

P4HA3 promotes gastric cancer cell proliferation and invasion via the PI3K/AKT/mTOR signaling pathway and regulates glycolytic metabolic reprogramming

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Abstract: Background: Dysregulated expression of prolyl 4-hydroxylase subunit alpha 3 (*P4HA3*) has been identified in Colon cancer, breast cancer, head and neck cancers, etc. and is closely associated with tumor progression. However, its specific roles in gastric cancer (GC), particularly regarding alterations in metabolic pathways, are remain unknown. **Objectives:** This study investigated how *P4HA3* influences glycolytic metabolic reprogramming and the proliferation and invasion of gastric cancer cells via the PI3K/AKT/mTOR signaling pathway. **Methods:** To determine *P4HA3* levels in GC tissues and corresponding cell lines, bioinformatics methods, immunohistochemical (IHC) staining and Western blotting (WB) were conducted. Furthermore, gain- and loss-of-function experiments, including cell viability assays (CCK-8), colony-forming tests, scratch wound assays and Transwell invasion experiments, were utilized to explore the influence of *P4HA3* on GC cell growth, motility and invasive capacity. Changes in cellular metabolism were assessed using the Seahorse system (under normoxic conditions), including measurements of basal metabolism, glycolytic capacity and mitochondrial respiration (oxidative phosphorylation), as well as Western blot analysis of key glycolytic proteins. Additionally, the involvement of the PI3K/AKT/mTOR signaling axis was examined by gene set enrichment analysis (GSEA), WB analysis and pharmacological inhibition studies. **Results:** GC tissues and cell lines exhibited markedly increased levels of *P4HA3*, correlating significantly with decreased overall survival (OS). Experimental data indicated that *P4HA3* facilitated GC cell proliferation, migration and invasion. In addition, *P4HA3* enhanced glycolytic activity and increased the expression of key glycolysis-related proteins. Unexpectedly, *P4HA3* also increased mitochondrial respiration under the assay conditions, suggesting that *P4HA3* may induce a broader metabolic activation rather than an exclusively glycolytic shift. Mechanistically, the glycolysis-enhancing effects of *P4HA3* were found to require PI3K/AKT/mTOR signaling (as shown by inhibitor studies). **Conclusion:** *P4HA3* promotes glycolysis via the PI3K/AKT/mTOR signaling pathway, thereby promoting the progression of gastric cancer.

Keywords: Gastric cancer; Glycolytic reprogramming; Invasion; *P4HA3*; PI3K/AKT/mTOR signaling pathway; Tumor proliferation

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INTRODUCTION

Gastric cancer (GC) represents a significant global health burden, contributing notably to worldwide cancer incidence and mortality (Thrift *et al.*, 2023) and ranking prominently among causes of cancer-related deaths (Bray *et al.*, 2024). Despite substantial progress in surgical techniques, systemic treatments and personalized medicine, the prognosis for advanced and metastatic GC patients remains unsatisfactory, with persistently low five-year survival rates (Choo *et al.*, 2026). Consequently, comprehensive identification and characterization of critical molecular mechanisms driving GC progression, along with novel therapeutic targets, remain urgent clinical priorities. Metabolic reprogramming is a core feature of tumorigenesis and tumor progression (Otakhor and Soladoye, 2024). Specifically, aerobic glycolysis (the Warburg effect) is widespread in various tumor types. Even with adequate oxygen supply, tumor cells preferentially use glycolysis for energy production (Kooshan *et al.*, 2024). In addition to ATP production, glycolysis provides metabolic intermediates to support rapid proliferation and biosynthetic demands of tumor cells (Patti and Wang, 2023). Earlier research has indicated that principal enzymes involved in glycolysis, including HK2 and

LDHA, are consistently elevated in numerous malignant tumors and significantly contribute to metabolic reprogramming (Ni *et al.*, 2024; Chelakkot *et al.*, 2023). Nonetheless, the regulatory mechanisms upstream of these metabolic changes are notably intricate and vary widely among cancer types, resulting in uncertainty regarding key control points. The PI3K/AKT/mTOR axis is crucially involved in regulating cell proliferation, survival and metabolism, thereby significantly contributing to tumor initiation and advancement (Rascio *et al.*, 2023). Accumulating evidence highlights aberrant activation of this pathway across multiple malignancies, facilitating enhanced tumor growth and metastatic dissemination, including breast cancer (Rzepecka *et al.*, 2025), ovarian cancer (Broege *et al.*, 2024), lung cancer (Sanaei *et al.*, 2022) and bladder cancer (Rezaei *et al.*, 2023). Nonetheless, the specific connections linking the PI3K/AKT/mTOR pathway with glycolytic metabolic reprogramming in GC are well established, but the involvement of *P4HA3* within this axis remains underexplored. Prolyl 4-hydroxylase subunit alpha 3 (*P4HA3*), a member of the prolyl 4-hydroxylase family (Hsu *et al.*, 2022), is abnormally expressed in various malignant tumors. Aberrant *P4HA3* expression is closely associated with aggressive tumor phenotypes (Zhang *et al.*,

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2024) and plays significant roles in tumor progression (Wu *et al.*, 2023), invasion and metastasis (Zhang *et al.*, 2025). Previous research predominantly emphasized the involvement of *P4HA3* in tumor invasive behaviors, whereas its potential impact on tumor metabolism is underexplored. Given that *P4HA3* is transcriptionally activated by hypoxia-inducible factor-1 α (HIF-1 α), a key driver of glycolytic reprogramming, it is plausible that *P4HA3* may influence glycolysis through intersecting pathways. In particular, whether *P4HA3* modulates glycolysis-related metabolic reprogramming in a manner requiring PI3K/AKT/mTOR signaling and the underlying molecular events governing these processes, remain unresolved. In response to this gap, the current study comprehensively characterizes the expression patterns and biological activities of *P4HA3* within GC contexts. Specifically, the present study aims to establish whether *P4HA3* promotes GC cell proliferation, migration and invasion via modulation of glycolytic metabolism mediated through the PI3K/AKT/mTOR signaling pathway. The broader goal is to clarify the molecular pathways implicated in *P4HA3*-associated GC progression, thus laying groundwork for its potential targeting in therapeutic strategies.

MATERIALS AND METHODS

Clinical tissue samples

This retrospective study included 80 patients with gastric cancer who underwent radical gastrectomy at Yixing People's Hospital between August 2024 and June 2025. Eligible patients were identified by screening the hospital's pathology archives (A flow diagram illustrating patient inclusion and exclusion is provided in Supplementary Fig. S1). Matched sets of primary tumor tissue and adjacent non-tumorous gastric mucosal tissue were collected from each patient. The adjacent non-tumorous tissue was obtained from a site at least 5 cm from the tumor margin and was histologically confirmed to be free of malignant cells.

Inclusion criteria: (1) Histologically verified primary gastric adenocarcinoma; (2) Availability of matched tumor and adjacent normal gastric samples; (3) Absence of previous systemic anticancer therapies; and (4) Comprehensive clinical and pathological documentation.

Exclusion criteria: (1) Diagnosis with another malignancy; (2) Prior systemic treatment; and (3) Insufficient specimen availability for analysis.

Tumor staging followed the Tumor-Node-Metastasis (TNM) classification guidelines outlined in the 8th edition of the American Joint Committee on Cancer (AJCC). Patient clinical details utilized in this study are summarized comprehensively in table 1.

Bioinformatics analysis

RNA-seq data and related clinical information for gastric adenocarcinoma (STAD) were retrieved from The Cancer

Genome Atlas (TCGA) database. Translation units per million (TPM) were used to normalize gene expression levels, which were then log₂-transformed before additional analysis. To determine if there were any differences in *P4HA3* mRNA expression between normal gastric tissues and tumors, The "limma" R package was used to identify differential expression of *P4HA3* between normal gastric tissues and tumors. The effects of *P4HA3* expression on survival were examined with the use of Kaplan-Meier curves and compared using log-rank testing. Statistically significant terms were identified by Gene Ontology (GO) enrichment studies that were carried out using the "clusterProfiler" software, with adjusted P-values <0.05. Molecular Signatures Database (MSigDB) curated gene sets were subjected to gene set enrichment analysis (GSEA), which evaluated nominal P-values, FDR q-values and normalized enrichment scores (NES). Gene sets were deemed highly enriched when their nominal P-values were less than 0.05 and their FDR q-values were less than 0.25. Pathways related to *P4HA3* expression were highlighted in the analysis, with a special emphasis on glycolysis and the PI3K/AKT/mTOR pathway.

Cell culture

The cell lines AGS, MKN-45, MKN-28 and HGC-27 were derived from GC and were kept in modified DMEM (Gibco, USA) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS). The human gastric epithelial cells (GES-1) and these cell lines were acquired from the Cell Bank of the Chinese Academy of Sciences in Shanghai, China. These cells were cultured in modified DMEM medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin. All cells were cultured at 37°C in a 5% CO₂ atmosphere. The culture medium was changed every 1 to 2 days. When the cell density reached 80–90%, trypsin (0.25%)–EDTA mixture was immediately added and the cells were passaged. In subsequent experiments, all cells were in the logarithmic growth phase. Initially, *P4HA3* protein levels were quantified in these cell lines. Based on the measured expression, AGS cells exhibiting higher *P4HA3* expression were chosen for knockdown studies, whereas HGC-27 cells with lower expression were selected for overexpression experiments.

Cell transfection

AGS cells were transfected with *P4HA3*-specific siRNAs or negative control siRNA (GenePharma, China) using Lipofectamine 3000 (Invitrogen, USA). For *P4HA3* overexpression, HGC-27 cells were transfected with pcDNA3.1-*P4HA3* expression plasmids or the empty pcDNA3.1 vector. The siRNA sequences targeting *P4HA3* were as follows:

si-P4HA3-1: 5'-GCAGUCUUACUGGAGGAAAUU-3',
si-P4HA3-2: 5'-GGACGUGUACAUGCACAAAUU-TT-3',
si-NC: 5'-UUCUCCGAACGUGUCACGUTT-3'.

Transfection was conducted using Lipofectamine 3000 (Invitrogen, USA). Briefly, plasmid vectors or siRNA were

diluted in Opti-MEM serum-free medium, gently mixed with Lipofectamine 3000 reagent and incubated for approximately 10-15 minutes to facilitate complex formation. Transfection complexes were subsequently added to cell cultures. The cells were cultured for an extra 24-48 hours before examination, after which the medium was changed to fresh culture medium with 10% FBS. Separate areas of *P4HA3* were targeted by two different *siRNAs* (*si-P4HA3-1* and *si-P4HA3-2*) to reduce off-target effects. The gene silencing tests were proven to be specific and reliable by the consistent findings achieved from these two *siRNAs*.

Colonial formation assay

Twenty-four hours after transfection, cells were seeded into 6-well plates at a density of 800–1,000 cells per well. Culture media was changed every two to three days and colonies were left to grow for about ten to fourteen days until they were easily visible. After a gentle two-step rinsing with PBS, the cells were fixed with 4% glutaraldehyde for 15-20 minutes. After that, they were stained with 0.1% crystal violet (CMA) for another 15-30 minutes. Following staining, the plates were carefully rinsed under running water, allowed to air dry and then the colonies were counted using a microscope.

CCK-8 cell proliferation test.

Cell proliferation rates were measured utilizing the Cell Counting Kit-8 (CCK-8; Dojindo, Japan). In 96-well plates, transfected cells were placed. Every well was inoculated with 10 μ L of CCK-8 solution at specific intervals (0, 24, 48 and 72 h) and then left to incubate for 1-2 hours. Then, in order to test the cell viability, the absorbance was taken at a wavelength of 450 nm using a microplate reader. The capacity of the cells to migrate was assessed using a wound healing test. In a nutshell, transfected cells were added to 6-well plates until they reached 90-100% confluence. With the help of a sterile 200 μ L pipette tip, a straight line was drawn over the cell monolayer. Fresh serum-free media (SFM) was added after a gentle rinsing of the cells twice with PBS to remove debris. Using an inverted microscope, pictures of the scratch area were taken both before and after 24 hours of incubation. ImageJ software was used to quantitatively evaluate wound closure, and the change in wound area was used to calculate cell migration.

Transwell migration and invasion assays

The capacities of cell migration and invasion were studied by employing Transwell chambers that were fitted with 8- μ m pore membranes (Corning, USA). In order to conduct migration tests, cells were firstly collected by trypsin digestion and then resuspended in serum-free media at 1×10^5 cells/mL. Then, 200 μ L of this suspension was put to the top compartment. To serve as a chemoattractant, the lower chamber was filled with 600 μ L of media that contained 10% FBS. In invasion tests, the cell solution was added to Transwell membranes that had been pre-coated with Matrigel (BD Biosciences, USA) and polymerized for

30 minutes. Gently wiping off any non-migrated cells on the top surface with a cotton swab was done after incubation for about 24 hours. Cells that had invaded or migrated onto the bottom surface of the membrane were fixed with 4% paraformaldehyde for 15-20 minutes and then stained with 0.1% crystal violet solution for 15 minutes. After rinsing with PBS, labeled cells were microscopically counted in five fields per chamber at random; the mean cell count was then calculated for statistical purposes.

Seahorse cell metabolic investigation.

The metabolic activity of cells was assessed with the use of an Agilent Technologies, USA, Seahorse XF analyzer. Seahorse XF microplates were used for cell seeding and overnight incubation after transfection. The cells were allowed to equilibrate for 45-60 minutes before the experiment and Seahorse XF Base Medium was substituted for the culture medium. Seahorse XF Glycolytic Stress Kit measurements of extracellular acidification rate (ECAR) were used to ascertain glycolytic function. Glycolytic reserve, maximal glycolytic capacity and basal glycolysis were measured by administering glucose, oligomycin and 2-deoxy-D-glucose (2-DG) in a sequential fashion. Oxygen consumption rate (OCR), was also determined using the Seahorse XF Mitochondrial Stress Kit. Measurements of ATP-linked respiration, maximal respiratory capacity and spare respiratory capacity were taken in this experiment by administering oligomycin, FCCP and a mix of antimycin A/rotenone consecutively. Experiments were conducted in triplicate or more, with results analyzed by Seahorse Wave software. ECAR and OCR values, along with glucose uptake, lactate secretion and ATP production data, were normalized to cell counts to ensure consistency among experimental groups.

Western blotting analysis

In a mixture of protease and phosphatase inhibitors, cell and tissue samples were lysed. A Beyotime, China-based BCA Protein Assay Kit was used for protein measurement. The protein samples, ranging from 20 to 40 μ g in weight, were separated using 10-12% SDS-PAGE gels before being deposited onto PVDF membranes from Millipore, USA. The following proteins were targeted by the primary antibodies: P4HA3, phosphorylated (p-)PI3K, total PI3K, p-AKT, total AKT, p-mTOR, total mTOR, Cyclin D1, MMP-2 and β -actin. The membranes were blocked in solutions containing either 5% skim milk or 5% BSA for 1 hour and then incubated overnight at 4°C. Prior to incubating membranes with secondary antibodies for 1 hour, they were washed three times in TBST for 5-10 minutes each. A gel imaging equipment was used to collect pictures and ECL reagents from Thermo Fisher Scientific, USA, were used for protein detection. The intensity of the bands was measured using the ImageJ program, with β -actin serving as the standard. Pharmacological inhibitors were used to determine whether the effects induced by P4HA3 were mediated through the PI3K/AKT/mTOR signaling pathway. Both the PI3K inhibitor LY294002 (10

μM) and the mTOR inhibitor rapamycin (100 nM) were applied to the cells for 24 hours after transfection with P4HA3-overexpressing vectors. Then, total protein lysates were obtained and Western blotting was used to identify p-AKT, total AKT, p-S6 and total S6 protein levels.

Immunohistochemistry IHC

The paraffin-embedded GC tissue samples were cut into 4 μm sections, deparaffinized with xylene and rehydrated using a graduated ethanol series. In a citrate buffer (pH 6.0), the antigen retrieval process was executed. To reduce the amount of nonspecific antibody binding, sections were incubated with 3% BSA at room temperature for 30 minutes after being blocked with a 3% hydrogen peroxide solution for 10 minutes. After that, a primary antibody that targets P4HA3 was added to the sections and left to incubate overnight at 4°C. After three rounds of washing with PBS, the sections were left to incubate with the suitable secondary antibody for half an hour. Nuclear counterstaining was carried out using hematoxylin and was observed with DAB chromogen. Dehydration, cleaning, mounting and microscopic analysis were the last steps for the stained sections. Two seasoned pathologists, each blinded to the patient's clinical data, independently evaluated the strength of immunostaining and the percentage of positive cells.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0. Before parametric analyses, data normality and homogeneity of variance were assessed using the Shapiro–Wilk test and Levene's test, respectively. Differences between two independent groups were analyzed using an unpaired Student's *t*-test. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. To examine the categorical variables, chi-square tests were used. Effect sizes were reported along with their 95% confidence intervals (CIs) to provide insight into the magnitude and reliability of observed differences. A threshold of $P < 0.05$ was defined as statistically significant. Survival analyses were conducted using Kaplan-Meier curves, with group comparisons assessed by log-rank testing. All data are presented as mean $\pm$ standard deviation (SD).

RESULTS

P4HA3 is upregulated in GC tissues and cell Lines and associated with poor prognosis

Transcriptomic data acquired from TCGA were examined to learn more about the expression patterns and clinical significance of P4HA3 in GC. Results from unpaired studies showed that P4HA3 mRNA expression was higher in GC samples (Fig. 1A). Similarly, P4HA3 was consistently upregulated when comparing tumor tissues with matched surrounding normal tissues from the same individuals (Fig. 1B). Fig. 1C shows that Kaplan-Meier survival curves showed a significant correlation between

higher P4HA3 expression and lower overall survival in GC patients ($p < 0.01$). The results of the WB and IHC investigations corroborated the greater levels of P4HA3 protein expression in the GC tissues (Fig. 1D, E). At the cellular level, GC-derived cell lines also exhibited increased expression of P4HA3 (Fig. 1F). Quantitative analysis further confirmed the increased P4HA3 protein expression in GC tissues and cell lines compared with their respective normal controls (Fig. 1G, H). The association between P4HA3 expression and the clinicopathological characteristics of patients with gastric cancer was analyzed. Based on expression levels, gastric cancer patients were divided into two groups. The data show that patients with high P4HA3 expression had more advanced cancer stages (Table 1).

P4HA3 Promotes GC Cell Proliferation

To evaluate the role of P4HA3 in regulating GC cell proliferation, two distinct siRNAs were utilized to silence P4HA3 in AGS cells, whereas overexpression of P4HA3 was induced in HGC-27 cells (Fig. 2A, B). Western blot analysis demonstrated that P4HA3 protein expression was markedly reduced in AGS cells transfected with P4HA3-specific siRNAs and significantly increased in HGC-27 cells transfected with the P4HA3 overexpression plasmid. (Fig. 2C, D) CCK-8 assays showed that P4HA3 knockdown significantly reduced AGS cell proliferation, whereas P4HA3 overexpression significantly increased HGC-27 cell proliferation (Fig. 2E, F). Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test for the AGS groups and an unpaired two-tailed Student's *t*-test for the HGC-27 groups. Data are presented as the mean $\pm$ SD from three independent experiments, with $p < 0.05$ considered statistically significant. Colony formation assays further confirmed these findings. P4HA3 knockdown significantly reduced the number of colonies formed by AGS cells, whereas P4HA3 overexpression significantly increased colony formation in HGC-27 cells (Figs. 2G–J). Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test for the AGS groups and an unpaired two-tailed Student's *t*-test for the HGC-27 groups. Data are presented as the mean $\pm$ SD from three independent experiments, with $p < 0.05$ considered statistically significant. These observations suggest a promotive role for P4HA3 in GC cell proliferation.

P4HA3 enhances migration and invasion of GC cells

The influence of P4HA3 on GC cell migration and invasion was further explored through additional knockdown and overexpression experiments. Wound-healing assays indicated that silencing P4HA3 impaired migratory capabilities of AGS cells, whereas its overexpression notably accelerated wound closure in HGC-27 cells (Fig. 3A, B). Quantitative analysis further confirmed that P4HA3 knockdown significantly reduced, whereas P4HA3 overexpression increased, the wound-healing rate of GC cells (Fig. 3C, D).

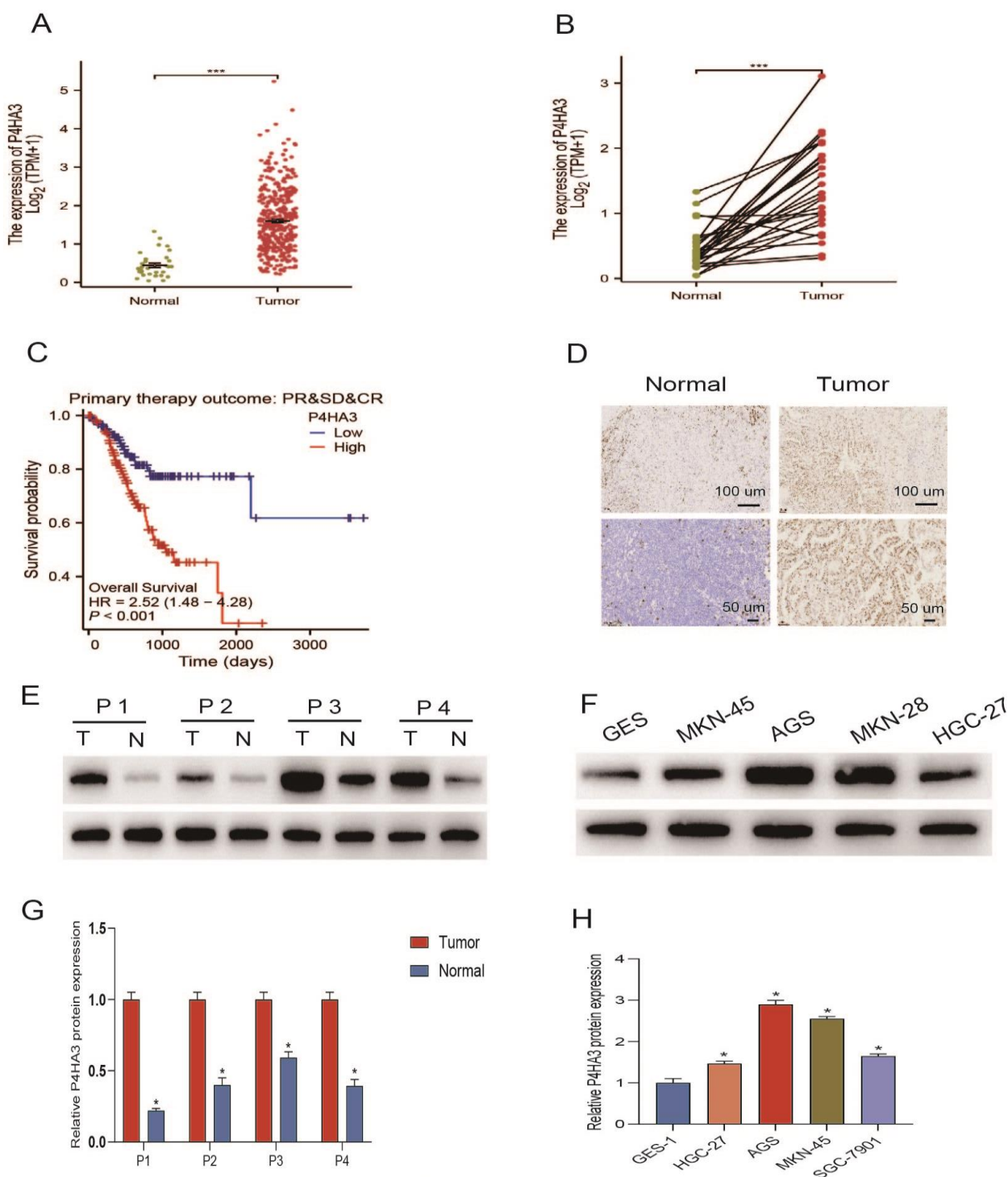


Fig. 1: P4HA3 expression in gastric cancer tissues and cells. (A) Sequencing data on P4HA3 expression in gastric cancer and normal unpaired tissues from the TCGA database; (B) Sequencing data on P4HA3 expression in gastric cancer and normal paired tissues from the TCGA database; (C) Kaplan–Meier survival curves for overall survival (OS) in patients with high and low P4HA3 expression; (D) Representative images of P4HA3 staining in gastric cancer and normal tissues at 100 $\times$ and 400 $\times$ magnification, respectively; (E) Representative Western blot analysis of P4HA3 expression in paired gastric cancer tissues and adjacent normal tissues; (F) Representative Western blot analysis of P4HA3 expression in gastric epithelial and gastric cancer cell lines; (G) Quantification of the Western blot signals shown in panel E; (H) Quantification of the Western blot signals shown in panel F. Data are presented as mean $\pm$ SD from three independent experiments (n = 3). (* p <0.05, ** p <0.01, *** p <0.001).

Table 1: Relationship between P4HA3 protein expression and clinical and pathological characteristics in human gastric cancer.

Characteristics	Number, n (%)	P4HA3 expression		P value
		High group, n (%)	Low group, n (%)	
Age				0.179
<60	38(47.50)	16(42.10)	22(57.90)	
≥60	42(52.50)	24(57.10)	18(42.90)	
Gender				0.485
Male	51(63.75)	27(52.90)	24(47.10)	
Female	29(36.25)	13(44.83)	16(55.17)	
Stage				0.001
I-II	41(51.25)	16(39.02)	25(60.98)	
III-IV	39(48.75)	26(66.67)	13(33.33)	
comorbidities				0.325
Yes	30(37.50)	16(53.33)	14(36.67)	
No	50(62.50)	21(42.00)	29(58.00)	

Consistent results were obtained from Transwell assays: knockdown of P4HA3 significantly reduced migration and invasion capacities of AGS cells, while enhanced P4HA3 expression significantly promoted these abilities in HGC-27 cells (Figs. 3E–J). Taken together, these data imply that P4HA3 positively regulates GC cell migration and invasion, thereby contributing to tumor advancement and metastatic potential.

P4HA3 regulates glycolysis-associated metabolism in GC cells

The role of *P4HA3* in metabolic reprogramming was subsequently investigated using Seahorse metabolic flux analysis. Following P4HA3 knockdown in AGS cells, extracellular acidification rate (ECAR) decreased, indicating reduced basal glycolysis, glycolytic capacity and glycolytic reserve (Fig. 4A, D). Additionally, glucose uptake, lactate production and ATP levels declined (Fig. 4C), indicating suppressed glycolytic flux. In contrast, mitochondrial respiration (OCR) increased upon P4HA3 silencing (Fig. 4B, E).

WB revealed that HK2, LDHA and PKM2 expression levels were downregulated in the P4HA3 knockdown group, whereas these glycolytic enzymes were upregulated following P4HA3 overexpression (Figs. 4F–I). These results demonstrate that P4HA3 promotes glycolysis through upregulation of key glycolytic enzymes, thereby driving GC cells toward a metabolic state favorable for tumor progression.

P4HA3 promotes tumor progression via activation of the PI3K/AKT/mTOR pathway

The molecular pathways by which *P4HA3* contributes to tumor progression were investigated by conducting GO enrichment analysis on differentially expressed genes (DEGs). Figure. 5A shows that these genes were more abundant in biological pathways related to glucose metabolism and PI3K/AKT signaling. In addition, GSEA found that samples with significant P4HA3 expression had

considerable activation of the PI3K/AKT pathway, lending credence to these findings (Fig. 5B). These findings, taken as a whole, point to the PI3K/AKT signaling cascade as a key mediator of *P4HA3*'s carcinogenic activities in GC.

To experimentally validate these observations, WB analysis was performed to examine protein expression within the PI3K/AKT/mTOR signaling axis. Knockdown of P4HA3 in AGS cells markedly reduced the phosphorylation levels of PI3K, AKT and mTOR, whereas total PI3K, AKT and mTOR levels remained unaffected (Fig. 5C, E). Conversely, overexpression of P4HA3 in HGC-27 cells resulted in significantly increased phosphorylation of PI3K, AKT and mTOR (Fig. 5D, F). Additionally, downstream effector proteins such as Cyclin D1 and MMP2 exhibited decreased expression after P4HA3 depletion and increased expression following P4HA3 overexpression. These observations collectively indicate that *P4HA3* facilitates GC cell proliferation and invasion by modulating this pathway. To further confirm the essential role of the PI3K/AKT/mTOR pathway in *P4HA3*-associated effects, GC cells overexpressing P4HA3 were treated with LY294002 (a PI3K inhibitor) or rapamycin (an mTOR inhibitor). Treatment with either inhibitor significantly attenuated the phosphorylation of AKT and S6 induced by elevated P4HA3 expression (Fig. 5G). Quantitative analysis of these results also confirmed the inhibitory effects of these compounds on pathway activation mediated by *P4HA3* (Fig. 5H). In summary, these findings demonstrate that *P4HA3* promotes GC progression through activation of the PI3K/AKT/mTOR cascade.

DISCUSSION

Tumor progression is a complex, prolonged process during which cells gradually acquire enhanced proliferation and survival capacities in adverse environments (Zhang *et al.*, 2024).

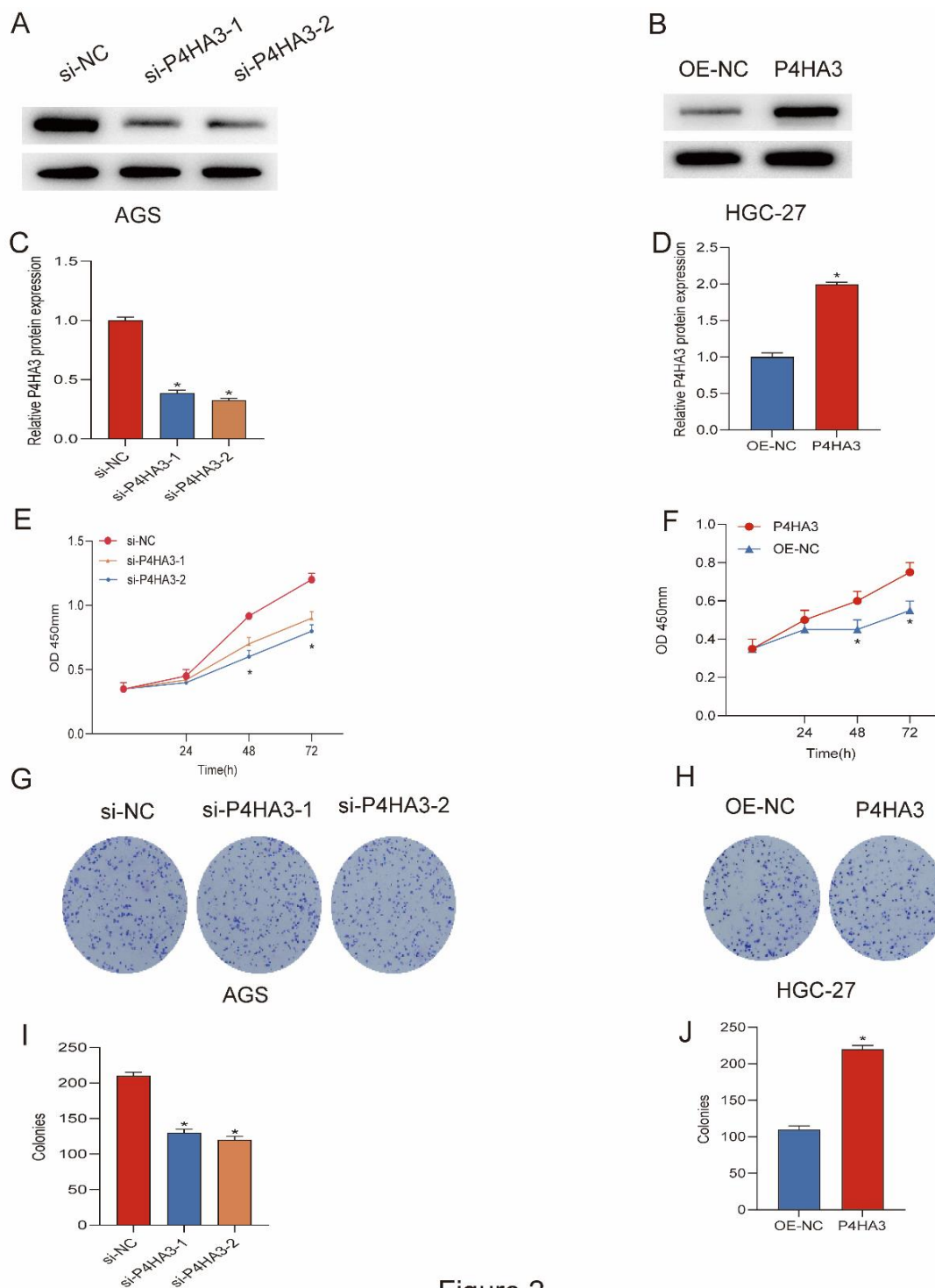


Figure 2

Fig. 2: P4HA3 regulates the proliferation of gastric cancer cells *in-vitro*. (A) Representative Western blot showing P4HA3 knockdown in AGS cells transfected with siRNA. (B) Representative Western blot showing P4HA3 overexpression in HGC-27 cells. (C) Quantification of the Western blot results shown in panel A. (D) Quantification of the Western blot results shown in panel B. (E) CCK-8 assay of AGS cells following P4HA3 knockdown. (F) CCK-8 assay of HGC-27 cells following P4HA3 overexpression. (G) Representative colony formation images of AGS cells. (H) Representative colony formation images of HGC-27 cells. (I) Quantification of colony formation in AGS cells shown in panel G. (J) Quantification of colony formation in HGC-27 cells shown in panel H. Data are presented as mean $\pm$ SD from three independent experiments ($n = 3$). (* $p < 0.05$).

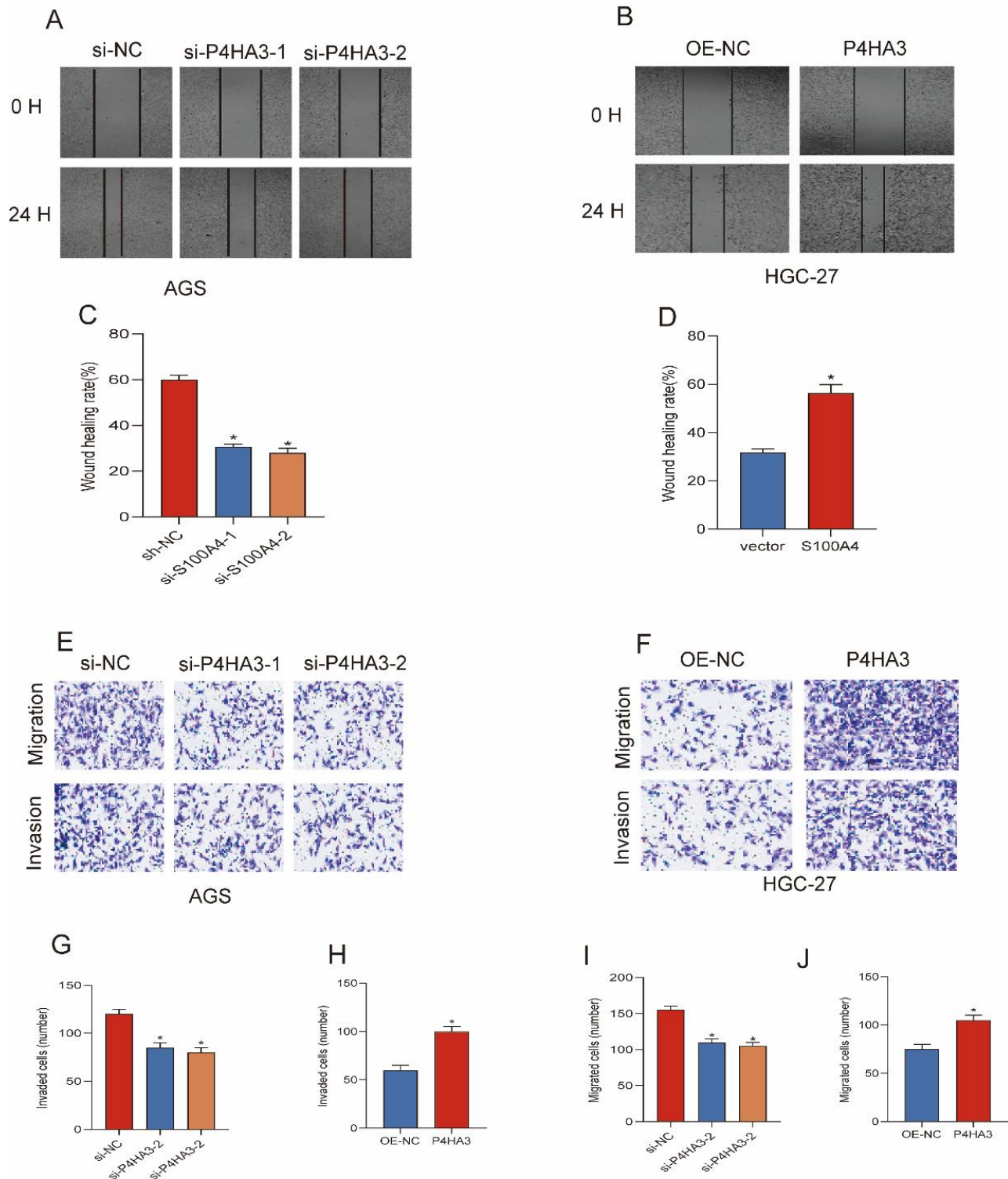


Fig. 3: P4HA3 regulates the migration and invasion of gastric cancer cells *in-vitro*. (A, B) Wound-healing assays demonstrated that P4HA3 knockdown significantly impaired wound closure in AGS cells, whereas P4HA3 overexpression significantly accelerated wound closure in HGC-27 cells. (C, D) Quantitative analysis of wound-healing rates confirmed these findings; (E, G, I) Images from Transwell migration and invasion assays in P4HA3-knockdown AGS cells, along with quantitative analysis of migrating and invading cells; (F, H, J) Images from Transwell migration and invasion assays in P4HA3-overexpressing HGC-27 cells, along with quantitative analysis of migrating and invading cells. Data are presented as mean $\pm$ SD from three independent experiments ($n = 3$). (* $p < 0.05$).

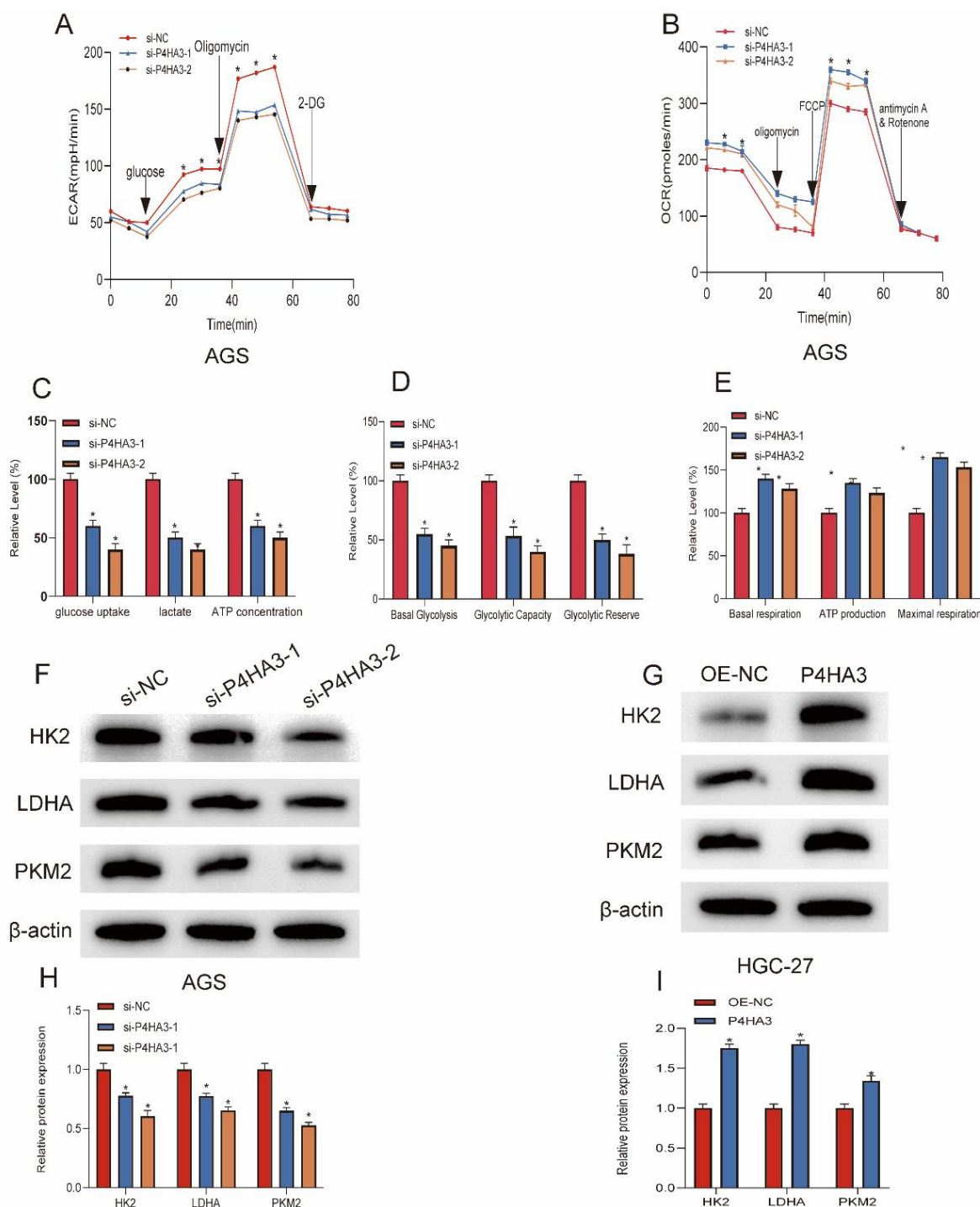


Fig. 4: P4HA3 knockdown affects glycolysis and mitochondrial respiration in gastric cancer cells. (A, B) Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) in AGS cells transfected with si-P4HA3-1, si-P4HA3-2, or si-NC. Key inhibitors and activators are indicated by arrows; (C) Quantitative analysis of glucose uptake, lactate production, and ATP levels in AGS cells; (D) Quantitative analysis of glycolytic parameters in AGS cells; (E) Quantitative analysis of mitochondrial respiratory parameters; (F, H) Western blot analysis and quantitative protein expression analysis of the glycolytic enzymes HK2, LDHA and PKM2 in AGS cells following P4HA3 knockdown; (G, I) Western blot analysis and quantitative protein expression analysis of the glycolytic enzymes HK2, LDHA and PKM2 in HGC-27 cells following P4HA3 overexpression. Data are presented as mean $\pm$ SD from three independent experiments ($n = 3$). (* $p < 0.05$).

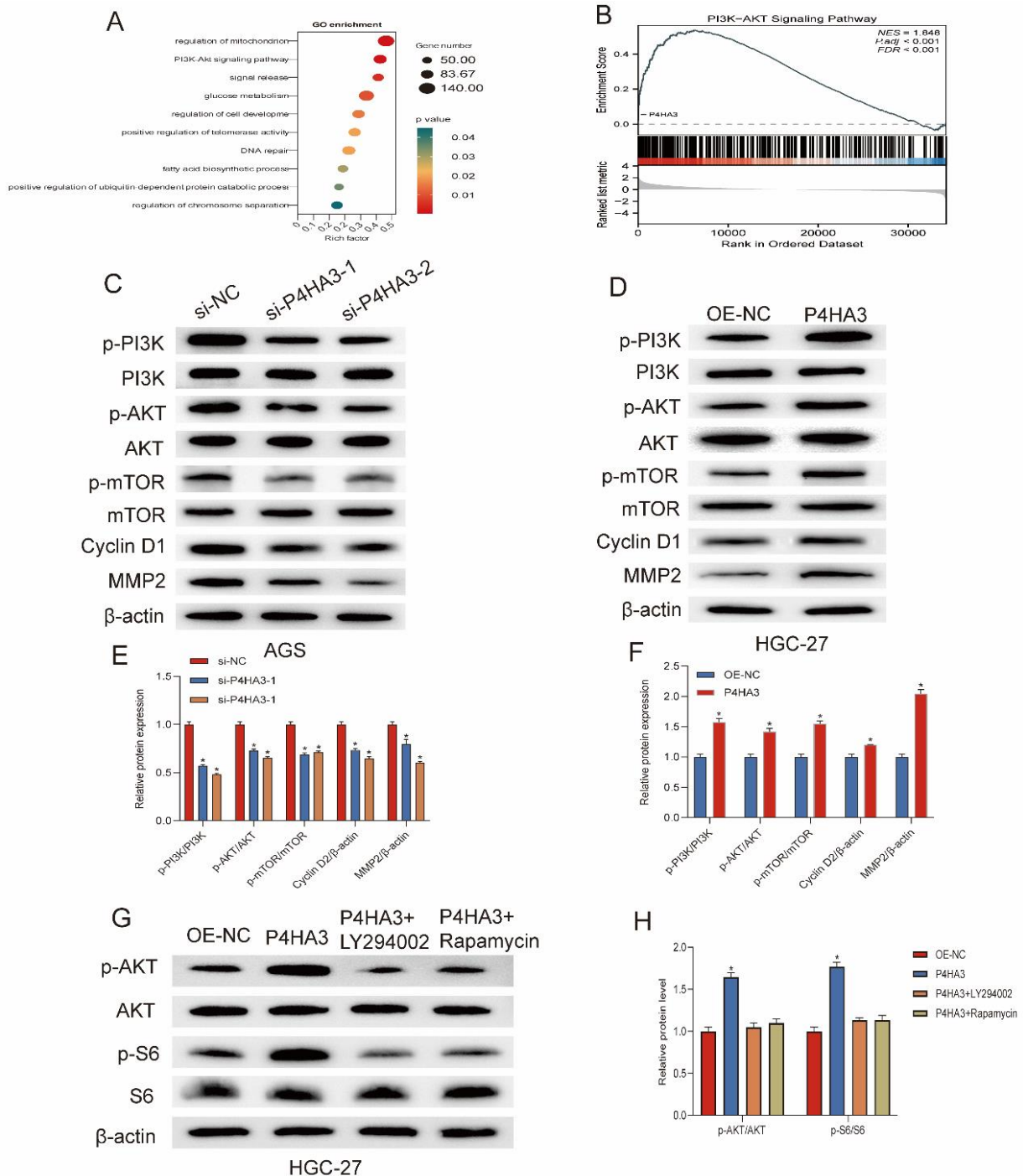


Fig. 5: P4HA3 regulates the PI3K-AKT signaling pathway in gastric cancer cells. (A) Gene Ontology (GO) enrichment analysis showing the most significant biological processes associated with P4HA3 knockdown in AGS cells; (B) Gene Set Enrichment Analysis (GSEA) of the PI3K-AKT signaling pathway in P4HA3-knockdown AGS cells; (C, E) Western blot analysis of the PI3K/AKT/mTOR signaling pathway and quantitative data on protein expression in AGS cells following P4HA3 knockdown (si-P4HA3-1 and si-P4HA3-2) or si-NC treatment; (D, F) Western blot analysis and quantitative protein expression data of the PI3K/AKT/mTOR signaling pathway in P4HA3-overexpressing HGC-27 cells (P4HA3) compared to control cells (OE-NC) (G, H) Western blot analysis and quantitative protein expression data of the PI3K/AKT/mTOR signaling pathway in P4HA3-overexpressing HGC-27 cells treated with LY294002 (PI3K inhibitor) and rapamycin (mTOR inhibitor). Data are presented as mean $\pm$ SD from three independent experiments ($n = 3$). ($*p < 0.05$).

This progression typically involves metabolic reprogramming (Navarro *et al.*, 2022), abnormal activation of multiple signaling pathways (Mai *et al.*, 2024) and altered expression of molecules such as SREBP1 (Su and Koeberle, 2024), PGC-1 α (Akasha *et al.*, 2025) and FOXM1 (Raghuwanshi and Gartel, 2025). Although ongoing studies continue to identify clinical implications and potential drivers, the molecular mechanisms underlying glucose metabolic reprogramming and its functional role in GC progression remain incompletely understood.

Prolyl-4-hydroxylase $\alpha 3$ (*P4HA3*) is a key member of the prolyl-4-hydroxylase enzyme family and is encoded by a conserved gene located on chromosome 11q13. Previous studies primarily focused on its role in mediating post-translational modifications of collagen. For instance, *P4HA3* enhances tumor cell invasiveness in hepatocellular carcinoma by promoting extracellular matrix (ECM) remodeling (Chaulagain *et al.*, 2025). In glioma, *P4HA3* promotes tumor cell proliferation and migration (Jang and Park, 2025). Additionally, *P4HA3* upregulation in pancreatic cancer increases tumor microenvironment stiffness by facilitating collagen hydroxylation and ECM remodeling, thereby promoting tumor progression and invasion. This process may synergize with hypoxia-related signaling, further driving adaptive tumor growth and poor prognosis (Shi *et al.*, 2025). Although previous studies have highlighted the significance of *P4HA3* in various malignancies, its functional role and underlying molecular pathways in GC remain inadequately understood. The present study systematically investigated the involvement of *P4HA3* in GC progression. Initially, elevated *P4HA3* expression was observed in GC tissues and multiple GC cell lines. Clinical and pathological correlation analyses indicated that increased expression of *P4HA3* strongly associates with advanced disease stages and unfavorable patient prognosis. Moreover, functional assays demonstrated that overexpressed *P4HA3* enhanced GC cell proliferation, migration and invasion capabilities, whereas its silencing markedly suppressed these malignant phenotypes. Taken together, the current data suggest a pivotal oncogenic role of *P4HA3* in GC.

Glucose metabolic reprogramming results from interactions among multiple oncogenic pathways. HK2, a critical rate-limiting glycolytic enzyme (Bhamboo *et al.*, 2026), catalyzes the conversion of glucose to glucose-6-phosphate, functioning as a metabolic gatekeeper and influencing downstream metabolic flux (Fang *et al.*, 2023). Altered HK2 expression suggests *P4HA3* involvement in regulating early glycolytic steps. LDHA, another key glycolytic enzyme, converts pyruvate to lactate (Claps *et al.*, 2022), thus maintaining glycolysis by regenerating NAD⁺ and supporting high metabolic flux (Erdem *et al.*, 2025). Similarly, PKM2 catalyzes phosphoenolpyruvate conversion to pyruvate (Toller-Kawahisa *et al.*, 2023) and

balances metabolic flux between energy production and biosynthesis (Liu *et al.*, 2025). It is widely recognized as a central metabolic regulator in cancer (Zamudio-Martinez *et al.*, 2025).

The PI3K/AKT pathway plays a pivotal role in tumor metabolic reprogramming, particularly glycolysis enhancement. Activation of this pathway promotes glucose uptake and utilization, regulating metabolism-related gene expression via downstream effectors. AKT also regulates the mTOR signaling axis, influencing transcription factors associated with hypoxia responses, thus upregulating glycolysis-associated proteins and driving tumor cells toward a glycolytic metabolic phenotype. The experimental results support this model, showing that *P4HA3* overexpression increases the expression of glycolysis-related proteins in association with activation of the PI3K/AKT pathway. Moreover, the PI3K-specific inhibitor LY294002 reduced *P4HA3*-mediated signaling activation, confirming the critical involvement of this pathway. Collectively, these findings indicate that *P4HA3*-associated glucose metabolic reprogramming is closely linked to enhanced proliferation, migration and invasion of GC cells.

Limitation

This study has several limitations. First, although WB analysis provided molecular evidence of enhanced glycolysis, protein expression levels do not fully reflect enzyme activity or actual metabolic flux (Walhout *et al.*, 2025). Second, due to the limited specificity of LY294002, the current findings serve primarily as supportive evidence. Third, this study lacks *in vivo* validation of metabolic mechanisms, including assessment of tumor growth and glycolytic activity using xenograft or *in situ* tumor models. Finally, the clinical sample size is relatively small, which may limit the generalizability of the findings.

Future directions

Future studies should employ advanced approaches, such as isotope tracing or direct enzyme activity assays (Bartman *et al.*, 2023), to determine whether *P4HA3* directly regulates carbon flux through glycolysis. Functional rescue experiments, such as genetic activation of the AKT/mTOR pathway, are also warranted to establish a more definitive causal relationship (Grüniger *et al.*, 2022). In addition, *in vivo* studies using xenograft or *in situ* tumor models are needed to validate the effects of *P4HA3* on tumor growth and glycolytic activity. Validation in larger independent patient cohorts will further clarify the translational potential of *P4HA3* as a prognostic biomarker and therapeutic target in GC.

CONCLUSION

The core objective of this investigation was to clarify the function of *P4HA3* in regulating metabolic processes,

especially glycolysis, within GC cells. Findings from the current study demonstrate that *P4HA3* significantly augments proliferation, migration and invasiveness of GC cells by activating the PI3K/AKT pathway and enhancing the expression of proteins associated with glycolytic metabolism. Collectively, these results clarify the mechanisms underlying *P4HA3*-mediated glucose metabolic reprogramming in GC, providing new insights for developing targeted therapeutic strategies.

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Author's contributions

Tao Wang: Conceptualization, Methodology, Investigation, Data curation, Writing original draft; Xiao Xu: Methodology, Formal analysis, Validation, Writing review & editing; Chen Jin: Investigation, Resources, Data curation; Yubing Zhong: Conceptualization, Supervision.

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Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethical approval

This retrospective human cohort study involving 80 gastric cancer tissue specimens was conducted and reported in accordance with the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines. *In-vitro* experimental procedures were conducted in accordance with standard laboratory protocols. This study was performed in adherence with the STROBE guidelines. See supplementary file for the STROBE checklist.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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