

Original Research

S100A4 Promotes the Proliferation and Invasion of Gastric Cancer Cells and Regulates the Reprogramming of Fatty Acid Metabolism via the Wnt/ β -catenin Signaling Pathway

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Abstract

Background: Related studies have shown that S100A4 is dysregulated in various types of cancer and is closely associated with tumor progression. However, its specific role in gastric cancer (GC), particularly its effects on metabolic pathways, has not yet been fully elucidated. **Methods:** We analyzed S100A4 expression levels in gastric cancer tissues and cell lines using bioinformatics data from The Cancer Genome Atlas (TCGA), immunohistochemistry (IHC), and western blotting (WB). We then conducted gain-of-function and loss-of-function experiments, including Cell Counting Kit-8 (CKK-8) assays, colony formation assays, scratch assays, and Transwell migration and invasion assays, to evaluate the effects of S100A4 on gastric cancer cell proliferation, invasion, and migration; Changes in lipid metabolism were assessed through lipid droplet staining (BODIPY), intracellular triglyceride quantification, and Western blotting analysis of key lipid synthesis enzymes, including ATP citrate lyase (ACLY), acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FASN). The involvement of the Wnt/ β -catenin pathway was investigated using Western blot analysis of relevant proteins, nuclear and cytoplasmic β -catenin expression analysis, and pharmacological inhibition with XAV939 and ICG-001. **Results:** S100A4 was significantly upregulated in gastric cancer tissues and cell lines and was associated with poorer overall survival. Functional experiments demonstrated that S100A4 enhanced the proliferation, migration, and invasion capabilities of gastric cancer cells. S100A4 also promoted the reprogramming of fatty acid metabolism, including increased lipid droplet formation, elevated intracellular triglyceride levels, and upregulation of lipid synthase expression. Mechanistically, these effects depended on the activation of Wnt/ β -catenin signaling pathway, as pharmacological inhibition of this pathway reversed the S100A4-induced malignant phenotype and alterations in lipid metabolism. **Conclusion:** S100A4 may promote gastric cancer progression through regulation of fatty acid metabolism via the Wnt/ β -catenin pathway and is associated with enhanced proliferation, migration, and invasion.

Keywords: S100A4 protein; stomach neoplasms; Wnt signaling pathway; fatty acids/metabolism; cellular metabolism

1. Introduction

Gastric cancer (GC) continues to be among the deadliest malignancies globally and represents a considerable public health challenge [1], particularly in East Asia [2]. Despite notable progress in diagnostic techniques and treatment options, the outlook for individuals with advanced GC continues to be unfavorable, primarily due to the aggressive nature of tumor proliferation, swift invasion, and the spread of metastases [3]. Increasing evidence underscores metabolic reprogramming as an essential characteristic of malignancies, crucial for supporting tumor growth and survival [4]. Among various metabolic shifts, altered lipid metabolism, particularly fatty acid (FA) biosynthesis, has been recognized as a critical driver of tumor invasiveness and resistance to treatment in GC [5].

Additionally, the Wnt/ β -catenin signaling pathway serves as a pivotal oncogenic mechanism involved in cellular proliferation, differentiation, and tumor progression within GC [6]. Aberrant activation of this pathway promotes tumor growth and metastatic potential, with

accumulating evidence indicating its role in metabolic regulation [7,8]. Notably, the Wnt/ β -catenin pathway bridges oncogenic signaling with lipid metabolism by inducing transcriptional activation of key enzymes involved in FA biosynthesis [9]. However, the upstream molecular mechanisms connecting Wnt/ β -catenin signaling to FA metabolism remodeling in GC are not well defined.

S100A4, a member of the S100 calcium-binding protein family, significantly influences tumor invasion [10], metastasis [11], and epithelial-mesenchymal transition [12] across various malignancies. Elevated S100A4 expression correlates strongly with aggressive disease characteristics and poorer clinical outcomes [13]. Previous research predominantly emphasized the involvement of S100A4 in cytoskeletal dynamics and cellular motility; nonetheless, its contribution to metabolic regulation has not been extensively investigated. Specifically, the underlying mechanisms by which S100A4 modulates the Wnt/ β -catenin signaling pathway and fatty acid metabolism remain to be further elucidated.



In this study, we evaluated the expression and biological roles of S100A4 in GC and explored the associated molecular mechanisms. Our findings demonstrate that S100A4 promotes gastric cancer progression by activating the Wnt/ β -catenin signaling pathway. Additionally, S100A4 induces fatty acid metabolic reprogramming, laying the groundwork for further experiments—such as investigating whether the S100A4-Wnt/ β -catenin signaling pathway influences gastric cancer proliferation and invasion through fatty acid metabolic reprogramming—and providing a theoretical basis and potential therapeutic strategies for the treatment of gastric cancer.

2. Materials and Methods

2.1 Bioinformatics Analysis

Access to the TCGA-STAD dataset's RNA-seq expression data (HTSeq-FPKM) and associated patient clinical information was facilitated by the Genomic Data Commons (GDC) database. For further analysis, the FPKM values that were downloaded were transformed into TPM format and then subjected to a $\log_2(\text{TPM} + 1)$ transformation. R software (version 4.3.2; R Foundation for Statistical Computing, Vienna, Austria) was used to identify genes that were differentially expressed between malignant and nearby normal samples. The Benjamini-Hochberg adjustment was applied with thresholds of $|\log_2\text{FC}| > 1$ and false discovery rate (FDR) < 0.05 . Patients were divided into high- and low-expression groups according to the median S100A4 expression value ($\log_2[\text{TPM} + 1] = 2.17$) in the TCGA-STAD cohort. To quantify variations in patient overall survival (OS), Kaplan-Meier curves with log-rank test comparisons were utilized. A hazard ratio (HR) and a 95% confidence interval (CI) were also computed using Cox proportional hazard regression. Using Pearson's correlation ($|r| > 0.3$, $p < 0.05$), genes that exhibited substantial associations with S100A4 expression were discovered. An FDR q-value less than 0.05 was used to determine the significance of functional enrichment studies conducted using GSEA with gene sets obtained from the MSigDB Hallmark and KEGG databases.

2.2 Patient Samples

In total, 30 pairs of GC tissues and corresponding adjacent normal tissues were obtained from surgically treated patients at our hospital, who had not undergone any pre-operative therapy. Informed consent was obtained in written form from all participants prior to sample procurement. Ethical approval was granted by the Ethics Committee of Yixing People's Hospital, and all experimental protocols adhered strictly to the ethical guidelines stipulated in the Declaration of Helsinki. Patient inclusion criteria included: (1) histologically verified primary gastric adenocarcinoma, (2) availability of paired tumor and corresponding normal gastric tissues, (3) absence of any prior chemotherapy, radiotherapy, targeted treatments, or immunotherapy, and (4)

complete clinical and pathological documentation. Exclusion criteria encompassed: (1) presence of concurrent malignancies, (2) recurrent gastric carcinoma, (3) previous neoadjuvant treatments, and (4) insufficient tissue samples for comprehensive analysis. Tumor staging adhered to the guidelines outlined in the 8th edition of the AJCC TNM classification system.

2.3 Cell Culture

STR profiling was performed to authenticate the human gastric cancer cell lines, including AGS (SCSP-5262), HGC-27 (SCSP-5263), MKN-45 (SCSP-5473), and the normal gastric epithelial cell line GES-1 (SCSP-308), which were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cell lines were routinely tested for mycoplasma contamination and confirmed to be negative before experiments. While MKN-45, and GES-1 were cultivated in RPMI-1640, AGS and HGC-27 were cultured in DMEM. Each medium was supplemented with 100 units of penicillin per milliliter, 100 micrograms of streptomycin per milliliter, and 10% fetal bovine serum (FBS). Cells were maintained at 37 °C in an incubator with 5% CO₂ and humidified conditions, and experimental procedures commenced once cells reached approximately 70%–80% confluency.

2.4 Immunohistochemistry (IHC)

The GC tissues underwent fixation in formalin, embedding into paraffin blocks, and were then sliced into sections approximately 4 μm thick. After deparaffinization using xylene, sections were gradually rehydrated with descending ethanol solutions. Microwaving the sections in a citrate buffer solution (pH 6.0) for 15 minutes allowed the antigen retrieval to take place. At room temperature for 10 minutes, 3% hydrogen peroxide inhibited endogenous peroxidase activity. This was followed by an overnight incubation at 4 °C with an anti-S100A4 primary antibody (#13018, Cell Signaling Technology, Danvers, MA, USA; diluted 1:200). The sections were then treated for an additional hour at room temperature in a blocking solution that contained 5% bovine serum albumin. After washing with PBS, the sections were incubated with an HRP-linked goat anti-rabbit IgG secondary antibody (Cat. No. 7074, Cell Signaling Technology, Danvers, MA, USA; diluted 1:5000) for 1 h at room temperature. Under a microscope, the staining was seen for 2–5 minutes using a DAB chromogenic kit (Vector Laboratories, USA; Cat. No. SK-4105). The last step was to mount the slices with coverslips, dehydrate them using rising ethanol gradients, counterstain them with hematoxylin, and then photograph them under a BX53 microscope (Olympus Corporation, Tokyo, Japan).

2.5 Cell Transfection

Small interfering RNAs (siRNAs) targeting S100A4 were synthesized by GenePharma (Shanghai, China), the

siRNA sequences are shown in Table 1 (Target sequence of siRNAs is shown in DNA format). The inhibitors XAV939 and ICG-001 were purchased from Thermo Fisher Scientific (Shanghai, China) for the inhibition of the Wnt/ β -catenin signaling pathway. Cell transfections were performed utilizing Lipofectamine® 2000 (Invitrogen, USA; Cat. No. 11668019). Specifically, two distinct small interfering RNAs (siRNAs, 50 nM each) designed to silence S100A4 or a nonspecific negative control siRNA were introduced into the cells. Additionally, a plasmid vector (pcDNA3.1) containing the full-length coding sequence of S100A4 was employed for overexpression experiments. The full-length coding sequence of human S100A4 was cloned into the pcDNA3.1 vector by GenePharma (Shanghai, China). The construct was validated by Sanger sequencing prior to use. An empty pcDNA3.1 vector was used as a negative control. Cells were collected for subsequent analysis within 24–48 hours after transfection. Western blot (WB) assays validated successful downregulation or overexpression. Each siRNA specifically targeted separate sequences within the S100A4 transcript.

Table 1. Target sequences of siRNAs.

Gene	Target sequence (5'–3')
<i>si-S100A4-1</i>	TGTAACGAATTCTTTGAAG
<i>si-S100A4-2</i>	ACGAATTCTTTGAAGGCTT
<i>si-NC</i>	TTCTCCGAACGTGTCACGT

siRNAs, small interfering RNAs.

2.6 Western Blot (WB)

To prepare the protein samples, tissues and cultured cells were extracted using RIPA lysis buffer (Beyotime, China; Cat. No. P0013B), which contained cocktails of phosphatase and protease inhibitors (Beyotime, Shanghai, China). A BCA assay kit (Thermo Fisher Scientific, USA; Cat. No. 23225) was used to measure the amounts of proteins. Using NE-PERTM Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher, USA; Cat. No. 78833), proteins of the nucleus and the cytoplasm were independently extracted. Protein extracts were then transferred onto PVDF membranes (Millipore, USA) and electrophoresed on a 10% SDS-polyacrylamide gel. This is how they were examined. Membranes were blocked with 5% BSA or skim milk solution for an hour before being incubated overnight at 4 °C with the proper primary antibodies (Cell Signaling Technology, USA, 1:1000). After three five-minute washes in PBS containing 0.1% Tween-20, Membranes were incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (Cell Signaling Technology, USA; Cat. No. 7074; 1:5000) or HRP-conjugated horse anti-mouse IgG secondary antibody (Cell Signaling Technology, USA; Cat. No. 7076; 1:5000) for 1–2 h at room temperature. A Bio-Rad ChemiDoc imaging system was used to detect

protein bands, and an ECL detection kit from Labscience in Wuhan, China, was used for chemiluminescence protein visualization. You can find β -actin in the cytoplasm (1:1000) and Histone H3 or Lamin B1 in the nucleus (1:2000) as internal reference proteins. The relative abundance of proteins was determined by quantifying band intensities using ImageJ software. The antibodies used are listed in Table 2.

2.7 Colony Formation Assay (CFA)

The GC cells' capacity to multiply over time was examined using a CFA. In summary, 500 cells per well were seeded into 6-well culture plates, which were then incubated for 14 days at 37 °C with 5% CO₂. The medium was replaced every three days. Following incubation, the colonies were cleaned with PBS, fixed for 15 minutes with a 4% paraformaldehyde solution, and then stained for another 15 minutes with a 0.5% crystal violet solution. Excess stain was removed by rinsing with distilled water, and plates were allowed to air-dry. Colonies containing ≥ 50 cells were counted manually under microscopy. All assays were independently repeated three times, and the mean number of colonies was calculated for statistical evaluation.

2.8 Wound Healing Assay (WHA)

A scratch assay was used to assess the motility of the cells. A 90% cell density was attained by cultivated cells that had been placed onto 6-well plates. Each cell monolayer was uniformly scratched with a sterile pipette tip (200 μ L). Gently washing the cells with PBS was followed by the addition of serum-free culture media. Microscopic pictures were taken at both the initial (0 h) and final (24 h) time periods. Scratch widths were measured at each interval using ImageJ software (version 1.54, National Institutes of Health, Bethesda, MD, USA) to calculate the wound healing percentage.

2.9 Cell Viability Assay (CCK-8)

After the initial 24 hours of culture, the cells were cultured at 37 °C in 5% CO₂ conditions for an additional 2 hours before 10 μ L of CCK-8 (Dojindo, Japan; Cat. No. CK04) reagent was added to each well. A microplate spectrophotometer (Bio-Rad, Hercules, CA, USA) was used to measure the absorbance at 450 nm. The procedure was independently repeated three times, and absorbance values were recorded at 0, 24, 48, and 72 hours to plot cell growth curves representing relative cell viability.

2.10 Transwell Migration and Invasion Assay

Cell migration and invasion were examined utilizing Transwell chambers (24-well, 8 μ m pore diameter; Corning, USA). To evaluate migration, cells (2×10^4 per 200 μ L) were suspended in serum-free medium after trypsinization and seeded into the top chamber. As a chemoattractant, 600 μ L of full growth media with 10% FBS was introduced to the lower chamber. Cotton swabs were used to carefully re-

Table 2. Antibodies used for western blotting analysis.

Name	Company	Catalog number	Antibody concentration
P-GSK3 β (Ser9)	Cell Signaling Technology, USA	#5558S	1:1000
GSK3 β	Cell Signaling Technology, USA	#12456S	1:1000
Histone H3	Cell Signaling Technology, USA	#4499S	1:2000
p- β -catenin (Ser33/37/Thr41)	Cell Signaling Technology, USA	#9561S	1:1000
β -catenin	Cell Signaling Technology, USA	#9562S	1:1000
c-MYC	Cell Signaling Technology, USA	#9402S	1:1000
β -actin	Cell Signaling Technology, USA	#4970S	1:1000
ACC1	Boster, China	A01802-2	1:2000
ACLY	Boster, China	PB10024	1:2000
FASN	Boster, China	PB9865	1:2000

ACC1, acetyl-CoA carboxylase 1; ACLY, ATP citrate lyase; FASN, fatty acid synthase.

move non-migrating cells from the top surface following a 24-hour incubation period at 37 °C and 5% CO₂. After the cells reached the membrane's underside, they were fixed for 15 minutes with 4% paraformaldehyde and then stained for an additional 15 to 20 minutes with a 0.1% crystal violet solution. The number of cells in five different microscopic areas was taken at random. Before incubating the plates at 37 °C for 1 hour to let the gel solidify, the upper membrane was coated with Matrigel (Corning, USA; Cat. No. 356234) diluted 1:8 for invasion study. Cells were subsequently seeded into the gel-coated chamber, with the assay performed similarly to migration tests but incubated for 24–48 hours.

2.11 Triglyceride Content and Cellular Lipid Quantification

Intracellular triglyceride contents were quantified using a triglyceride assay kit (J22127; Wuhan Jilid Biotechnology Co., Ltd., China). Additionally, lipid droplets within cells were visualized by staining with the fluorescent dye BODIPY 493/503 (Invitrogen, USA; Cat. No. D3922). Following fixation in 4% paraformaldehyde for 15 min, cells were stained using fluorescent dyes (BODIPY 493/503 diluted 1:500 and Hoechst 33342 diluted 1:1000) for 15 min in darkness. Excess dye was subsequently washed off using PBS. Quantitative analyses of lipid droplet numbers and fluorescence intensities were performed using ImageJ (Fiji). Lipid droplet areas and fluorescence intensities per cell were normalized for comparative analysis.

2.12 Quantitative and Statistical Analysis

Statistical analyses were performed using GraphPad Prism (version 9.0; GraphPad Software, San Diego, CA, USA). All quantitative data from cellular experiments and protein assays are expressed as mean $\pm$ standard deviation (SD). The number of independent biological replicates (n) is indicated for each experiment; all Western blot intensity measurements represent the mean of at least three independent biological experiments. Comparisons between two groups were performed using Student's *t*-test, while

comparisons among multiple groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. The normality of data distribution was assessed using the Shapiro-Wilk test, and homogeneity of variances was assessed using the Levene test. When the assumptions of normality or homogeneity of variances were not met, appropriate nonparametric tests were used.

For survival analysis, Kaplan-Meier survival curves were plotted, and the Log-rank test was used to assess differences between groups. Hazard ratios (HR) and 95% confidence intervals (CI) were calculated using the Cox proportional hazards regression model. All statistical tests were two-sided; *p*-values < 0.05 were considered statistically significant.

3. Results

3.1 S100A4 Is Highly Expressed in GC and Is Associated With Poor Prognosis

Initially, the levels of S100A4 expression were examined by comparing GC samples with normal gastric tissues utilizing TCGA-derived datasets. The results indicated a markedly higher expression of S100A4 in GC tissues than in normal controls (Fig. 1A). Further evaluation identified increased S100A4 expression correlating with higher T stages (T2–T4) relative to the T1 stage, highlighting its potential role in tumor advancement (Fig. 1B). Kaplan-Meier survival curves demonstrated notably reduced OS in patients exhibiting elevated S100A4 levels, indicating its prognostic significance for unfavorable outcomes (Fig. 1C). IHC staining corroborated these findings by showing substantial increases in S100A4 protein expression in GC tissues versus normal gastric mucosa (Fig. 1D). Subsequently, validation via WB was performed, confirming that the S100A4 protein was expressed at increased levels in cancer tissues (Fig. 1E,F). Furthermore, the expression of S100A4 was notably elevated across several GC cell lines (MKN-45, AGS, HGC-27) (Fig. 1G,H). Collectively, these data illustrate the aberrant upregulation of S100A4 in GC, correlating with disease progression and adverse clinical outcomes.

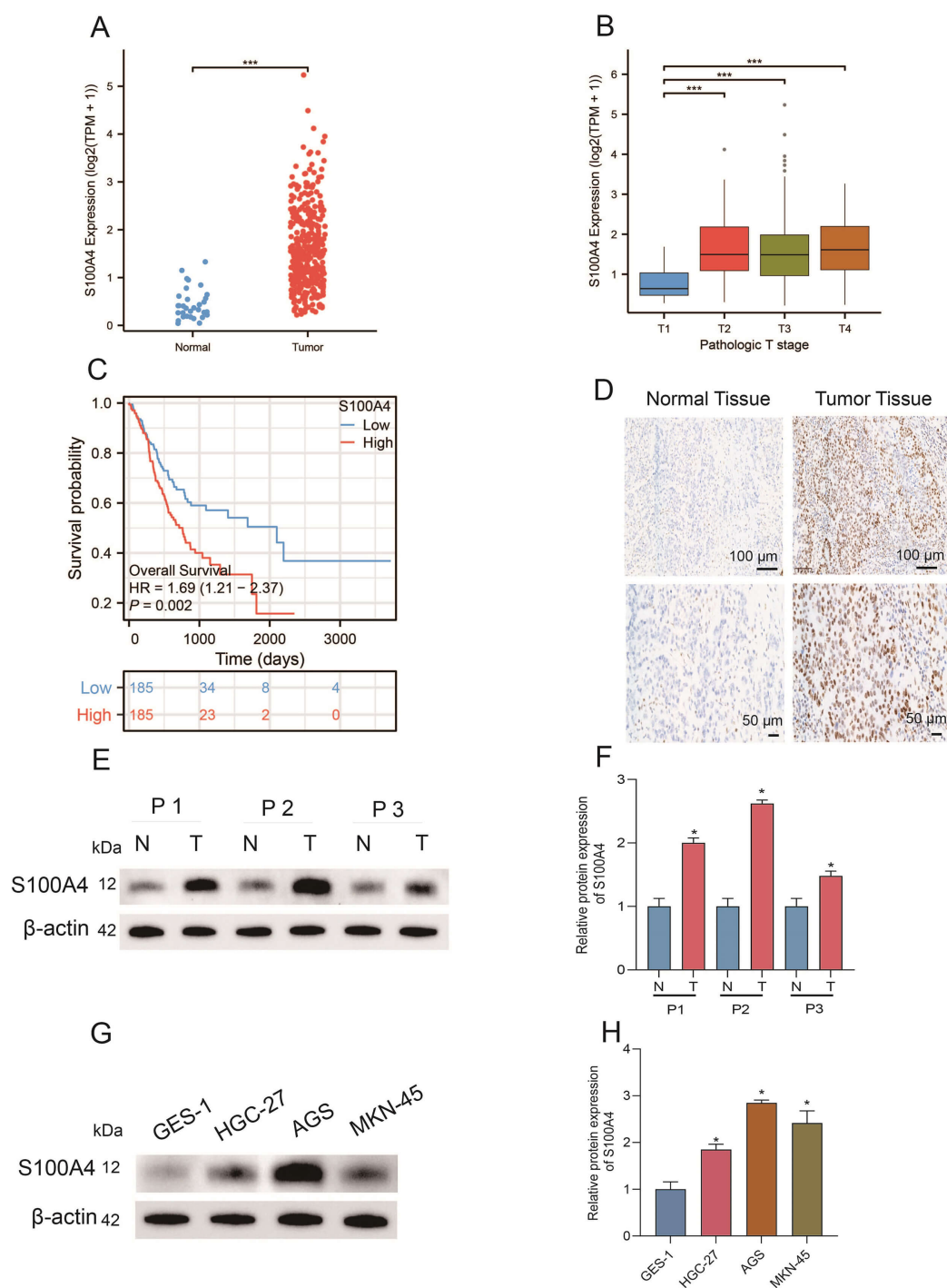


Fig. 1. S100A4 is overexpressed in gastric cancer and is associated with poor prognosis. (A) Analysis of the TCGA dataset shows that S100A4 expression levels are elevated in gastric cancer. (B) Stratification of S100A4 expression by pathological T stage in the TCGA-STAD cohort. (C) Kaplan-Meier survival analysis shows that high S100A4 expression is associated with shorter overall survival (OS) in gastric cancer patients. (D) Representative immunohistochemical images demonstrating the staining patterns of S100A4 in gastric cancer tissue and normal tissue (Positive S100A4 staining is shown in brown, and nuclei are counterstained with hematoxylin (blue)). Scale bars: 100 μ m (top) and 50 μ m (bottom). (E,F) Western blot results showing S100A4 protein expression and quantitative analysis in paired samples of gastric cancer tissue (T) and adjacent normal tissue (N). (G,H) Western blot results and quantitative analysis comparing S100A4 protein expression between different gastric cancer cell lines and GES-1 normal gastric epithelial cells. For the experimental data (panels D–H), data are presented as mean $\pm$ SD from three independent experiments (n = 3). (* $p < 0.05$, *** $p < 0.001$ compared with Normal Tissue. Horizontal lines indicate the specific groups being compared).

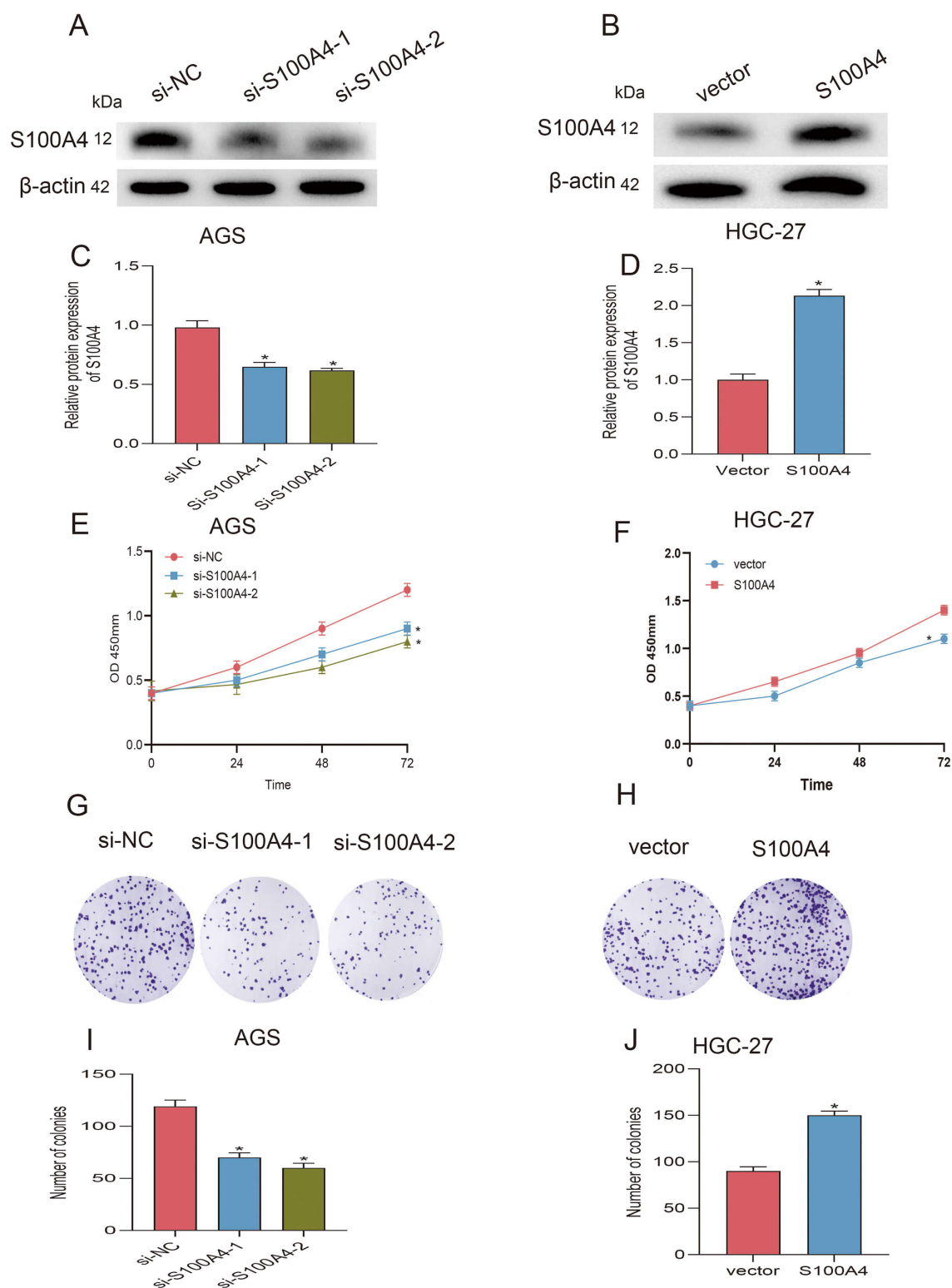


Fig. 2. S100A4 promotes GC cell proliferation. (A) WB analysis verifying knockdown of S100A4 in AGS cells transfected with si-S100A4-1 or si-S100A4-2. (B) WB analysis confirming S100A4 overexpression in HGC-27 cells following transfection with S100A4 plasmid. (C,D) Densitometric quantification of S100A4 protein expression levels shown in (A) and (B). (E,F) Cell viability evaluated using CCK-8 assays in AGS and HGC-27 cells after S100A4 knockdown or overexpression. (G,H) Representative CFA images for AGS and HGC-27 cells. (I,J) Colony count quantification corresponding to (G) and (H). Data are presented as mean $\pm$ SD from three independent experiments (n = 3). (* $p < 0.05$ versus the corresponding control group (si-NC or vector)). GC, gastric cancer; CFA, colony formation assay.

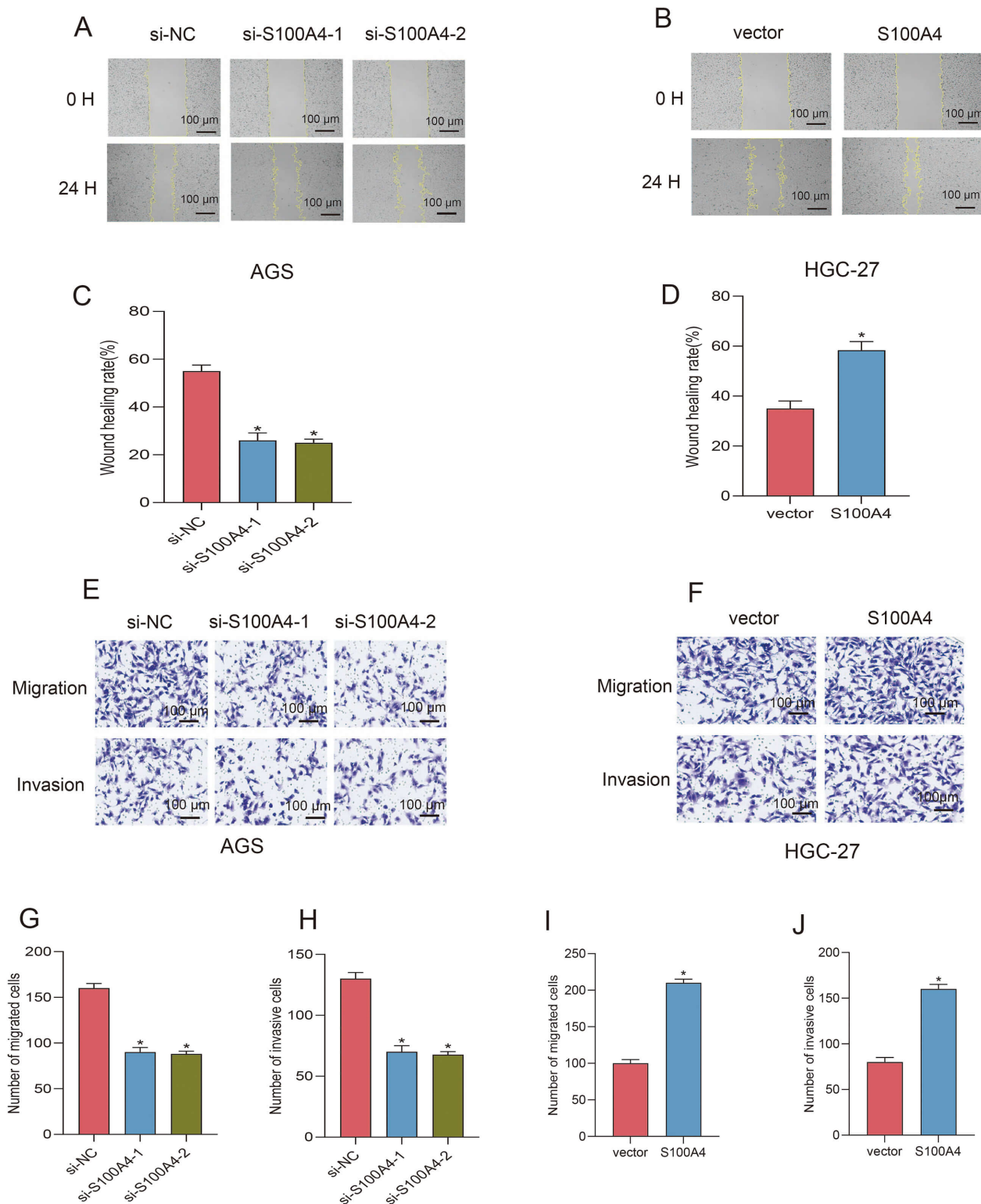


Fig. 3. S100A4 promotes migration and invasive properties in GC cells. (A,B) Representative wound-healing assay images captured at 0 h and 24 h after silencing or overexpressing S100A4 in AGS and HGC-27 cells. Scale bars: 100 μ m. (C,D) Quantitative results for the rate of wound closure calculated from images shown in (A,B). (E,F) Representative Transwell images demonstrating cell migration and invasion of AGS and HGC-27 cells following S100A4 knockdown or overexpression. (G,H) Numbers of migrated and invaded AGS cells quantified from the images presented in (E). Scale bars: 100 μ m. (I,J) Numbers of migrated and invaded HGC-27 cells quantified from images depicted in (F). Scale bars: 100 μ m. Data are presented as mean $\pm$ SD from three independent experiments ($n = 3$). (* $p < 0.05$ versus the corresponding control group (si-NC or vector)).

3.2 The Proliferative Role of S100A4 in GC Cells

To investigate the role of S100A4, its expression was silenced in AGS cells using siRNA, and an overexpression model was established in HGC-27 cells. WB results showed that si-S100A4-1 and si-S100A4-2 effectively reduced S100A4 expression, whereas overexpression significantly increased its protein levels (Fig. 2A–D). Cell proliferation was then evaluated using a CCK-8 assay. S100A4 knockdown significantly suppressed AGS cell proliferation, while overexpression markedly enhanced proliferation in HGC-27 cells (Fig. 2E,F). In addition, CFAs demonstrated that S100A4 knockdown reduced clonogenic ability, whereas overexpression increased colony formation capacity (Fig. 2G–J). Collectively, these findings indicate that S100A4 promotes proliferation and clonogenicity in GC cells and acts as a tumor-promoting factor in GC progression.

3.3 S100A4 Enhances Migration and Invasion Capabilities of GC Cells

Considering that migration and invasion play critical roles in cancer progression, the influence of S100A4 expression on these processes was evaluated using wound healing and Transwell assays. The WHA revealed that silencing S100A4 significantly impaired cell migration abilities in AGS cells, whereas its enforced expression substantially promoted migration capabilities in HGC-27 cells (Fig. 3A–D). Additionally, Transwell assays confirmed these findings; S100A4 knockdown notably decreased migration and invasion in AGS cells, while its overexpression significantly elevated these capabilities in HGC-27 cells compared to control groups (Fig. 3E–J). Collectively, these results indicate that S100A4 substantially promotes migration and invasion, highlighting its critical role in GC metastasis.

3.4 S100A4 Facilitates GC Progression by Modulating Lipid Metabolism

To explore the potential molecular mechanisms of S100A4 in gastric cancer, the researchers conducted bioinformatics analyses. GO analysis revealed that genes associated with S100A4 are primarily involved in lipid metabolism, the Wnt signaling pathway, and cell growth regulation (Fig. 4A). GSEA further revealed that S100A4 expression was significantly associated with the HALLMARK_XENOBIOTIC_METABOLISM pathway (Fig. 4B), suggesting its potential involvement in tumor metabolic regulation (Fig. 4B), suggesting its involvement in tumor metabolic regulation. Fluorescent staining of lipid droplets revealed that S100A4 knockdown significantly reduced lipid droplet formation, whereas its overexpression increased lipid accumulation (Fig. 4C–F). Quantitative analysis of fluorescence intensity confirmed these observations (Fig. 4E,F). Triglyceride assays showed that S100A4 knockdown significantly reduced

triglyceride levels in AGS cells, whereas S100A4 overexpression significantly increased them in HGC-27 cells (Fig. 4G,H). Western blot analysis revealed that S100A4 knockdown in AGS cells significantly reduced the expression of lipid synthesis-related proteins ACC1, ACLY, and FASN, whereas S100A4 overexpression in HGC-27 cells significantly increased the levels of these proteins (Fig. 4I–L). In summary, these results indicate that S100A4 enhances lipid synthesis and lipid droplet accumulation in gastric cancer cells, suggesting that S100A4 plays a role in reprogramming tumor lipid metabolism.

3.5 S100A4 Activates the Wnt/ β -catenin Signaling Pathway in Gastric Cancer Cells

To investigate the mechanisms by which S100A4 promotes GC progression, expression changes of Wnt/ β -catenin signaling-related proteins were analyzed. WB results showed that in AGS cells, S100A4 knockdown significantly reduced p-GSK3 β (ser9) and c-MYC expression while increasing p- β -catenin(Ser33/37/Thr41) levels. Conversely, S100A4 overexpression in HGC-27 cells significantly elevated p-GSK3 β (ser9) and c-MYC protein levels and decreased p- β -catenin(Ser33/37/Thr41) expression (Fig. 5A–D). Additionally, β -catenin nuclear localization assays revealed that S100A4 knockdown significantly decreased nuclear β -catenin levels in AGS cells, while its overexpression markedly increased nuclear β -catenin accumulation in HGC-27 cells (Fig. 5E–H). However, the levels of β -catenin in the cytoplasm showed the opposite trend: they were significantly increased in AGS cells with S100A4 knocked down, whereas they were significantly reduced in HGC-27 cells with S100A4 overexpression (Fig. 5I–L). However, total β -catenin expression levels remained unchanged regardless of S100A4 manipulation (Fig. 5M–P). Collectively, these data support a role for the Wnt/ β -catenin pathway in mediating the pro-tumorigenic effects of S100A4 in GC cells.

3.6 Inhibition of the Wnt/ β -catenin Pathway Partially Reverses S100A4-induced Tumor Promotion

To validate the involvement of the Wnt/ β -catenin pathway, HGC-27 cells overexpressing S100A4 were treated with pathway inhibitors XAV939 (Selleck Chemicals, USA; Cat. No. S1180) and ICG-001 (Selleck Chemicals, USA; Cat. No. S2662). CFAs showed that S100A4 overexpression promoted colony formation, whereas treatment with XAV939 or ICG-001 effectively reversed this effect (Fig. 6A,C). Transwell assays revealed that S100A4 overexpression enhanced cell migration and invasion, but inhibition of the Wnt/ β -catenin pathway notably reduced these activities (Fig. 6B,D,E). Furthermore, lipid droplet fluorescence staining demonstrated that S100A4 overexpression significantly increased lipid accumulation, while inhibitors markedly reduced lipid droplet formation (Fig. 6F,H). WB analysis further showed that S100A4 overex-

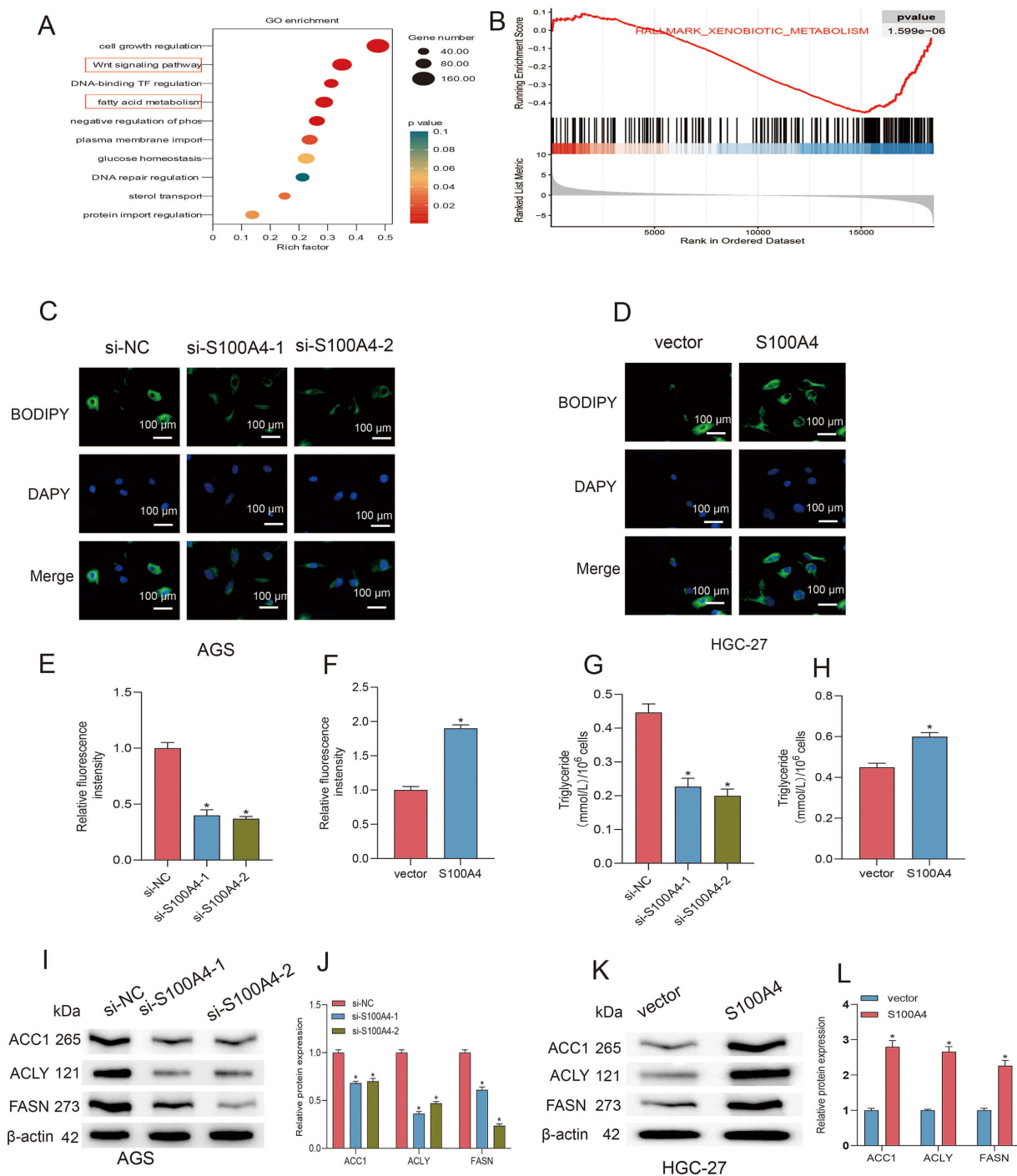


Fig. 4. S100A4 regulates lipid metabolism in gastric cancer cells. (A) GO enrichment analysis of S100A4-correlated genes identified by Pearson correlation analysis. (B) GSEA showing significant enrichment of the HALLMARK_XENOBIOTIC_METABOLISM pathway in cells with high S100A4 expression. (C,D) Representative fluorescence images of lipid droplets in AGS and HGC-27 cells after S100A4 knockdown or overexpression. Lipid droplets were stained with BODIPY (green) and nuclei with DAPI (blue). Merged images are shown. Scale bars: 100 μ m. (E,F) Quantitative Analysis of Relative Fluorescence Intensity. (G,H) Triglyceride levels were measured in AGS cells following S100A4 knockdown and in HGC-27 cells following S100A4 overexpression. (I,K) Western blot analysis of lipid metabolism-related proteins (ACC1, ACLY, FASN) in AGS and HGC-27 cells with altered S100A4 expression. (J,L) Densitometric quantification of protein levels from (I,K), normalized to β -actin. Data are presented as mean $\pm$ SD from three independent experiments ($n = 3$). (* $p < 0.05$ versus the corresponding control group (si-NC or vector)).

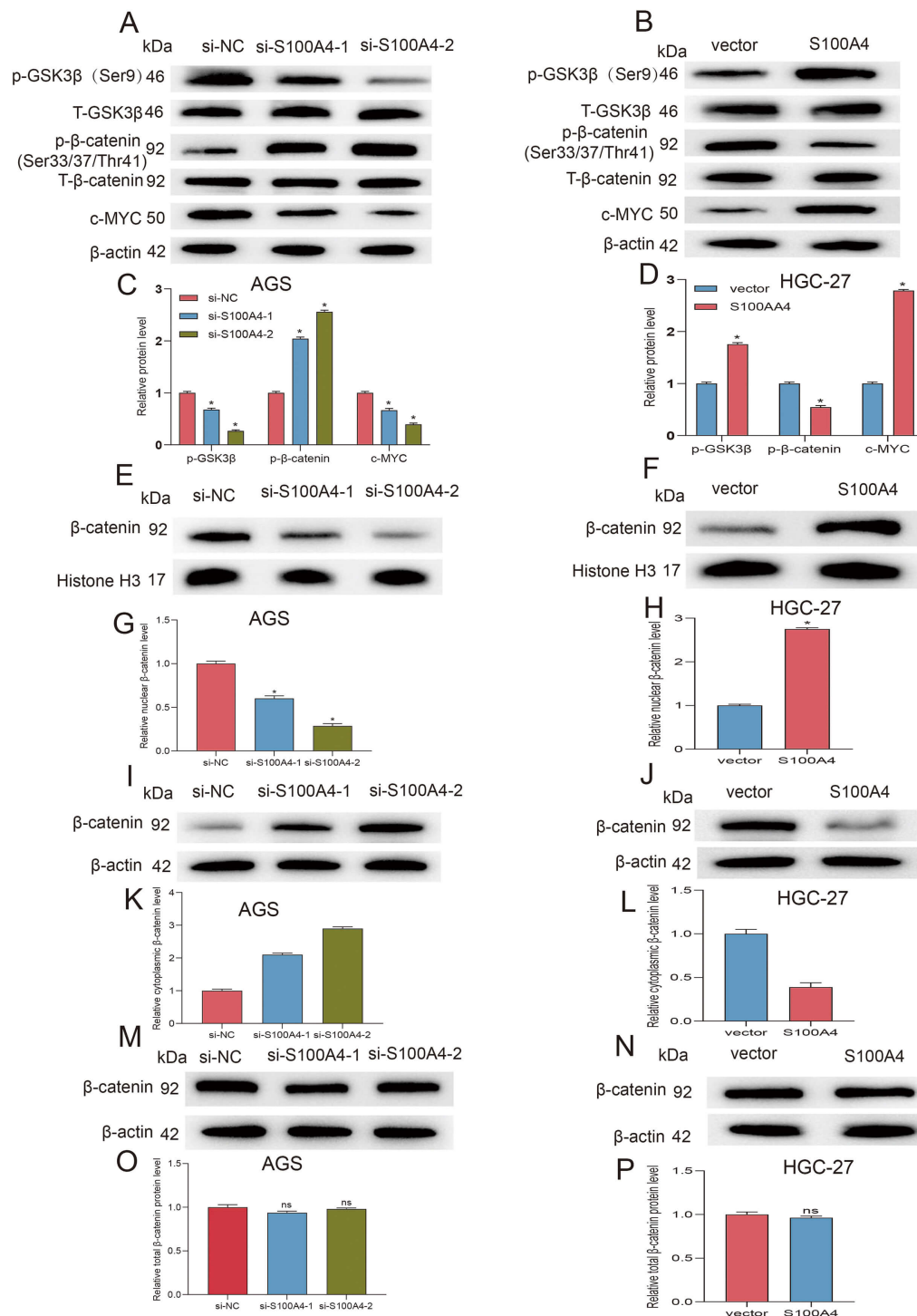


Fig. 5. S100A4 regulates the β -catenin signaling pathway in gastric cancer cells. (A,B) Western blot analysis of p-GSK3 β (Ser9), total GSK3 β , p- β -catenin (Ser33/37/Thr41), total β -catenin, and c-MYC in S100A4-knockdown AGS cells and S100A4-overexpressing HGC-27 cells. (C,D) Quantitative analysis of p-GSK3 β , p- β -catenin, and c-MYC protein levels shown in (A,B). (E–H) Western blot results and quantitative analysis of nuclear β -catenin levels in AGS and HGC-27 cells with altered S100A4 expression. (I–L) Western blot results and quantitative analysis of cytoplasmic β -catenin levels in AGS and HGC-27 cells following S100A4 knockdown or overexpression. (M–P) Western blot results and quantitative analysis of total β -catenin levels in AGS and HGC-27 cells with altered S100A4 expression. Data represent the mean $\pm$ SD from three independent experiments ($n = 3$). (* $p < 0.05$ versus the corresponding control group (si-NC or vector). ns, not statistically significant).

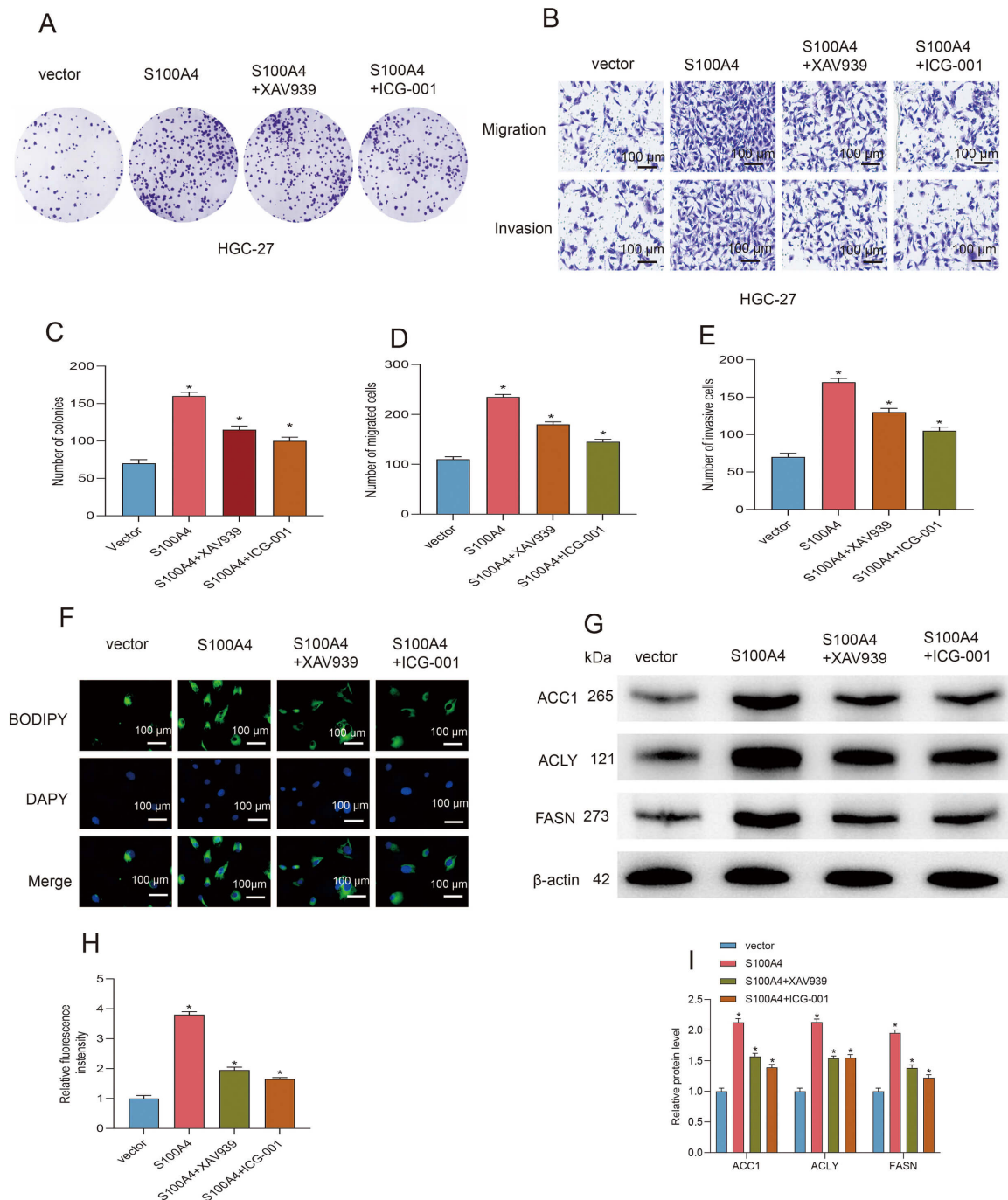


Fig. 6. Targeted pharmacological blockade of the Wnt/β-catenin pathway diminishes malignant characteristics driven by S100A4 in GC cells. (A) Representative CFA images of HGC-27 cells transfected with either S100A4 or empty vector, treated in the presence or absence of the Wnt/β-catenin signaling inhibitors XAV939 and ICG-001. (B) Illustrative images of cell migration and invasion assessed through Transwell assays under the specified experimental conditions. Scale bars: 100 μm. (C) Quantitative colony counts derived from the images shown in (A). (D,E) Statistical analysis quantifying migrated and invaded cells depicted in (B). (F) Representative images of lipid droplet accumulation visualized by BODIPY staining in HGC-27 cells overexpressing S100A4, with or without XAV939 or ICG-001 treatment (Cells were stained with BODIPY (green) and DAPI (blue)). (G) WB analysis of lipid synthesis-related proteins (ACC1, ACLY, and FASN) under indicated conditions. (H) Quantitative analysis of lipid fluorescence intensity from (F). Scale bars: 100 μm. (I) Densitometric analysis of ACC1, ACLY, and FASN protein levels from (G). Data are presented as mean ± SD from three independent experiments (n = 3). (* $p < 0.05$ versus the corresponding control group).

pression upregulated lipid synthesis proteins ACC1, ACLY, and FASN, whereas pathway inhibition significantly decreased their expression (Fig. 6G,I). These results collectively confirm that inhibition of the Wnt/ β -catenin pathway significantly attenuates S100A4-mediated effects on proliferation, migration, invasion, and lipid metabolic reprogramming, highlighting the pathway's critical role in GC progression driven by S100A4.

4. Discussion

The findings from this study underscore the critical involvement of S100A4 in facilitating GC advancement, both functionally and at the underlying molecular level. Substantial increases in S100A4 were identified in GC tissue specimens, correlating with enhanced tumor cell proliferation and invasive abilities. In contrast, targeted knockdown of S100A4 effectively mitigated these aggressive cellular behaviors. Previous studies have shown that MKN45 cells are generally considered more malignant than AGS cells [14]. The Kaplan-Meier curves in this study also demonstrate that elevated S100A4 levels in gastric cancer are associated with a poorer prognosis. However, Western blot (WB) results indicate that S100A4 expression is lower in MKN45 cells than in AGS cells. Nevertheless, this does not contradict the clinical finding that “high S100A4 expression is associated with a poor prognosis”. Rather, this highlights that multiple factors collectively contribute to the malignant phenotype [15]. At the same time, these clinical results are primarily based on tumor tissue [16]. At the mechanistic level, S100A4 augments tumor malignancy by activating the Wnt/ β -catenin signaling cascade, thus driving FA metabolic alterations. Overall, these data confirm that S100A4 is a key molecular mediator in oncogenic signaling pathways and in the reprogramming of lipid metabolism in gastric cancer.

Accumulating evidence highlights that dysregulated Wnt/ β -catenin signaling critically influences cancer initiation and progression [17,18]. Beyond its traditional role in promoting cell proliferation, the Wnt/ β -catenin pathway has emerged as a central regulator linking oncogenic signals with metabolic reprogramming [19]. It regulates lipid synthesis-related gene expression via β -catenin/TCF-mediated transcriptional activation [20], thereby supplying tumor cells with vital biosynthetic precursors and sustaining malignancy [21]. Our findings demonstrate that elevated S100A4 correlates with enhanced Wnt/ β -catenin signaling activity, increased FA metabolism-related protein expression, and lipid accumulation. Moreover, inhibition of Wnt/ β -catenin signaling using XAV939 or ICG-001 significantly reversed S100A4-induced lipid metabolism, cell proliferation, and invasion, confirming this pathway's essential role in mediating S100A4's tumor-promoting effects. Although our study provides clear evidence that S100A4 activates Wnt/ β -catenin signaling, the precise upstream regulatory mechanisms remain to be further ex-

plored. Given the observed increase in inhibitory phosphorylation of GSK3 β at Ser9 and enhanced nuclear accumulation of β -catenin, S100A4 may stabilize β -catenin by modulating GSK3 β activity. Since inhibitory phosphorylation of GSK3 β prevents β -catenin degradation, S100A4 may enhance β -catenin signaling through regulation of upstream kinases or phosphatases involved in GSK3 β phosphorylation. These findings expand our understanding of S100A4's oncogenic functions from phenotypic regulation to metabolic signaling dependency. Next, we will continue to conduct follow-up experiments to investigate whether S100A4 promotes phenotypic changes in gastric cancer through the Wnt/ β -catenin signaling pathway as a result of fatty acid metabolic reprogramming. If this hypothesis is confirmed, it would demonstrate that targeting the S100A4-Wnt/ β -catenin-FA metabolic axis has a solid theoretical foundation, thereby offering a novel and promising therapeutic strategy for clinical practice.

FA metabolic reprogramming in tumor cells is essential for supporting rapid growth and invasive behaviors [22]. This reprogramming sustains malignant characteristics by continuously providing substrates necessary for membrane synthesis, signaling molecules, and energy production [23,24]. Therefore, understanding the upstream regulatory networks driving lipid metabolic reprogramming is crucial to unraveling metabolic dependencies involved in tumor invasiveness [25]. The Wnt/ β -catenin pathway connects oncogenic signaling to lipid metabolic reprogramming by enhancing FA synthesis and regulating lipid homeostasis. This study demonstrates that activation of Wnt/ β -catenin signaling by S100A4 significantly increases expression of ACLY, ACC1, and FASN, as confirmed by WB analysis. This signaling activation also promotes lipid droplet formation and alters droplet-organelle contacts [26], enabling lipid droplets to function effectively as rapidly mobilizable lipid storage pools integrating uptake, resynthesis, and recycling fluxes [27]. These metabolic alterations support two essential processes necessary for sustained migratory and invasive capabilities. Firstly, they provide immediate substrates for membrane phospholipid synthesis and remodeling. Secondly, they reduce lipotoxicity and mitigate oxidative or nutritional stress by sequestering and mobilizing FAs through lipid droplets [28]. When necessary, FAs are released into mitochondria for energy production via lipolysis or lipophagy, thereby enhancing cellular adaptability and motility [29]. Although this study reveals that S100A4 regulates fatty acid metabolism and promotes the malignant phenotype of gastric cancer via the Wnt/ β -catenin pathway, its role in mitochondrial metabolism remains to be elucidated. Given that fatty acid β -oxidation is closely associated with ATP production, ROS homeostasis, and apoptosis sensitivity [30], S100A4-mediated metabolic reprogramming may influence mitochondrial energy metabolism and cellular adaptability. Future studies could further validate these findings by incorporating as-

says for mitochondrial respiration, oxidative phosphorylation, ATP production, and ROS levels, thereby providing a basis for combined metabolic interventions in gastric cancer.

Upregulated expression of key enzymes involved in fatty acid metabolism, including fatty acid synthase (FASN) [31], acetyl-CoA carboxylase 1 (ACC1), and ATP citrate lyase (ACLY), has been demonstrated to be associated with reduced sensitivity to commonly used gastric cancer chemotherapeutic agents such as fluorouracil and platinum-based drugs [32]. Studies in the relevant literature suggest that this may be related to enhanced fatty acid metabolism, which can alleviate chemotherapy-induced oxidative stress, maintain cell membrane integrity, and regulate apoptosis through lipid-mediated signaling pathways [33]. Furthermore, intermediates of lipid metabolism can promote immune evasion by modulating the tumor microenvironment, thereby further diminishing treatment efficacy [34]. In light of the findings of this study, S100A4 significantly upregulates key fatty acid metabolism enzymes such as ACLY, ACC1, and FASN and increases lipid droplet accumulation. This metabolic reprogramming may provide gastric cancer cells with additional stress resistance and an energy buffer, thereby potentially enhancing their tolerance to chemotherapy. Therefore, we hypothesize that S100A4 may not only act as a driver of tumor invasion but also participate in the metabolic adaptation of tumor cells to chemotherapy. Future studies could further validate the role of S100A4 in gastric cancer chemotherapy resistance through drug sensitivity assays, lipid flux analysis, and *in vivo* chemotherapy models. This would help provide a theoretical basis for combination therapy strategies based on the S100A4-Wnt/fatty acid metabolism axis and may offer new targets for personalized treatment.

Furthermore, intermediates of lipid metabolism can promote immune evasion by modulating the tumor microenvironment, thereby further diminishing treatment efficacy. Therefore, targeting fatty acid metabolic pathways holds promise as a potential strategy to overcome chemotherapy resistance in gastric cancer and improve clinical outcomes, particularly when combined with conventional chemotherapy. In this study, BODIPY 493/503 staining showed significantly increased lipid droplet accumulation in GC cells following S100A4-mediated Wnt/ β -catenin activation. These findings further support that enhanced FA synthesis promotes lipid droplets as critical lipid and energy reservoirs, facilitating invasive migration. Our findings suggest that S100A4 plays a key role in regulating the metabolic reprogramming of fatty acids in gastric cancer.

Beyond mechanistic insights, our results suggest that S100A4 may define a subset of GCs characterized by concurrent activation of Wnt signaling and FA biosynthesis. This signaling-metabolic coupling may confer a dependency on *de novo* lipogenesis, potentially rendering tumors with high S100A4 expression more vulnerable to ther-

apeutic interventions targeting lipid synthesis. Thus, inhibitors against key lipogenic enzymes, especially FASN, or combined targeting of Wnt/ β -catenin signaling and lipid metabolism, may represent rational therapeutic strategies against this integrated oncogenic pathway. Further *in vivo* and clinical studies are required to evaluate the therapeutic benefits of targeting the S100A4-Wnt-lipogenesis pathway.

5. Limitations

This study has several limitations. First, the study primarily utilized *in vitro* cell models, which cannot fully replicate the tumor microenvironment and immune responses [35]. Second, the cell lines used in this study were derived from different stages of differentiation and may not fully represent the functions of S100A4. Therefore, future studies should conduct bidirectional functional validation in a broader range of gastric cancer models to further elucidate whether the oncogenic role of S100A4 depends on subtypes. Third, although the study investigated how S100A4 regulates fatty acid metabolism via the Wnt/ β -catenin pathway, it lacks metabolic flux analysis [36] and direct immunofluorescence evidence of β -catenin nuclear translocation. Fourth, this study utilized two partially overlapping siRNAs to validate their roles in phenotypic changes and fatty acid metabolic reprogramming. Although this design improved silencing efficiency and reproducibility, these two siRNAs are not entirely independent and may exhibit similar off-target effects; future experiments should employ completely independent siRNAs or utilize CRISPR technology to further validate the results. Finally, the upstream mechanism by which S100A4 activates the Wnt/ β -catenin pathway remains unclear and requires further validation in future studies.

6. Conclusion

In summary, this study highlights the critical role of S100A4 in gastric cancer progression and metabolic reprogramming through signaling pathways. This suggests potential applications in the treatment of gastric cancer. It extends the understanding of S100A4's oncogenic role from phenotypic regulation to signaling-driven metabolic processes. Although our findings are primarily based on *in vitro* models and require further validation through functional experiments and metabolic flux analysis, they clearly highlight the biological significance of the S100A4-Wnt/ β -catenin signaling axis in gastric cancer and its critical role in fatty acid metabolic reprogramming. This may provide new theoretical insights for the development of targeted metabolic therapies for gastric cancer.

Availability of Data and Materials

The datasets used in this article are accessible from the corresponding author upon request.

Author Contributions

TW: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft. XX: Methodology, Formal analysis, Validation, Writing – review & editing. CJ: Investigation, Resources, Data curation. YZ: Conceptualization, Supervision, Writing – review & editing. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was reviewed and approved by the Institutional Ethics Committee of Yixing People's Hospital, with approval number No.: 2024-031-01. All procedures were conducted in accordance with relevant guidelines. The study was conducted in accordance with the guidelines of the Declaration of Helsinki. Written informed consent was obtained from all participants.

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Conflicts of Interest

The authors declare no conflicts of interest.

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