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Malignant ascites supernatant enhances the proliferation of gastric cancer cells partially via the upregulation of asparagine synthetase

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Abstract. Malignant ascites (MA) is a common manifestation of advanced gastric cancer (GC) with peritoneal metastasis (PM), which usually indicates a poor prognosis. The present study aimed to explore the effects of MA, a unique microenvironment of PM, on the proliferation of cancer cells and investigate the underlying mechanisms. *Ex vivo* experiments demonstrated that GC cells treated with MA exhibited enhanced proliferation. RNA sequencing indicated that asparagine synthetase (*ASNS*) was one of the differentially expressed genes in GC cells following incubation with MAs. Furthermore, the present study suggested that MA induced an upregulation of *ASNS* expression and the stimulatory effect of MA on cancer cell proliferation was alleviated upon *ASNS* downregulation. Activating transcription factor 4 (ATF4), a pivotal transcription factor regulating *ASNS*, was upregulated when cells were treated with MA supernatant. After ATF4 knockdown, the proliferation of MA-treated GC cells and the expression of *ASNS* decreased. In addition, the decline in the proliferation of the ATF4-downregulated AGS GC cell line was rescued by *ASNS* upregulation. The findings indicated that MA could promote the proliferation of GC cells via activation of the ATF4-ASNS axis. Hence, it may be a potential target for treating GC with PM and MA.

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Introduction

Gastric cancer (GC) ranks as the fifth most commonly diagnosed cancer and the third most common cause of cancer-related mortality worldwide (1). Peritoneal metastasis (PM) is a major cause of the development of recurrence and distant dissemination in patients with GC (2). The median survival time of patients with GC and PM is ~four months and such patients can barely survive beyond five years (3,4). Malignant ascites (MA) is a common manifestation of advanced GC with PM, which implies a shorter life expectancy in patients with GC (5). Among all patients with GC and PM, ~40% of them have MA fluid (6). Although intraperitoneal chemotherapy combined with systemic paclitaxel chemotherapy has shown promising effects on the survival of patients with advanced GC and PM, the prognoses of these patients are still poor (7).

An imbalance between the production and outflow of fluid in the abdominal cavity results in ascites, which is primarily reported in liver cirrhosis and a variety of malignancies (8), such as ovarian (9) and gastrointestinal cancer with PM (10). Accumulated ascites in the abdominal cavity, which are composed of cellular and acellular components, constitute a unique microenvironment for PM and the tumor cells suspended in the peritoneal fluid (11). Non-cancer cells in ascites, such as macrophages (12) and cancer-associated fibroblasts (13), interact with cancer cells and serve a pivotal role in the progression of PM. In addition, the acellular fraction of ascites, such as exosomes (14), metabolites (15), soluble growth factor (16) and chemokines (17) is involved in peritoneal dissemination, epithelial-to-mesenchymal transition (EMT), chemoresistance and cell proliferation (14,18). Previous research indicated that pleural effusion and ascites from breast, lung and ovarian cancer may induce EMT and manifestation of cancer stem cell traits via activation of the PI3K/Akt/mTOR pathway (19). However, little is known about the effects of MA supernatant on GC cells, which needs further exploration.

Asparagine synthetase (ASNS), which catalyzes the synthesis of asparagine and glutamate using aspartic acid and glutamine, is ubiquitous in mammalian cells (20). Upregulation of the expression of ASNS is associated with

poor prognosis in patients with colorectal cancer (21), hepatocellular carcinoma (22) and malignant gliomas (23), suggesting a prominent role in cancer progression. In addition, the downregulation of ASNS expression inhibits the growth of GC (24). Nevertheless, a consensus has not been established on the correlation between ASNS expression and cancer evolution (25,26). Few studies have explored the role of the ASNS in mediating cancer cell progression associated with ascites or peritoneal lesions in patients with GC.

The current study investigated the effects of MA on the proliferation of GC cells. RNA sequencing was used to explore the differentially expressed genes (DEGs) between MA-treated GC cells and benign ascites-treated GC cells. *ASNS*, one of the DEGs, was assessed its role in proliferation-promoting effects of MA on GC cells. Notably, MA may initiate activation of the activating transcription factor 4 (ATF4)-ASNS axis to promote the proliferation of GC cells.

Materials and methods

Ascites samples. MA fluid was obtained from GC patients with large volumes (>1,000 ml) of MA, who were admitted to Changhai Hospital (Shanghai, China) between May 1, 2020 and Jan 31, 2021. All patients were free from peritonitis, life-threatening complications and secondary cancers. The clinicopathological features of the patients are shown in Table I. A sample of benign ascites was collected from a 46-year-old female patient diagnosed with liver cirrhosis. Ascites (50-100 ml) were collected from the enrolled patients who underwent peritoneal paracentesis. Ascites samples were processed within 24 h after collection. After being dispensed into 50 ml sterile centrifuge tubes, the samples were centrifuged at 400 x g at 4˚C for 10 min. The cell pellets were then used for organoid construction and the supernatant was transferred into a new centrifuge tube after being filtered through a 0.22 μm sterilizing filter. All ascites supernatants were cryopreserved at -80˚C and heat-inactivated prior to use.

Cell culture. The human GC cell lines, AGS, SNU5 and SNU16, were purchased from the Cell Bank of Chinese Academy of Science and Bena Culture Collection. AGS is an adherent cell line, while SNU5 and SNU16 are semi-adherent and suspended cells respectively. AGS cells were cultivated in high-glucose DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 ng/ml streptomycin. SNU5 and SNU16 cells were maintained in RIPM1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. All cell lines were subjected to STR analysis and the absence of mycoplasma contamination was confirmed. The three cell lines were cultivated at 37˚C with 5% CO_2. Organoids derived from MA were constructed as previously described (27).

Cell proliferation assay. Cell proliferation was measured using Cell Counting Kits-8 (CCK-8) according to the instructions of the manufacturer (Beyotime Institute of Biotechnology). Briefly, 2,000 cells per well (100 μl) were seeded in a 96-well plate. After incubation with or without 10% MA for 24, 48 and 72 h, the normalized proliferation of cells was determined. A total of 10 μl of CCK-8 was added to each well. After 2 h of incubation, the optical density was measured at 450 nm.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from $1x10^6$ cells using RNAiso Plus reagent (Takara Bio, Inc.) according to the manufacturer's protocol. Reverse transcription was then performed to obtain cDNA using PrimeScript RT Master Mix (Takara Bio, Inc.) according to manufacturer's instructions. Next, qPCR was performed in a total reaction volume of 20 μl to determine the relative mRNA expression of the target genes using the Powerup SYBR Green Master Mix (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The parameters of qPCR were as follows: Initial denaturation at 95˚C for 120 sec; followed by 40 cycles of denaturation at 95˚C for 10 sec, and annealing and extension at 60˚C for 30 sec. The primers used are listed in Table SI. Relative mRNA expression of genes was calculated using the $2^{-\Delta\Delta Cq}$ formula (28) and β-actin was used as an internal control. The experiments were performed in triplicate.

Western blotting (WB). Total protein of GC cells was extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology) containing phenylmethylsulfonyl fluoride and phosphatase inhibitors. The concentration of protein was determined using the Pierce BCA protein kit (Thermo Fisher Scientific, Inc.). A total of 40 μg of the total protein was loaded in each gel lane and proteins with different molecular weights were separated by 10% SDS-PAGE. After the proteins were transferred onto PVDF membranes, the membranes were blocked with 5% skimmed milk at 25˚C for 1 h. The membranes were then incubated with ASNS (Santa Cruz Biotechnology, Inc.; 1:800; cat. no. 365809; mouse), ATF4 (Proteintech Group, Inc.; 1:1,000; cat. no. 10835-1-AP; rabbit) or β-actin antibody (Cell Signaling Technology, Inc.; 1:1,000; cat. no. 3700; mouse) at 4˚C overnight. The membrane was then incubated with the corresponding HRP-conjugated secondary antibodies (Biosharp Life Sciences; cat. nos. BL001A and BL003A; 1:5,000; goat) at 25˚C for 1 h. Protein expression was detected by the Amersham imager 680 (Cytiva). Quantification of the strips was performed using ImageJ software (National Institutes of Health; version: 1.8.0).

Immunofluorescence. Cells at a density of $3x10^5$ per well were seeded onto the 6-well plate. AGS cells treated with or without ascites were fixed using 4% paraformaldehyde for 10 min at 25˚C. After blocking with 5% donkey serum (cat. no. ab7475; Abcam) for 30 min, the cells were incubated with a Ki-67 antibody (Abcam; 1:200; cat. no. ab15580; mouse) at 4˚C overnight. After washing with PBST three times, the cells were cultured with secondary antibodies conjugated to Alexa-Fluor 488 (Abcam; 1:1,000; cat. no. ab150113; goat) at 25˚C for 1 h. The cells were subsequently stained with DAPI at 25˚C for 10 min. Finally, images were captured using a fluorescence microscope (magnification, x200; Olympus Corporation). A total of five random fields were selected to capture images for calculating Ki-67 positive cells.

RNA isolation, library preparation and RNA sequencing (RNA-seq). RNA-seq of MA-treated AGS and untreated AGS

Table I. Clinicopathological characteristics of gastric cancer patients with malignant ascites.

No.	Sex	Age (years)	Disease	Primary cancer sites	Pathology type	Clinical stage	Her-2 status
1	Male	36	Gastric cancer	Body	Poorly differentiated	IV	Positive
2	Male	61	Gastric cancer	Antrum	Medium differentiated	IV	Negative
3	Female	69	Gastric cancer	Cardia	Poorly differentiated	IV	Not detected

cells was performed in duplicate. Total RNA was isolated using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The purity and quantification of RNA were evaluated using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). RNA integrity assessment was conducted using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). Libraries were then constructed using a TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, Inc.) and the libraries were sequenced on an Illumina HiSeq X Ten platform (Illumina, Inc.) by 150 bp paired-end sequencing. Then clean data were mapped to the human genome (GRCh38) using HISAT2 (http://daehwankimlab.github.io/hisat2/). Transcriptome sequencing and data analysis were conducted by Shanghai OE Biotech Co., Ltd. P<0.05 and foldchange >2 or <0.5 were set as differentially expressed genes (DEGs) threshold. DEGs were identified using the DESeq R package (http://www.bioconductor.org/; version: 1.34.1) (29).

Small interfering (si) RNA transfection. ASNS siRNA, ATF4 siRNA and negative control (NC; non-targeting siRNA) were designed and constructed by Shanghai GenePharma Co., Ltd. and the sequences are shown in Table SII. A density of $5x10^5$ cells per well were seeded into 6-well plates to achieve 60-80% confluence after 12 h of cultivation. RNA iMAX (Thermo Fisher Scientific, Inc.)-siRNA mix (final siRNA concentration, 50 nM) was then added to each well. Cells were transfected at 37˚C for 8 h. After 48 h, transfected cancer cells were collected to detect the silencing effect on the target genes at the mRNA and protein levels. The siRNA with the highest efficacy was used for further experiments. The subsequent experiments were performed 48 h after transfection.

Stably transfected cells. Control vector and ASNS-overexpression ($ASNS^{oe}$) lentiviral vector were constructed using pcSlenti-EF1-EGFP-P2A-puro-CMV-3xFLAG-WPRE. The lentivirus was constructed and packaged by OBiO Technology (Shanghai) Corp., Ltd. A total of 40 μl lentivirus ($4x10^8$ TU/ml) was added to each well to infect the targeted cells. GPF fluorescence was detected using a fluorescence microscope (Olympus Corporation) after 48 h. Transduced cells were selected using 2 μg/ml puromycin to obtain stable cell lines. The overexpression of ASNS was then verified by RT-qPCR and WB.

Chromatin immunoprecipitation (ChIP) assay. This was conducted according to the manufacturer's instructions using an EZ-Magna CHIP A/G kit (cat. no. 17-10086; MilliporeSigma). AGS cells (density, $1.3x10^5/cm^2$ in a 75-cm^2 cell culture flask) treated with MA or benign ascites were cross-linked using formaldehyde at 25˚C for 10 min and then lysed with ChIP lysis buffer. Then nuclear lysates were sonicated (20 kHz frequency at 4˚C for 10 min) on ice to generate 100-500 bp DNA fragments. After the sonication and centrifugation (10,000 x g at 4˚C for 10 min), the supernatant was removed to a new tube with CHIP dilution buffer (part no. CS200624; MilliporeSigma) containing protease inhibitors (part no. 20-283; MilliporeSigma). Subsequently, 10 μl of the supernatant was removed as input control, and the input control was stored at 4˚C and then processed via elution and cross-link reversal, while the rest of the supernatant was divided into two aliquots and these two aliquots were immunoprecipitated at 4˚C for 2 h using anti-ATF4 (Cell Signaling Technology, Inc.; dilution, 1:200; cat. no. 11815S; rabbit) or IgG antibody (Cell Signaling Technology, Inc.; dilution, 1:200; cat. no. 2729S; rabbit) with magnetic protein A/G beads (part no. CS204457; MilliporeSigma). The magnetic beads were pelleted and the supernatants were removed. The bead-antibody/chromatin complexes were washed with the buffers in the following order: Low salt wash buffer (part no. CS200625; MilliporeSigma), one wash; high salt wash buffer (part no. CS200626; MilliporeSigma), one wash; LiCl wash buffer (part no. CS200627; MilliporeSigma), one wash; and TE buffer (part no. CS200628; MilliporeSigma), one wash. After elution and cross-link reversal of protein-DNA complex (including the immunoprecipitated samples and the input control samples), the DNA was purified using unique polypropylene spin columns which contains activated silica membrane filters that can capture DNA and separate the DNA from proteins in combination with the binding reagent A (part no. 20-292; MilliporeSigma) and washing reagent B (part no. 20-293; MilliporeSigma). The purified DNA was eluted using elution reagent C (part no. 20-294; MilliporeSigma) and used to perform qPCR. The enrichment ratio was assessed by qPCR which was performed as described for RT-qPCR. Primers for the *ASNS* promoter are shown in Table SI.

Statistical analysis. All experiments were performed in triplicate and the data are presented as mean ± standard deviation. One-way ANOVA and unpaired t-test were used to compare the differences between multiple groups or two groups, respectively. Tukey's honestly significant difference test was used after the ANOVA when comparing multiple groups. GraphPad Prism version 8 (Dotmatics) was used to generate graphs and perform statistical analyses. P<0.05 was considered to indicate a statistically significant difference.

Results

MA supernatant promotes the proliferation of GC cells. The survival and proliferation of GC cells in MA is a pivotal step

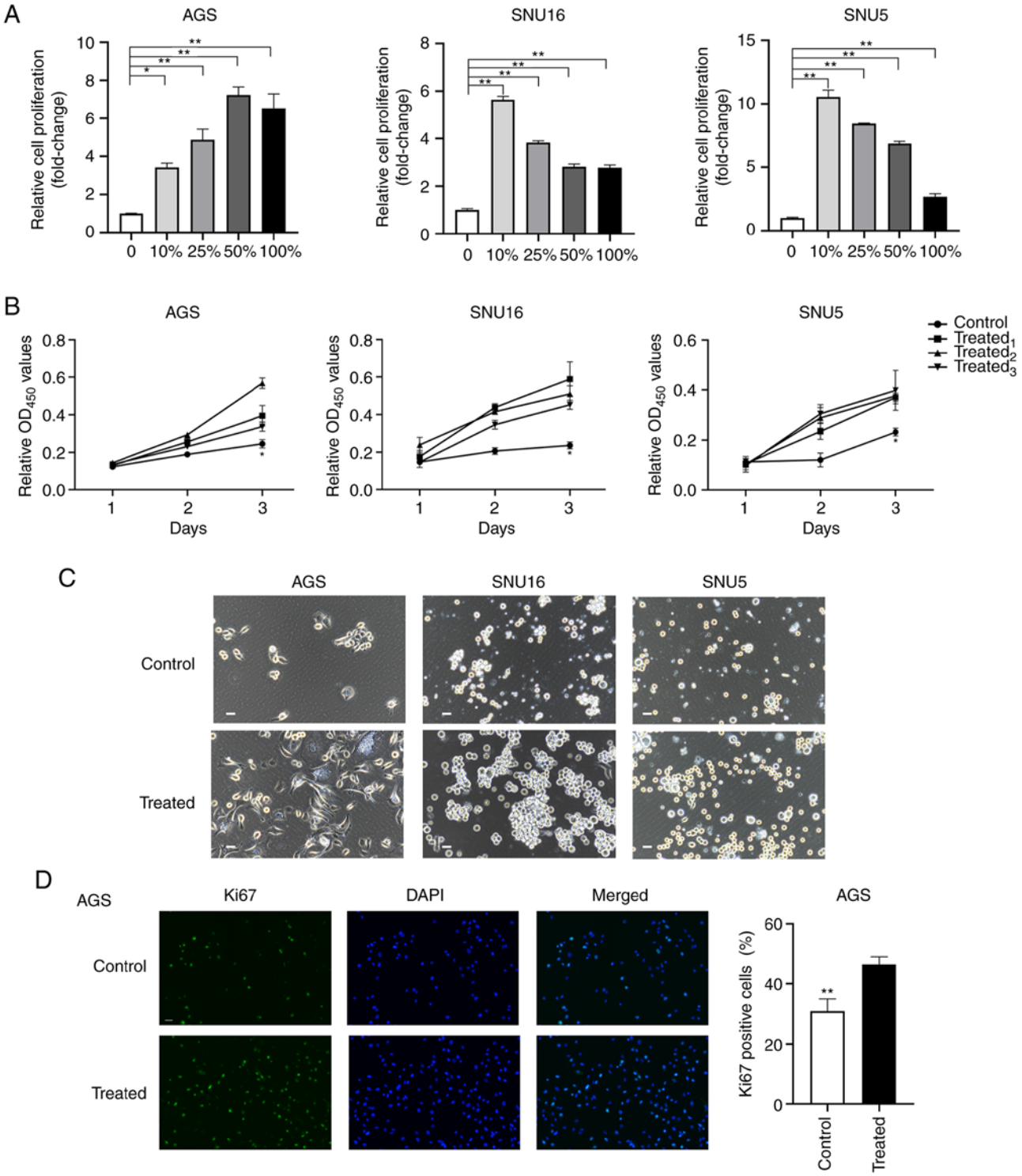

Figure 1. MA supernatant promotes the proliferation of gastric cancer cells. (A) Cell proliferation was assessed by CCK8 after AGS, SNU16 and SNU5 cells were treated with medium containing 0, 10, 25, 50, 100% gradient MA for 72 h. (B) The proliferation of AGS and SNU16, SNU5 cells grown in medium with 10% MA or 10% benign ascites at 24, 48 and 72 h was measured by CCK8 assay. Control: 10% benign ascites treatment; $treated_1$, $treated_2$ and $treated_3$ represent cells treated with 10% MA from three different patients with GC. (C) Phase contrast images of AGS, SNU5, SNU16 cells exposed to medium with or without MA in two dimensions were captured at 48 h (scale bar, 100 μm; MA was derived from the patient named No.1 in Table I). (D) Immunofluorescence staining of Ki67 was performed in AGS after being treated with MA or benign ascites for 24 h (magnification, x200). The quantification of Ki67 positive cells was shown in the bar graphs. Control: Benign ascites treatment; treated: Treated with MA which was collected from the patient named No.1 in Table I. Data are shown as mean ± standard derivation. P-value was analyzed by one-way ANOVA or two-tail unpaired Student's *t* test (*P<0.05; **P<0.01.). MA, malignant ascites; GC, gastric cancer.

in PM formation and progression (30). To evaluate the effects of MA on the proliferation of GC cells, AGS, SNU16 and SNU5 cells were incubated with different gradients of ascitic fluid (0, 10, 25, 50 and 100%) for 72 h. Then, the normalized proliferation of the cells was detected using the CCK-8. The results indicated that the proliferation of GC cells was

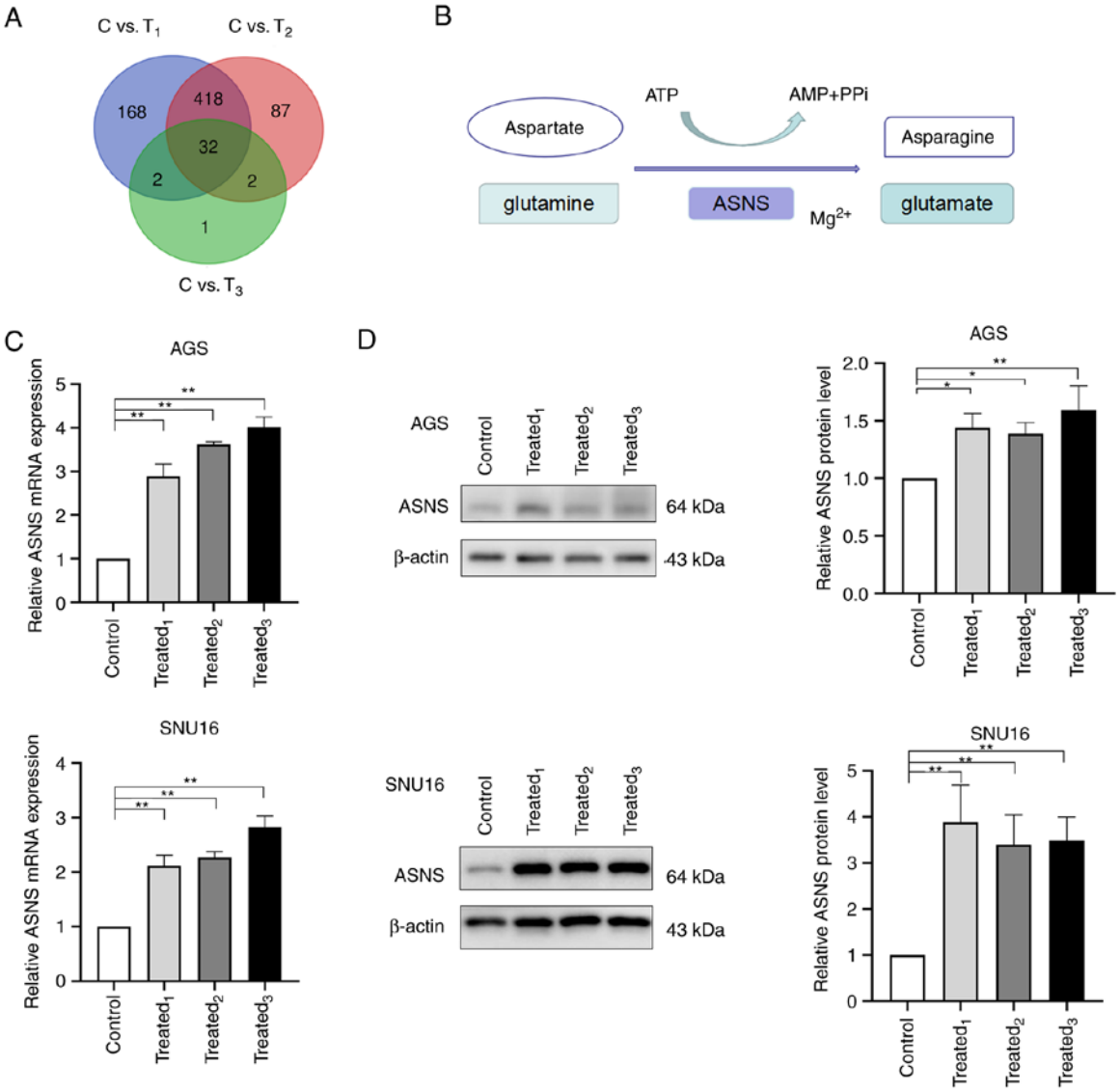

Figure 2. MA treatment induces the upregulation of ASNS expression in GC cells. (A) Venn graph showed differentially expressed genes between AGS cells treated with MA and those treated with benign ascites for 24 h using transcriptional sequencing. C is AGS treated with 10% benign ascites, while T_1, T_2 and T_3 represented AGS treated with MA from three different GC patients. (B) Schematic diagram showing the catalytic role of ASNS in mammalian cells. (C) Relative mRNA level of ASNS was quantified by reverse transcription-quantitative PCR in AGS and SNU16 cells following 24 h treatment with 10% MA or benign ascites (**P<0.01). (D) Protein levels and the quantification of ASNS were detected using western blotting in AGS and SNU16 cells exposed to medium with 10% MA or 10% benign ascites. Control: Treated with benign ascites; $treated_1$, $treated_2$ and $treated_3$: treated with MA from three different GC patients. Data represented the mean ± SD (*P<0.05; **P<0.01). ASNS, asparagine synthetase; GC, gastric cancer; MA, malignant ascites.

increased after treatment with gradient ascites compared with the untreated cells (P<0.05; Fig. 1A). However, no significant dose-dependent effects of MA were observed during the proliferation-promoting process. To exclude the different effects of variable gradient ascites on cell proliferation, cells treated with 10% ascites supernatant were selected as the treated group while cells treated with 10% benign ascites were regarded as the control group. To further verify the promotional effects of MA fluid on cancer cells, the proliferation of GC cells treated with 10% MA from different patients was evaluated. The results demonstrated that ascites supernatants from three representative patients promoted the proliferation of GC cells at 72 h (Fig. 1B). Phase contrast images of cell lines and organoids derived from MA (MADO) cultured in two dimensions at 48 h and three dimensions (3D) at 72 h were captured after treatment with MA or benign ascites (Figs. 1C and S1A). The volumes of the spheres of the treated group in the 3D culture system were larger than those of the control group (Fig. S1B). Furthermore, after treatment with 10% MA or benign ascites for 48 h, the proportion of $Ki67^+$ AGS cells was determined. A higher proportion of $Ki67^+$ cells was observed in the MA-treated group than in the untreated group (P<0.01; Fig. 1D). Taken together, these findings suggested that MA promoted the proliferation of GC cells *ex vivo*.

MA induces the upregulation of ASNS expression in GC cells. To further elucidate the underlying molecular mechanisms mediating the proliferation-promoting effects of MA on GC cells, transcriptome sequencing was performed to screen for DEGs. AGS cells, treated with MA (derived from three patients with GC) or benign ascites for 24 h, were collected for RNA-seq. As illustrated in the Venn graph shown in Fig. 2A, 32 DEGs

Table II. Differentially expressed genes of malignant ascites-treated AGS cells compared with the control group.

		Treated$_1$ vs. control		Treated$_2$ vs. control		Treated$_3$ vs. control	
Gene	Upregulated/Downregulated	Fold change	P-value	Fold change	P-value	Fold change	P-value
ASNS	Upregulated	5.47	<0.05	5.16	<0.05	2.47	<0.05
DDIT4	Upregulated	5.52	<0.05	4.50	<0.05	2.50	<0.05
FGF21	Upregulated	242.22	<0.05	177.80	<0.05	86.37	<0.05
INHBE	Upregulated	25.40	<0.05	20.78	<0.05	3.55	<0.05
IRAK1BP1	Upregulated	2.95	<0.05	2.75	<0.05	2.16	<0.05
KLHDC7B	Upregulated	50.10	<0.05	29.50	<0.05	4.51	<0.05
NUPR1	Upregulated	8.41	<0.05	6.87	<0.05	2.43	<0.05
PCK2	Upregulated	3.87	<0.05	3.47	<0.05	2.19	<0.05
S100P	Upregulated	8.28	<0.05	6.13	<0.05	2.66	<0.05
SLC43A1	Upregulated	5.32	<0.05	5.34	<0.05	2.66	<0.05
SPINK1	Upregulated	5.02	<0.05	4.52	<0.05	3.06	<0.05
TGM2	Upregulated	2.57	<0.05	2.57	<0.05	2.69	<0.05
TUBE1	Upregulated	4.26	<0.05	3.56	<0.05	2.17	<0.05
BATF2	Downregulated	0.10	<0.05	0.07	<0.05	0.18	<0.05
CCDC141	Downregulated	0.24	<0.05	0.22	<0.05	0.37	<0.05
CMPK2	Downregulated	0.21	<0.05	0.19	<0.05	0.44	<0.05
CREB3L3	Downregulated	0.24	<0.05	0.25	<0.05	0.40	<0.05
CXCL11	Downregulated	0.45	<0.05	0.36	<0.05	0.43	<0.05
HSD17B2	Downregulated	0.18	<0.05	0.23	<0.05	0.38	<0.05
ISG15	Downregulated	0.31	<0.05	0.30	<0.05	0.47	<0.05
KCNK2	Downregulated	0.03	<0.05	0.03	<0.05	0.19	<0.05
MSMO1	Downregulated	0.43	<0.05	0.25	<0.05	0.49	<0.05
PSG4	Downregulated	0.15	<0.05	0.14	<0.05	0.45	<0.05
REG4	Downregulated	0.24	<0.05	0.30	<0.05	0.43	<0.05
RSAD2	Downregulated	0.35	<0.05	0.32	<0.05	0.44	<0.05
SARM1	Downregulated	0.26	<0.05	0.29	<0.05	0.36	<0.05
SEMA3C	Downregulated	0.19	<0.05	0.21	<0.05	0.47	<0.05
SLC7A8	Downregulated	0.17	<0.05	0.25	<0.05	0.48	<0.05
SYNPR	Downregulated	0.37	<0.05	0.42	<0.05	0.49	<0.05
SYP	Downregulated	0.15	<0.05	0.17	<0.05	0.39	<0.05
TNNC1	Downregulated	0.43	<0.05	0.30	<0.05	0.46	<0.05
TTN	Downregulated	0.11	<0.05	0.10	<0.05	0.26	<0.05

Control: treated with benign ascites; treated$_1$, treated$_2$ and treated$_3$: treated with malignant ascites from 3 different gastric cancer patients.

were identified. According to the results of the sequencing, the mRNA levels of 13 genes were upregulated in the MA-treated groups whereas 19 genes were downregulated (Table II).

ASNS was one of the upregulated genes in MA-treated AGS cells. It is a universally expressed gene in almost all tissues in humans, with its encoded protein catalyzing the synthesis of asparagine and glutamate using glutamine and aspartate in the presence of ATP (Fig. 2B) (31). The aberrant expression of ASNS is correlated with cancer progression (32-34). As few studies have explored the role of *ASNS* in the progression of PM in GC, it was selected as the target gene for further study. To determine whether MA treatment could induce high expression of *ASNS*, the mRNA and protein levels of ASNS was detected in AGS and SNU16 cells after being treated with MA from three GC patients. Significant upregulation of ASNS expression at the mRNA level was observed in MA-treated AGS and SNU16 cells when compared with those in the control group (Fig. 2C). Besides, AGS and SNU16 cells exposed to a medium containing 10% MA exhibited an increase in ASNS protein levels (Fig. 2D). The aforementioned results suggested that GC cells exposed to MA had enhanced expression of ASNS.

MA exhibits proliferation-promotional effects on GC cells partially via upregulation of ASNS expression. Based on the RNA-seq and *in vitro* experiments, the findings of the present study implied that MA from different patients could mediate an increase in ASNS expression in GC cells. To further determine whether ASNS serves a pivotal role in mediating the effects of MA on GC cell proliferation, ASNS

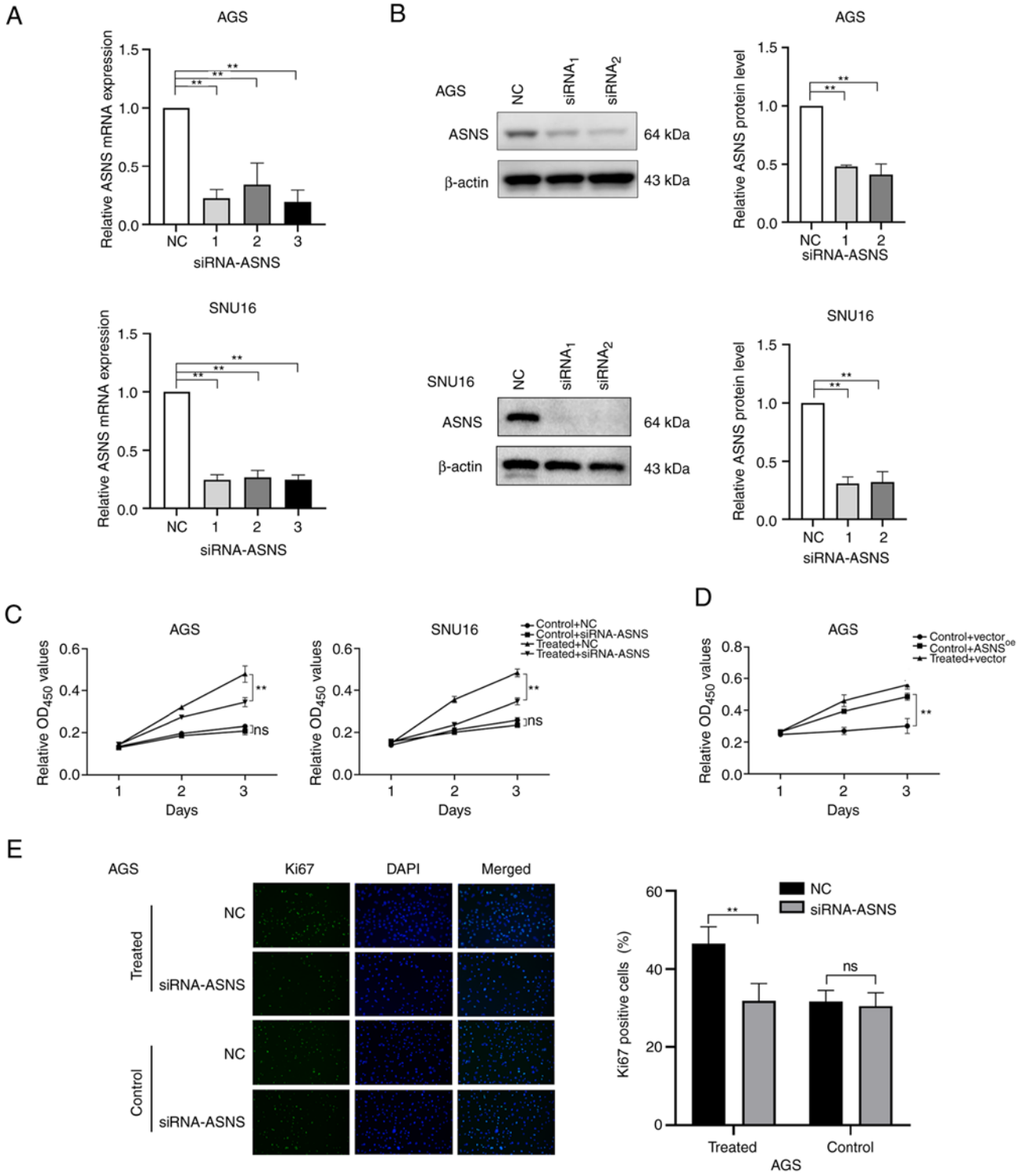

Figure 3. Enhanced proliferation of GC cells following MA treatment may be partially mediated by the upregulation of ASNS. (A) Knockdown efficacy of siRNA on the mRNA level of ASNS was quantified by reverse transcription-quantitative PCR. 1, 2, 3 represent three different siRNAs targeting ASNS. (B) Silencing efficacy of siRNA targeting ASNS was detected by western blotting at the protein level. (C) After cells were transfected with siRNA-ASNS or NC, the growth of AGS and SNU16 cells exposed to medium with or without MA (obtained from the No.1 patient in Table I) were analyzed using CCK8 on day 1, 2 and 3. (D) The proliferation of AGS cells with or without MA (from the No.1 patient in Table I) treatment, after being infected with negative control (vector)/ASNS-overexpression lentivirus (ASNS^{oe}), was determined by CCK8 at day 1, 2, 3. (E) Immunofluorescence staining was applied to detect the proportion of Ki67-positive cells in MA-treated/control AGS with siRNA-ASNS or NC (magnification, x200). MA was from the patient named No.1 in Table I. Control: With benign ascites treatment; treated: Treated with MA. **P<0.01; ns, no statistical significance. GC, gastric cancer; MA, malignant ascites; ASNS, asparagine synthetase; si, small interfering; NC, negative control.

knockdown and overexpression were performed. Since the baseline levels of ASNS in SNU16 cells were higher than those in AGS cells (P<0.01; Fig. S2A), the AGS cell line was chosen for overexpression of ASNS. The silencing efficacy of siRNA (Fig. 3A and B) and the upregulation of ASNS in ASNS^{oe} lentivirus-transduced cells (Fig. S2B) at the mRNA

and protein levels were assessed. Next, the relative proliferation of GC cells transfected with siRNA-NC/siRNA-ASNS combined with treatment with or without MA was determined. The results indicated that the relative proliferation of AGS cells treated with MA was partially inhibited upon a decrease in *ASNS* expression (Fig. 3C). In addition, AGS cells stably expressing upregulated ASNS levels exhibited higher proliferation ability (Fig. 3D). In addition, in MA-treated AGS, the number of $Ki67^+$ cells also decreased when *ASNS* expression was knocked down by siRNA, compared with AGS cells transfected with siRNA-NC ($P<0.05$; Fig. 3E). The above findings revealed that MA may promote the proliferation of GC cells partially via elevated ASNS expression and downregulation of ASNS could, in part, reverse the effects of MA on GC cell proliferation.

Malignant abdominal fluid of patients with GC promotes the proliferation of cancer cells via the activated ATF4-ASNS axis. ATF4 is the main transcription factor (TF) that regulates ASNS (35). Prediction results presented on JASPAR (http://jaspar.genereg.net/), an online TF database (36), indicated that ATF4 might bind to the *ASNS* promoter region with a relative score of 7.17. In addition, a ChIP-qPCR assay using AGS cells suggested that ATF4 binds to the promoter of *ASNS* and that MA treatment induced enhanced binding activity of ATF4 to the promoter region of *ASNS* (Fig. 4A). Considering the aforementioned results, it was hypothesized that MA might initiate the upregulation of ASNS via ATF4. To verify this hypothesis, the expression of ATF4 after MA treatment was detected. Relative protein levels of ATF4 increased in AGS and SNU16 cells (Fig. 4B) following treatment with MA derived from different patients. Then, siRNA (Table SIII) targeting ATF4 was constructed to knockdown the expression of *ATF4*. $siRNA_1$ and $siRNA_3$ targeting *ATF4* exhibited the highest silencing efficacy in AGS and SNU16 cells, respectively (Fig. S2C). After ATF4 expression was knocked down, the promoting effects of MA on cancer growth were partially mitigated at 72 h ($P<0.01$; Fig. 4C) and the proportion of Ki67-positive cells decreased in MA-treated AGS cells (Fig. 4D). These results suggested that MA could upregulate ATF4 expression, accelerating the proliferation of cancer cells.

To further elucidate whether ascites promotes cell proliferation by influencing the expression of ASNS via the upregulation of ATF4, the expression of ASNS after ATF4 silencing was determined. The results showed that ASNS levels were simultaneously downregulated when effective siRNA-ATF4 was applied (Fig. 4E). In addition, for AGS cells, no significant differences were observed in proliferation between siRNA-NC and siRNA-ATF4 groups when ASNS was overexpressed (Fig. 4F), suggesting that *ASNS* upregulation could reverse the inhibitory effects of siRNA-ATF4 on cell proliferation. Taken together, these results indicated that MA might enhance the proliferation of GC cells via activation of the ATF4-ASNS axis.

Discussion

MA fluid is often observed in advanced stage and invasive cancers which supports the view that MA is involved in disease progression (37). According to clinical data, the management of MA in patients with advanced GC would improve their prognoses (6). Survival and proliferation in the peritoneal cavity are critical steps for GC cells to form peritoneal metastases after cancer cells detach from the primary cancer sites (38). The tumor microenvironment has been a hotspot in cancer research for decades (39-41). MA is a special microenvironment for detached GC cells and the metastasis sites of the peritoneum and has garnered significant interest recently. Previous research has implied that components such as growth factors (IL6 and VEGF) (19), soluble proteins (16), lipids (15) and microRNAs (14) may accelerate the progression of PM. However, the precise mechanisms involved in the process by which MA influences the biological behaviors of cancer cells remain to be elucidated. In the present study, the results suggested that the proliferation of AGS and SNU16 cells treated with MA from different patients with GC (stage IV with PM) was significantly enhanced compared with that of the cells treated with benign ascites. This finding is consistent with prior studies that demonstrated that pleural fluid or MA can promote the proliferation of cancer cells (42,43). In the present study, no significant dose-dependent promotion effects of MA on the proliferation of cancer cells were observed, which may be attributed to the exhaustion of nutrients in the culture system. Therefore, a more complex and refined model may be needed for an improved representation of the actual conditions *in vivo*.

Most previous studies on pleural effusion or ascites have primarily explored the influence of one component or a certain type of substance on the progression of cancer (14,16,19). The present study aimed to elucidate the potential universal mechanisms underlying the effects of different MA on GC cell proliferation. It was found that MA treatment induced the upregulation of *ASNS* levels in GC cells, which serves a role in mediating the proliferation-promoting effects of MA on GC cells. In addition, the upstream TF ATF4, a main regulator of *ASNS*, was upregulated at the protein level following MA treatment. Finally, the findings indicated that the increased proliferation of MA-treated cells may be partially attributed to the activation of the ATF4-ASNS axis.

Previous research on ASNS has mainly focused on hematological malignancies (44). As acute lymphoblastic leukemia (ALL) tumor cells lack ASNS, L-asparaginase, which catalyzes the conversion of asparagine into aspartate, exhibits anti-tumor activity for ALL cell proliferation relying on exogenous asparagine (45). In the present study, MA promoted the upregulation of *ASNS*, which resulted in increased levels of asparagine and L-asparaginase, did not show antitumor effects in MA-treated cancer cells (data not shown). This is consistent with previous conclusions that upregulated ASNS can confer resistance to L-asparaginase (46,47). In addition, to the best of the authors' knowledge, no clinical drugs targeting ASNS have yet been developed. A recent study suggested that inhibition of the production of aspartate can sensitize $ASNS^{high}$ lymphoma to L-asparaginase (48). This conclusion may provide new insights into the treatment of GC with PM and MA.

The present study revealed a previously unknown mechanism of the proliferation-promoting effects of MA on GC cells which may be involved in the progression of PM. However, the substance in MA that specifically affects the

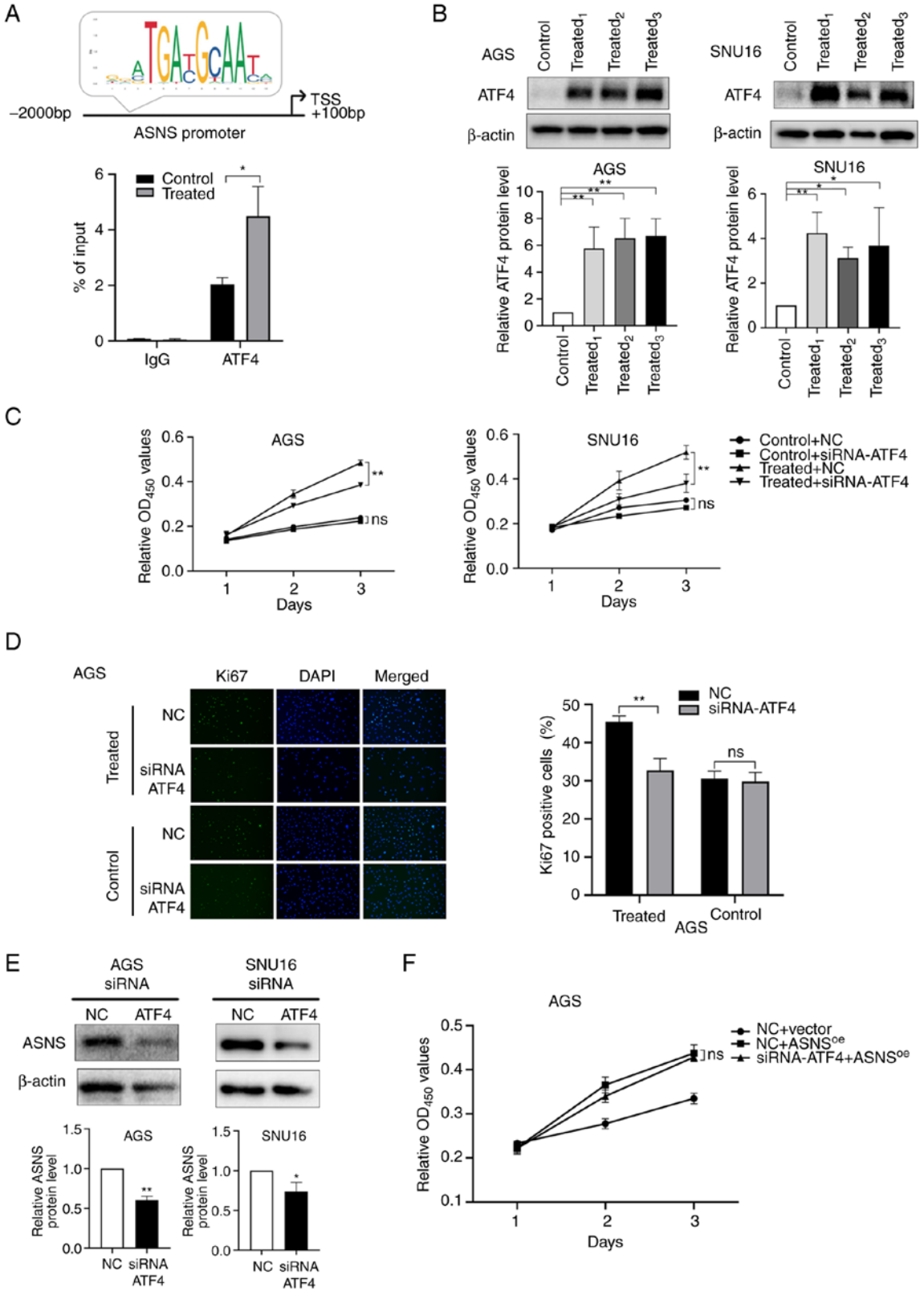

Figure 4. MA promotes the growth of GC cells partially via the activation of ATF4-ASNS axis. (A) Top: Predictive binding site of ATF4 at the promoter region of ASNS by JASPAR database and (bottom) chromatin immunoprecipitation assay of ATF4 on ASNS promoter in AGS cells treated with malignant ascites or benign ascites for 48 h. (B) The protein levels of ATF4 in AGS and SNU16 cells and the quantification were measured by western blotting after cells were treated with 10% benign ascites or 10% MA for 48 h. Control: Treated with benign ascites; treated$_1$, treated$_2$ and treated$_3$: Cells treated with MA from three different GC patients. (C) Cell viability of AGS and SNU16 cells, after cells were transfected with siRNA-NC/siRNA-ATF4, were detected on day 1, 2 and 3 by CCK8 when cells were treated with benign ascites (control) or MA (treated). (D) Ki67-positive cells were counted in MA-treated/control AGS after siRNA-ATF4 or NC was transfected (magnification, x200). MA used in (C) and (D) was from the No.1 patient in Table I. (E) ASNS expression at the protein level in AGS and SNU16 was detected by western blotting when ATF4 was downregulated using siRNA-ATF4. (F) In negative control lentivirus (vector) or ASNS-overexpressed lentivirus (ASNS^{oe}) transduced AGS, the proliferation of cells treated with siRNA-ATF4/NC were recorded at 24, 48 and 72 h by CCK-8 assay (ns, no statistical significance; *P<0.05; **P<0.01). MA, malignant ascites; GC, gastric cancer; ATF4, activating transcription factor 4; ASNS, asparagine synthetase; si, small interfering; NC, negative control.

proliferation of cancer cells via the activated ATF4-ASNS axis remains unclear. Owing to the heterogeneity and complexity of MA, which contains a variety of proteins, polypeptides, lipids and some small molecules, the authors are planning to use mass spectrometry and chemokine/cytokine arrays to explore the active substances in MA in a future study. In addition, previous research has demonstrated that the molecular components and cell contents of ascites change continuously during the course of disease, implying that a changing environment for cancer cells has emerged in MA (11). Therefore, ascites from patients with different stages, subtypes or even ascitic fluids derived from one patient at different times may exert different effects on the biological behavior of cancer cells. In addition, the number of ascites samples used in the present study was limited, so more representative samples are needed in a future study to further support our conclusions.

Of patients with cancer, ~10% develop MA during the course of the disease, which is primarily observed in patients with ovarian, pancreatic and gastric cancer (49). The effect of MA on tumor progression has not been thoroughly elucidated and the underlying molecular mechanisms remain obscure. In the present study, it was demonstrated that the MA supernatant from patients with GC promoted the proliferation of cancer cells. MA fluid may activate the ATF4-ASNS axis, which facilitates the enhanced proliferation of GC cells. These findings imply that, for advanced GC patients with PM and MA, successful management of MA may slow down the progression of PM and inhibition of the production of ASNS may be a potential target for treating GC with MA.

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Availability of data and materials

The RNA-seq datasets generated and/or analyzed during the current study are available in the Genome Sequence Archive for Human repository, https://ngdc.cncb.ac.cn/gsa-human/s/VUMxD15b. The other datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XZ and WL contributed to the conception, supervision of the experiments and revision of the manuscript. YJ designed, performed the experiments, conducted the data analysis and wrote the manuscript. XP performed experiments, revised the article and conducted data interpretation. YW, ZH, LC, MW, YZ and JL collected the clinical samples, clinicopathological information and conducted the experiments. XZ and YJ confirmed the authenticity of all the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was performed in accordance with the Declaration of Helsinki. All the enrolled patients signed informed consents to provide ascitic biospecimen, which was approved by the ethics committee of Navy Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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