR PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). qPCR was performed using SYBR Premix Ex Taq II (Takara Biotechnology Co., Ltd.) and measured in a LightCycler 480 system (Roche, Basel, Switzerland). Human GAPDH gene was amplified as an internal control. Each sample was tested three times and all other quantities were expressed as an n-fold difference relative to the corresponding control group.

Cell proliferation assay. AGS and SGC7901 cells (1×10^3) were seeded into 96-well plates. The number of viable cells was determined by Cell Counting Kit-8 (CCK-8; Dojindo

Molecular Technologies, Inc., Kumamoto, Japan) for 6 days. Briefly, 10 ml CCK-8 solution was added, and absorbance was measured at a wavelength of 490 nm using a microplate reader (Enspire 2300 M R; PerkinElmer, Inc., Waltham, MA, USA) after 2 h of incubation at 37°C. Each cell group was plated into three duplicate wells.

Cell cycle analysis. AGS and SGC7901 cells ($\sim 1 \times 10^6$) cells were trypsinized, washed twice with PBS and fixed in 70% ice-cold ethanol for 1 h. The samples were centrifuged at 300 x g for 5 min at 4°C, the ethanol removed and they were exposed to 100 mg/ml RNaseA (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 30 min at 37°C. Cellular DNA was stained with propidium iodide (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Cell-cycle distributions were determined by flow cytometry using a BD FACSCalibur system (BD Biosciences, Franklin Lakes, NJ, USA) and data was analyzed using the ModFit software version 4.1 (Verity Software House, Inc., Topsham, ME, USA).

In vitro apoptosis assay. AGS and SGC7901 cells were transfected with 50 nM oligo using Lipofectamine[®] 2000 according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were subsequently harvested and stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI), according to the manufacturer's protocol (BioVision, Inc., Milpitas, CA, USA). Annexin V-FITC/PI binding was analyzed by flow cytometry using a BD FACSCalibur system and data was analyzed using the CellQuest software version 5.1 (BD Biosciences, Franklin Lakes, NJ, USA).

In vivo tumorigenesis assay. To evaluate *in vivo* tumor growth, experimental mice were injected subcutaneously in to the left flank with 1×10^7 AGS cells carrying BAG4 overexpressing vector and the control mice were treated with 1×10^7 AGS cells carrying control lentivirus vectors (n=6 per group). The sizes of tumors were measured with calipers to estimate volumes by length x width x height from days 5 to 35 following injection. BAG4 expression was detected in xenograft tumors by western blotting. The proliferative index of Ki-67 was evaluated in xenograft tumors by immunohistochemistry (IHC). For tail vein metastasis assay, a total of 1×10^6 cells were injected into the tail veins of nude mice. After 35 days, the mice were sacrificed and the lung tissues dissected out and subjected to histological examination. Metastatic tumors were detected by H&E staining and quantified by counting metastatic lesions in each section. Images were captured by Olympus DP72 microscope and were analyzed by DP2-BSW software version 1.3 (Olympus Corporation, Tokyo, Japan).

IHC. After dissection, tissues were washed twice with PBS and fixed with 10% neutral formalin for 2 h and embedded in paraffin. Then paraffin-embedded specimens were cut into 4 mm sections. The sections were deparaffinized with xylene and rehydrated. Sections were submerged into EDTA antigenic retrieval buffer and microwaved for 10 min for antigenic retrieval. The sections were treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity. Rabbit monoclonal antibody against Ki67 (ab92742;

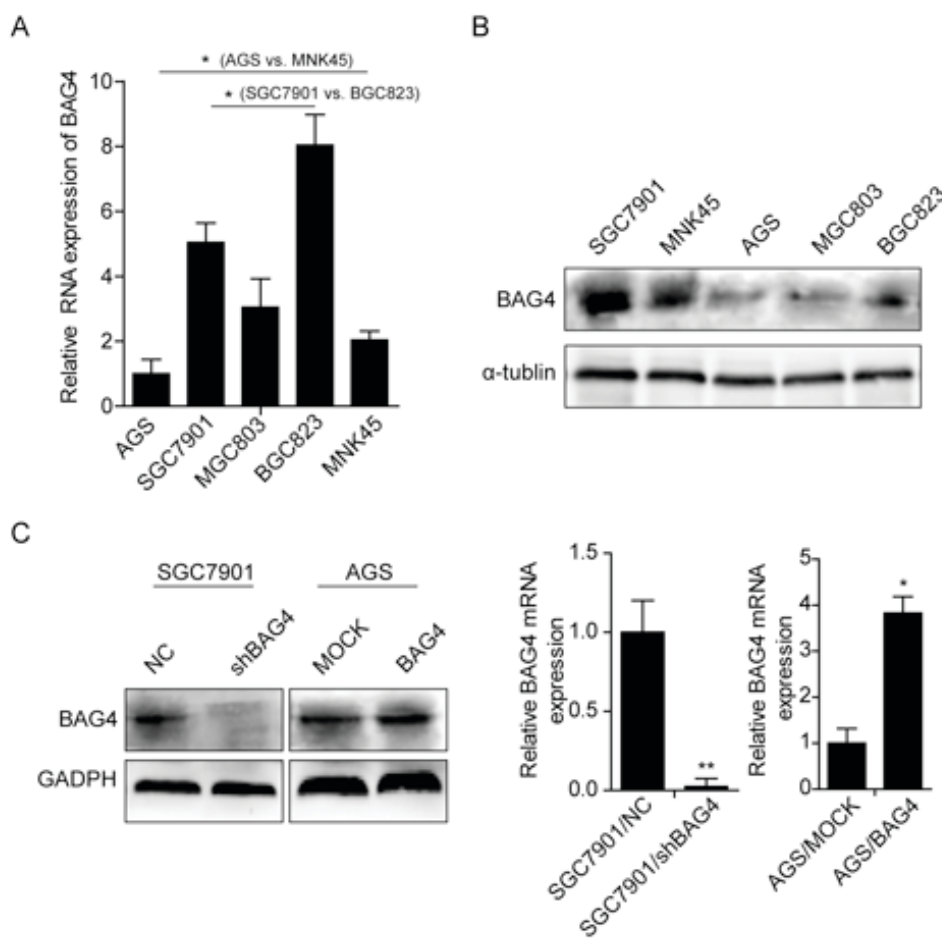


Figure 1. BAG4 is upregulated in human gastric cancer cell lines. (A) mRNA and (B) protein expression levels of BAG4 in the five GC cell lines, as assessed by RT-qPCR and western blot analysis, respectively. * $P < 0.05$ BGC823 vs. AGS; * $P < 0.05$ AGS vs. MNK45. (C) Protein and mRNA expression levels of BAG4 in BAG4-knockdown AGS and BAG4-overexpressing SGC7901 cells. α -tubulin and GAPDH served as internal controls. Data are presented as the mean \pm standard deviation of three independent experiments. * $P < 0.05$; ** $P < 0.01$. BAG4, Bcl-2, associated athanogene 4; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

1:1,000; Abcam, Cambridge, UK) were incubated with the sections overnight at 4°C. After incubation with 50 μ l per section goat anti-rabbit IgG/horseradish peroxidase (HRP) polymer secondary antibody for 30 min (PV-6001; ZSGB-Bio Co., Ltd., Beijing, China), the visualization signal was developed with 3,3'-diaminobenzidine tetrachloride (ZSGB-Bio Co., Ltd.) for 3 min. Images were captured by Olympus DP72 microscope (Olympus Corporation) and were analyzed with DP2-BSW software. The stained tissue sections were reviewed and scored separately by two pathologists blinded to the clinical parameters. The total Ki67 immunostaining score was calculated as the sum of the percent positivity of stained tumor cells.

Western blotting. Cells were collected and washed twice with PBS and then lysed with lysis buffer (Nanjing KeyGen Biotech Co., Ltd.) for 30 min on ice. Xenograft tissues were ground up in liquid nitrogen and lysed with 100-200 μ l lysis buffer (Nanjing KeyGen Biotech Co., Ltd.) for 30 min on ice. The proteins were then centrifuged at 11,000 \times g for 20 min at 4°C. The concentrations of proteins were detected by BCA kit (Bioworld, Guangzhou, China). Then, 30 μ g protein was separated by 10% SDS-PAGE and transferred

onto polyvinylidene difluoride membranes. Membranes were blocked with 5% BSA for 1 h and incubated with rabbit polyclonal anti-BAG4 (ab2048; 1:100; Abcam), mouse monoclonal anti- α -tubulin (T6199; 1:1,000; Sigma-Aldrich; Merck KGaA) and anti-GAPDH (G8795; 1:1,000; Sigma-Aldrich; Merck KGaA) primary antibodies for overnight at 4°C. The membranes were washed three times for 10 min with PBST (PBS 1,000:Tween-1) and incubated with HRP-conjugated goat anti-rabbit (FDR007; 1:10,000; Fdbio Science, Hangzhou, China) or anti-mouse (FDM007; 1:10,000; Fdbio Science) for 1 h at 37°C. The membranes were then washed three times for 10 min with PBST and visualized with Pico ECL (Fdbio Science) by tanon-5200 (Tanon Science and Technology Co., Ltd., Shanghai, China). α -tubulin and GAPDH served as internal controls.

Statistical analysis. Cell proliferation, *in vitro* and *in vivo* invasion assays were tested using one-way analysis of variance followed by LSD (equal variances assumed) or Dunnett's T3 (equal variances not assumed). Data were analyzed using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA) and are presented as the mean \pm standard deviation. $P < 0.05$ was considered to indicate a statistically significant difference.

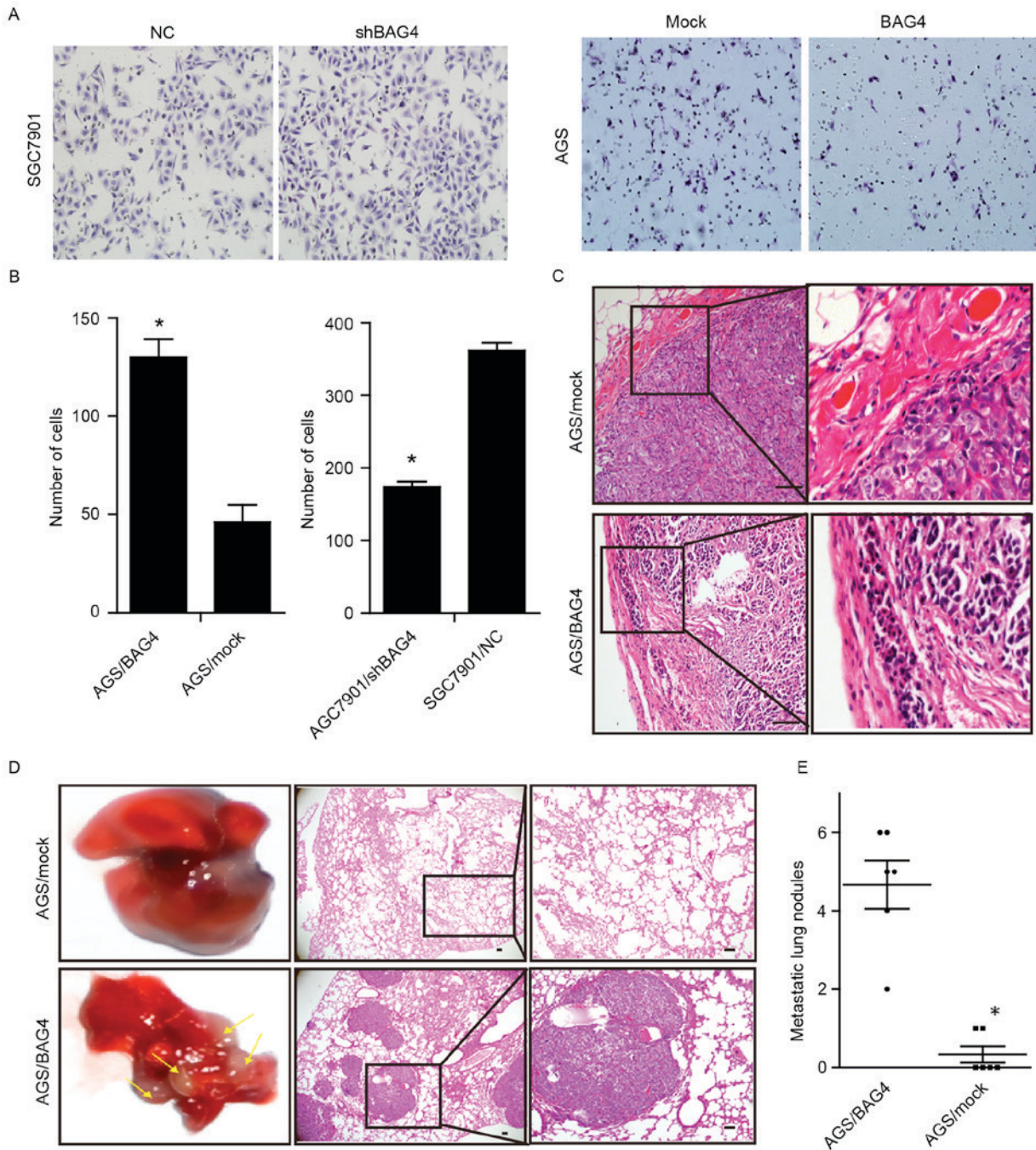


Figure 2. Effect of BAG4 on the migration and invasion of AGS and SGC7901 gastric cancer cells *in vitro* and *in vivo*. (A) Effect of BAG4 knockdown or ectopic on cell invasion *in vitro*. Morphological comparison of cells penetrating the artificial basement membrane is presented. Magnification, x200. (B) Quantification of invasive cells. (C) Effect of BAG4 overexpression on cell invasion *in vivo*. The tumor sections were stained with H&E. Scale bar, 20 μ m. (D) Representative immunohistochemistry images and (E) quantification of BAG4 overexpression on pulmonary metastases *in vivo*. The tumor sections were stained with H&E. Scale bar, 50 μ m. The number of metastatic nodules in individual mice were counted under a microscope. Data are presented as the mean \pm standard deviation. * P <0.05. H&E, hematoxylin and eosin; BAG4, Bcl-2, associated athanogene 4; sh, short hairpin; NC, negative control.

Results

BAG4 is upregulated in human GC cell lines. First, the expression of BAG4 in five human GC cell lines were assessed by RT-qPCR and western blotting. The mRNA (Fig. 1A) and protein (Fig. 1B) expression levels of BAG4 were increased in more aggressive GC cell lines (SGC7901 and MGC803) and lower in less aggressive GC cell lines (AGS and BGC823). Therefore, it was hypothesized that BAG4 may be associated with invasion and metastasis of GC.

Effect of BAG4 on migration and invasion of GC cells in vitro. To investigate the role of BAG4 in the progression of GC, loss and gain of function were performed to examine the effect of BAG4 on cell migration and invasion *in vitro* and *in vivo*. AGS cell lines were selected for stable transfection with a BAG4-expressing vector, and SGC7901 cell lines were selected for transient transfection with shRNA lentivirus vectors for BAG4. Transfection efficiency was validated by RT-qPCR and western blot analysis. The results demonstrated that shRNA successfully knocked down BAG4 protein

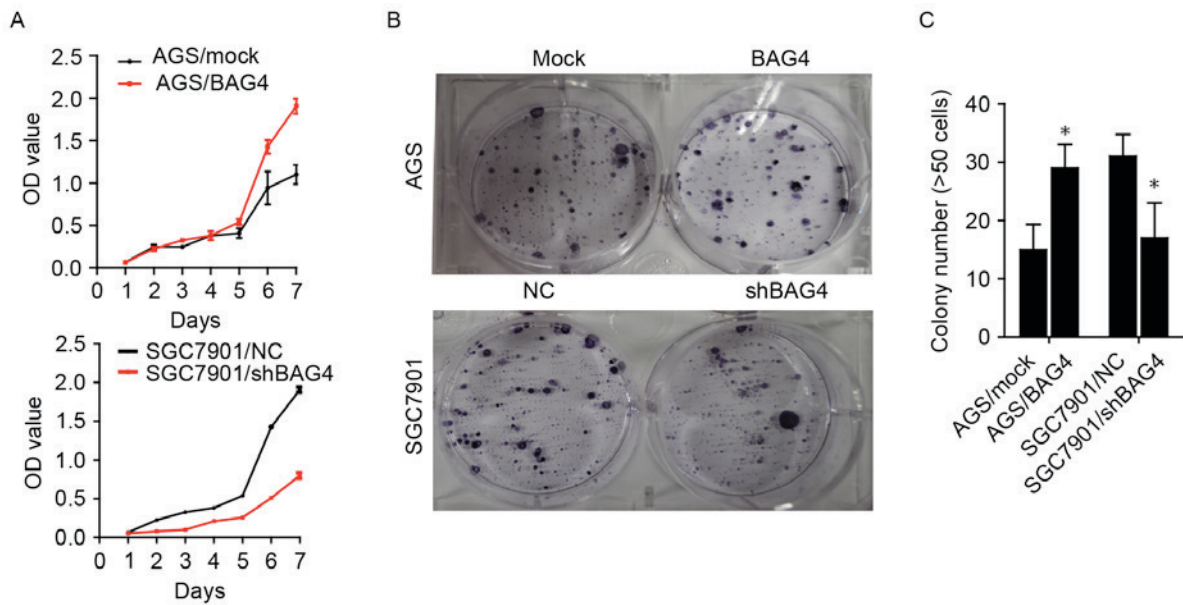


Figure 3. Effect of BAG4 on the proliferation of GC cells *in vitro* and *in vivo*. (A) Effect of BAG4 knockdown or overexpression on cell proliferation *in vitro*, as assessed by Cell Counting Kit-8 assay. (B) Effect of BAG4 knockdown or overexpression on cell proliferation *in vitro* by colony formation assay. (C) Quantification of colony numbers (cells >50). Data are presented as the mean \pm standard deviation. * $P < 0.05$ AGS/mock vs. AGS/BAG4 and SGC7901/NC vs. SGC790/shBAG4. BAG4, Bcl-2, associated athanogene 4; sh, short hairpin; NC, negative control; OD, optical density.

(Fig. 1C) and mRNA (Fig. 1D) expression levels. Furthermore, BAG4 was successfully overexpressed in AGS cells, compared with mock cells (Fig. 1C and D).

The results of the Boyden Chamber assay demonstrated that BAG4-depleted SGC7901 cells exhibited a marked decrease of invasive ability, whereas overexpression of BAG4 demonstrated the opposite effect (Fig. 2A and B; both $P < 0.05$). Furthermore, BAG4-expressing subcutaneous tumors exhibited evidence of local invasion, whereas control tumors were well encapsulated and noninvasive (Fig. 2C). To test the effect of BAG4 on GC metastasis *in vivo*, BAG4-overexpressing AGS cells were injected into the tail vein of nude mice to examine the role of BAG4 in lung colonization. BAG4 markedly promoted lung tumor metastases (Fig. 2D). In AGS cells, the numbers of metastatic lesions in the BAG4-expressing group was significantly increased compared with the control group ($P < 0.05$; Fig. 2E). The results indicated that BAG4 suppresses migration and invasion of GC cells.

Effect of BAG4 on the proliferation of GC cells *in vitro* and *in vivo*. The role of BAG4 on proliferation of GC cells was investigated. The results of the CCK-8 assay demonstrated that overexpression of BAG4 caused a significant increase of the proliferation rate in the AGS cell line, whereas BAG4 depletion decreased the proliferation of SGC7901 cell ($P < 0.05$; Fig. 3A). Similar results were observed in colony formation assays ($P < 0.05$, Fig. 3B and C). Furthermore, AGS cells overexpressing BAG4 exhibited a significant increase in the percentage of cells in the G1/G0 peak, and a decrease in the percentage of cells in the S and G2/M peak ($P < 0.05$, Fig. 4A). However, SGC7901 cells treated with BAG4 shRNA demonstrated the opposite effect (Fig. 4B). These results suggested that BAG4 promotes GC cell proliferation by eliciting G1/G0 phase arrest. Compared with control cells, AGS cells overexpressing BAG4 showed decreased rate of

apoptosis ($P < 0.05$; Fig. 4C), while the rate of apoptosis was remarkably increased when BAG4 was depleted in SGC7901 cells ($P < 0.05$; Fig. 4D). Additionally, BAG4-overexpressing or control cells were injected subcutaneously into nude mice, and the growth of the resultant primary tumors were measured. Tumors in mice injected with BAG4-overexpressing cells grew more rapidly compared with those injected with control cells (Fig. 5A and B). IHC staining confirmed that the tumors of BAG4-overexpressing group exhibited much higher Ki67 index compared with the control group (Fig. 5C and D). These results demonstrated that BAG4 promotes GC cell proliferation by upregulating cell cycle progression or decreasing apoptosis.

Discussion

Although the survival rate of GC has increased recently due to modern medical treatment, the mortality rate of GC remains high (17). Molecular therapy in recent years has become increasingly important for GC patients; the identification of biomarkers in GC etiology, progression and clinical behavior can lead to novel approaches in molecular therapy. The BAG4 protein family includes BAG1, -2, -3, -4, -5 and -6 (18). They share a conserved protein interaction module near the C-terminal end called the BD. The BD can bind to the ATPase domain of Hsp70/Hsc70 and regulate activity of these molecular chaperones (19,20). As an inhibitor of the death domain of the tumor necrosis factor receptor 1 (TNF-R1), BAG4 is also called Silencer of Death Domain (SODD). It prevents trimerization of the receptor subunits in the absence of specific signaling by binding with TNF- α (21,22). Subsequently, activated TNF receptors trigger apoptosis or nuclear factor (NF)- κ B activation. Numerous studies have reported that BAG4, as a member of BAG1-related protein family (5), can promote the development of breast, gastric and pancreatic cancer (10-13), and its upregulation may be associated with

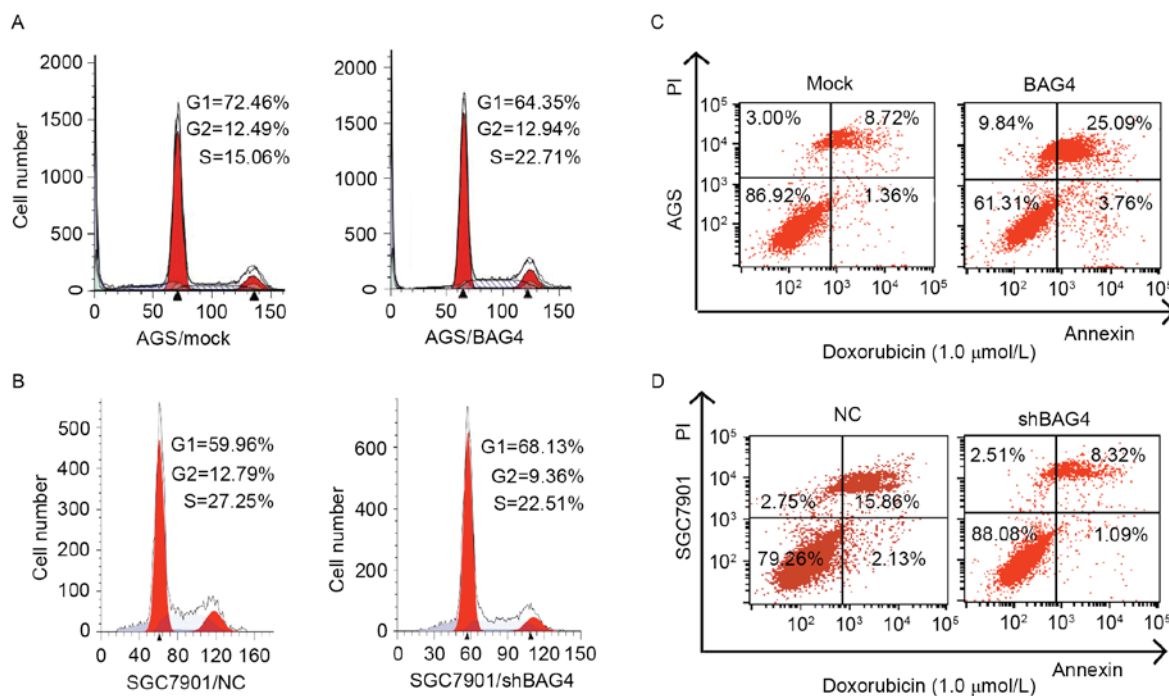


Figure 4. Effect of BAG4 on the cell cycle and apoptosis of GC cells. Representative histograms depicting cell cycle profiles of (A) BAG4-knockdown SGC7901 and (B) BAG4-overexpressing AGS cells. The percentage of apoptotic (C) SGC7901 and (D) AGS cells. BAG4, Bcl-2, associated athanogene 4; sh, short hairpin; NC, negative control; PI, propidium iodide.

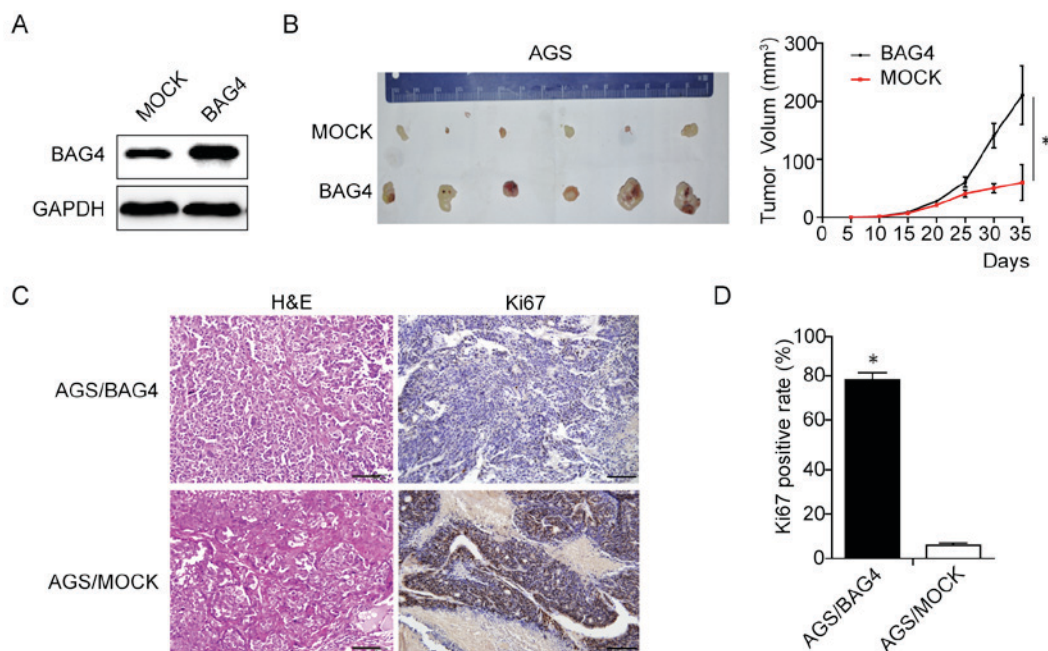


Figure 5. Effect of BAG4 on the proliferation of GC cells *in vivo*. (A) Protein expression levels of BAG4 in xenograft tissues, as assessed by western blot analysis. (B) Images and quantification of subcutaneous tumor sizes of mice injected with stable AGS/MOCK and AGS/BAG4 cells. AGS/MOCK group on day 35 vs. and AGS/BAG4 group on day 35 * $P < 0.05$. (C) The tumor sections were stained with hematoxylin and eosin or subjected to immunohistochemistry staining using an antibody against Ki-67. Scale bar, 20 μm . (D) Quantification of Ki-67-positive staining. Data are presented as the mean \pm standard deviation from three independent experiments. * $P < 0.05$. BAG4, Bcl-2, associated athanogene 4; H&E, hematoxylin and eosin.

the early recurrence of esophageal squamous cell carcinoma after definitive chemoradiation (23), and the severity of acute lymphoblastic leukemia in children (24).

The present study investigated the function of BAG4 in GC cells. Firstly, the expression levels of BAG4 in five gastric

cancer cell lines were examined by western blotting and RT-qPCR. BAG4 was demonstrated to be highly expressed in SGC7901 and MNK45 cells with higher metastatic potential, and less expressed in AGS and MGC 803 cells with lower metastatic potential. Based on these data, it was hypothesized

that BAG4 may be associated with the invasion and metastasis of GC cells. The Transwell assays indicated that downregulating BAG4 can decrease the invasiveness of SGC7901 cells, and overexpressing BAG4 served the opposite effect in AGS cells. Furthermore, subcutaneous tumor experiments demonstrated that BAG4 overexpression promotes local tumor cell invasion. The vein injection experiments demonstrated that cells overexpressing BAG4 can seed more lung localization. All these results demonstrated that BAG4 can promote invasion and localization of GC cells. Previous studies have indicated that BAG1 serves as an anti-apoptotic protein and interacts with other proteins to affect cell apoptosis and growth (14,17). BAG1 proteins can interact with B-cell lymphoma 2 Bcl-2 to enhance resistance to apoptosis (25-27). Interestingly, BAG1 also binds to Hsp70, and inhibits the chaperone activity of this molecule (7,8,28), and the downregulation of SODD and NF- κ B induces apoptosis. Due to the balance between pro- and anti-apoptotic signaling of TNF-R1 activation in melanoma (29), SODD may serve to tip the balance away from cell death and toward NF- κ B activation, which is constitutively active in melanoma and important in its progression (30,31). Therefore, SODD may serve a role in the anti-apoptotic functions of Bcl-2 family members (32,33). Therefore, the present study detected whether BAG4 promotes the proliferation of GC cells. CCK-8 and colony formation assays indicated that overexpression of BAG4 can markedly increase the proliferation of GC cells, whereas depletion of BAG4 demonstrated the opposite results. Furthermore, overexpression of BAG4 in the AGS cell line elicited G1/G0 phase arrest and inhibited cell apoptosis, whereas depletion of BAG4 in SGC7901 cells decreased the number of cells in the G1/G0 phase and promoted cell apoptosis. This observation was confirmed in nude mice; in the BAG4 overexpressing group, the subcutaneous tumors were increased compared with the control group. These results demonstrated that BAG4 promotes GC proliferation by eliciting G1/G0 phase arrest and inhibiting cell apoptosis.

In conclusion, the present study demonstrated that BAG4 promotes the invasion, metastasis and proliferation of GC cells. Further studies are required to verify the significance of BAG4, and whether it may represent a potential therapeutic target for therapies such as adjuvant chemotherapy and radiosensitization, or whether it may serve as a biomarker for the establishment of rational treatment selection criteria for patients GC.

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