学位論文

Metabolic shift to serine biosynthesis through 3-PG accumulation and PHGDH induction promotes tumor growth in pancreatic cancer
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Metabolic shift to serine biosynthesis through 3-PG accumulation and PHGDH induction promotes tumor growth in pancreatic cancer

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ABSTRACT

Cancer cells craftily adapt their energy metabolism to their microenvironment. Nutrient deprivation due to hypovascularity and fibrosis is a major characteristic of pancreatic ductal adenocarcinoma (PDAC); thus, PDAC cells must produce energy intrinsically. However, the enhancement of energy production via activating Kras mutations is insufficient to explain the metabolic rewiring of PDAC cells. Here, we investigated the molecular mechanism underlying the metabolic shift in PDAC cells under serine starvation. Amino acid analysis revealed that the concentrations of all essential amino acids and most nonessential amino acids were decreased in the blood of PDAC patients. In addition, the plasma serine concentration was significantly higher in PDAC patients with PHGDH-high tumors than in those with PHGDH-low tumors. Although the growth and tumorigenesis of PK-59 cells with PHGDH promoter hypermethylation were significantly decreased by serine starvation, these activities were maintained in PDAC cell lines with PHGDH promoter hypomethylation by serine biosynthesis through PHGDH induction. In fact, DNA methylation analysis by pyrosequencing revealed that the methylation status of the PHGDH promoter was inversely correlated with the PHGDH expression level in human PDAC tissues. In addition to PHGDH induction by serine starvation, PDAC cells showed enhanced serine biosynthesis under serine starvation through 3-PG accumulation via PGAM1 knockdown, resulting in enhanced PDAC cell growth and tumor growth. However, PHGDH knockdown efficiently suppressed PDAC cell growth and tumor growth under serine starvation. These findings provide evidence that targeting the serine biosynthesis pathway by inhibiting PHGDH is a potent therapeutic approach to eliminate PDAC cells in nutrient-deprived microenvironments.

1. Introduction

Pancreatic cancer is currently the seventh leading cause of cancer-related mortality, and the most common type of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC) [1]. Although the major treatment option for early-stage PDAC is surgery, there is no standard

Abbreviations: PDAC, pancreatic ductal adenocarcinoma; ATP, adenosine triphosphate; PPP, pentose phosphate pathway; HBP, hexosamine biosynthesis pathway; TCA cycle, tricarboxylic acid cycle; 3-PG, 3-phosphoglyceric acid; PGAM1, phosphoglycerate mutase 1.

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diagnostic tool to detect PDAC at an early stage [2]. In addition, the treatment strategies for advanced PDAC are extremely limited, and the clinical outcomes of patients with unresectable PDAC remain highly unsatisfactory. Thus, it is necessary to develop new therapeutic strategies targeting cancer-specific pathways to improve the prognosis of PDAC patients.

Cancer cells show unique metabolic alterations compared with their normal counterparts. Metabolic reprogramming is considered a hallmark of cancer cells that allows their survival under conditions of multiple nutrient and oxygen deprivation [3,4]. In general, nutritional problems are often encountered during the treatment of cancer patients, and patients with advanced PDAC often experience malnutrition, including amino acid deficiencies, resulting in cancer cachexia [5–7]. In fact, metabolomic analysis of human PDAC tumors revealed that multiple metabolites related to glucose and amino acid metabolism are depleted in tumor tissues compared to adjacent normal tissues, indicating the limited nutrient availability in PDAC cells, which is caused by poor vascularization and high interstitial pressure [8]. More than 90% of PDACs exhibit Kras mutation, which is a critical driver of PDAC initiation [9] and plays an important role in the enhancement of glycolytic pathway activity, i.e., the "Warburg effect" [10]. Moreover, the expression of the glucose transporter GLUT-1 is frequently increased in human PDAC and is associated with histological grade [11]. Based on this evidence, altered glycolysis has been recognized as one of the characteristics of PDAC.

Glycolysis is a universal catabolic pathway in cells and provides energy in the form of adenosine triphosphate (ATP) for cell division and growth. Multiple metabolic pathways, such as the pentose phosphate pathway (PPP), hexosamine biosynthesis pathway (HBP), serine biosynthesis pathway, and tricarboxylic acid cycle (TCA cycle), are branches of glycolysis [12]. The importance of each metabolic pathway in PDAC has been investigated, and enhancement of serine biosynthesis via oncogenic Kras explained the resistance of PDAC to serine- and glycine-depleted conditions in a mouse model of Kras mutant PDAC [13]. However, the process of the transition from glycolysis to serine biosynthesis has not yet been clarified. The current study was conducted to verify the molecular mechanism underlying the adaptive metabolic shift of PDAC cells to survive in harsh microenvironments with nutrient deprivation. We showed that the DNA methylation status of CpG islands in the *PHGDH* gene in PDAC cells plays an important role in the metabolic switch from glycolysis to serine biosynthesis and demonstrated the metabolic rewiring mechanism by which 3-phosphoglyceric acid (3-PG) accumulation and PHGDH induction synergistically enhance serine biosynthesis in PDAC cells under serine- and glycine-depleted conditions.

2. Materials and methods

2.1. Patients

All experimental protocols were approved by the Ethical Review Board of the Graduate School of Medicine, Kumamoto University (IRB approval no. 1291) and were performed in accordance with the committee guidelines and regulations. All subjects provided informed consent. We examined 213 subjects who underwent surgery except for R2 resection for PDAC at Kumamoto University Hospital between 2004 and 2019. Resected specimens were fixed with 10% formalin and processed for embedding in paraffin. We also prospectively collected blood samples from 20 pancreatic cancer patients and 20 healthy controls.

2.2. Cell lines and cell culture

The following human PDAC cell lines were obtained from the indicated sources: PK-8, MIAPaca2 and PK-59, RIKEN Bioresource Center Cell Bank (Tsukuba, Ibaraki, Japan); and S2-VP10 and S2-013, Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Miyagi, Japan). S2-VP10, S2-013 and MIAPaca2 cells were cultured in DMEM supplemented with 10% FBS, and the other cell lines were cultured in RPMI-1640 medium supplemented with 10% FBS. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Immunohistochemical (IHC) staining and analysis

Paraffin-embedded tissues were sectioned at a thickness of 4 μm, deparaffinized and soaked in distilled water. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide. Sections were incubated with a diluted primary antibody (anti-PHGDH/malate dehydrogenase antibody, 1:300 dilution, ab57030, Abcam, Cambridge, UK; anti-PGAM1 antibody, 1:100 dilution, ab2220, Abcam, Cambridge, UK). Staining was detected with a biotin-free HRP enzyme-labeled polymer in the Envision Plus detection system (Dako, Tokyo, Japan). All IHC staining was scored in a blinded manner by two independent investigators. Staining of PHGDH and PGAM1 was scored based on both the intensity and the extent of staining in cancer cells. The average positive staining intensity in cancer cells was determined, and an intensity score ranging from 0 to 3 was assigned: 0, absent; 1, weak; 2, moderate; and 3, strong (Sup. Figs. 1A and 4A). In addition, the average proportion of positively stained cancer cells was estimated, and a percentage score ranging from 0 to 3 was assigned: 0, <5%; 1, 5–20%; 2, 20–50%; and 3, >50% positive staining. The two scores were multiplied to characterize PHGDH or PGAM1 expression as low (scores of 0–3) or high (scores of 4–9) (Sup. Figs. 1B and 4B).

2.4. Serine starvation experiments

Serine starvation medium was made from Eagle's minimum essential medium (catalog no. 11095080, Thermo Fisher Scientific, Rockford, IL, USA) supplemented with additional 1 × Eagle's minimum essential medium vitamin solution (catalog no. 11120052, Thermo Fisher Scientific), 10% FBS, and additional D-glucose to 25 mM (catalog no. 079–05511, Wako, Osaka, Japan). For the control medium, serine and glycine (Wako) were readded to a final concentration of 0.4 mM, assuming that no FBS was added. FBS contains amino acids, and the amino acid analysis performed by SRL revealed that the concentrations of serine and glycine in the serine starvation medium were approximately 1/10 of those in the control medium (Sup. Fig. 2). A previous study [13] indicated that serine is the major factor in serine and glycine starvation; therefore, all starvation experiments are described as serine starvation.

2.5. Pyrosequencing

To measure the methylation status of the ATF4 binding site in the PHGDH promoter region, polymerase chain reaction (PCR) and pyrosequencing were performed using a PyroMark Q24 system (Qiagen). Genomic DNA was collected from PDAC cell lines using a QIAamp DNA Mini Kit (Qiagen). Unmethylated cytosine in genomic DNA was converted to uracil with sodium bisulfite using an EpiTect Bisulfite Kit

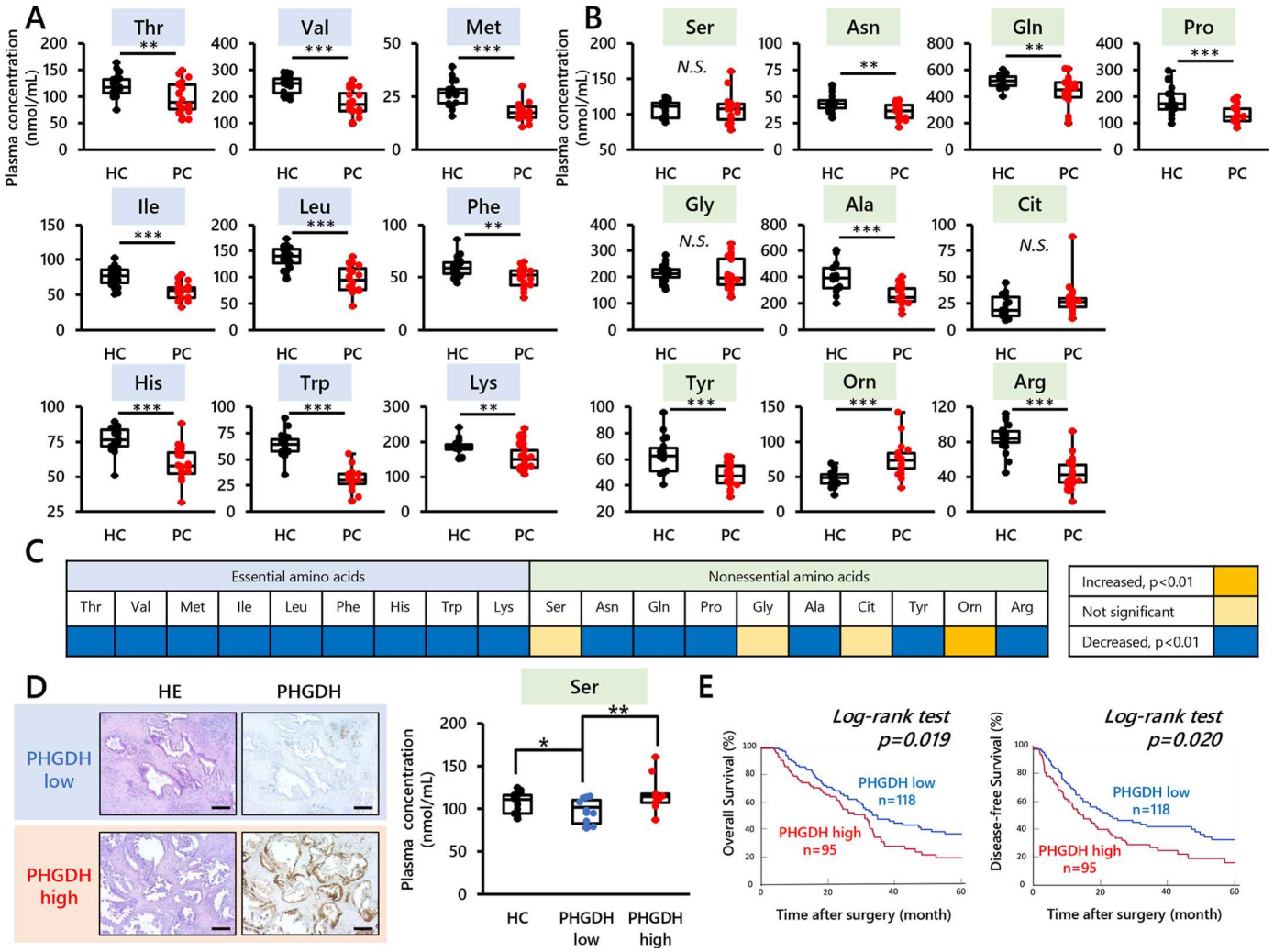

Fig. 1. High PHGDH expression in PDAC tissues is significantly correlated with a high plasma serine concentration and poor prognosis.
A. Analysis of plasma essential amino acids in healthy controls (HC, n = 20) vs pancreatic cancer patients (PC, n = 20). **B.** Plasma nonessential amino acid analysis of HCs (n = 20) vs PCs (n = 20). **C.** The results presented in Fig. 1A and B are summarized in this heatmap showing the comparison of plasma amino acid concentrations between HCs and PCs. Amino acids with lower plasma concentrations in PCs than in HCs are shown in blue. **D.** Left: Image of HE staining and representative IHC staining of PHGDH in resected PDAC specimens. Right: Plasma serine concentrations in HCs and PCs. The PC group was divided into a PHGDH-high group (n = 9) and a PHGDH-low group (n = 11) according to the expression level of PHGDH as evaluated by IHC staining. Scale bar, 200 μm. **E.** Overall and disease-free survival curves for all patients with PDAC based on PHGDH expression status. N.S., not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

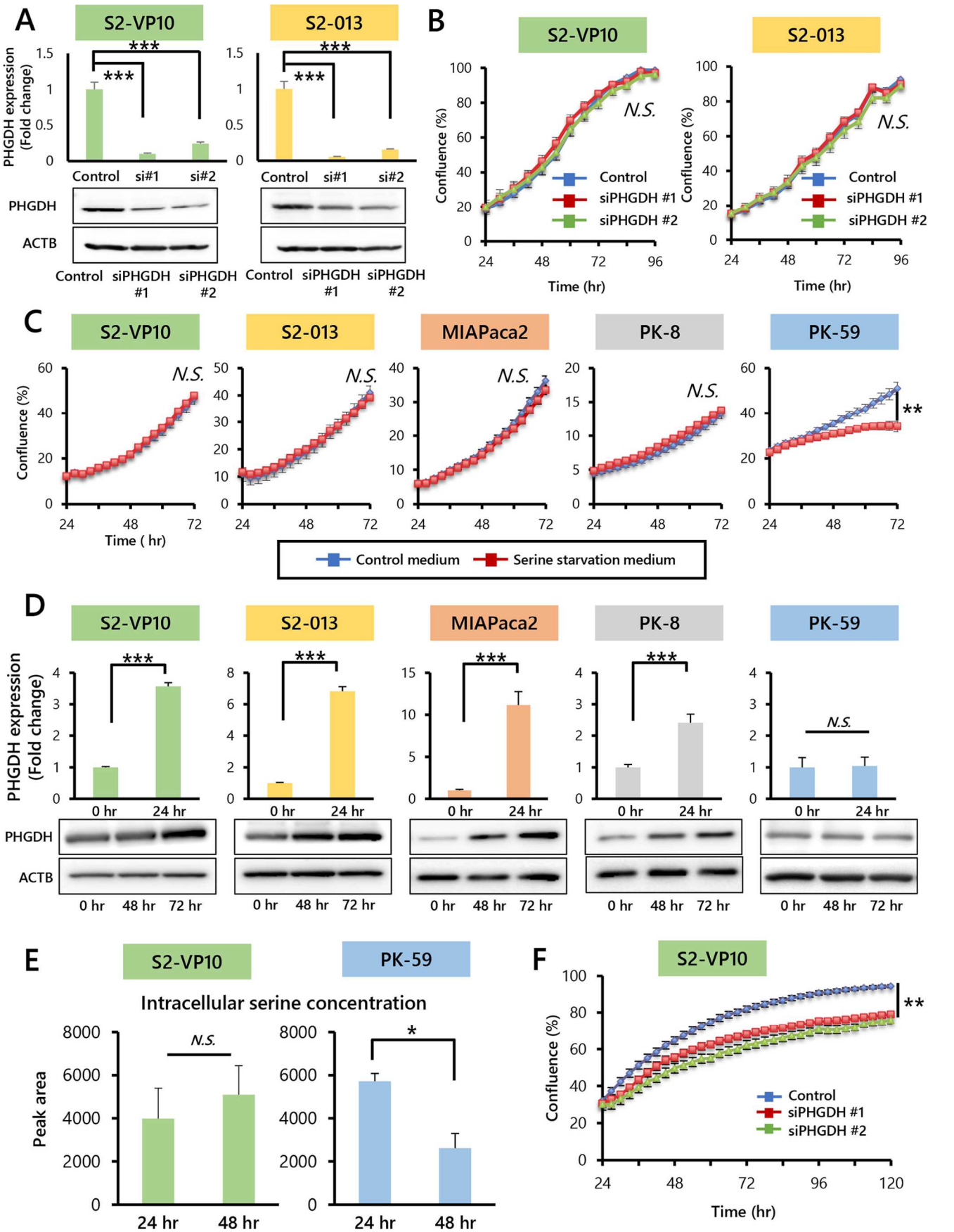

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Fig. 2. Most pancreatic cancer cell lines exhibit resistant to serine starvation via the induction of PHGDH expression.
A. The expression of PHGDH in S2-VP10 and S2-013 cells transfected with *PHGDH* siRNA (#1 or #2) relative to that in these cell lines transfected with the control siRNA was evaluated by qRT-PCR and Western blot analysis. **B.** S2-VP10 and S2-013 cells transfected with the control siRNA and *PHGDH* siRNA (#1 or #2) were plated into 96-well plates in normal medium, and a proliferation assay was conducted using IncuCyte technology. **C.** The same experiment was performed by plating 5 PDAC cell lines into 96-well plates in control or serine starvation medium. **D.** The changes in PHGDH expression in the 5 PDAC cell lines when cultured in serine starvation medium were evaluated by qRT-PCR and Western blot analysis. **E.** The intracellular serine concentration in two cell lines cultured in serine starvation medium was analyzed by LC/MS/MS at 24 and 48 h after replacement of the culture medium with serine starvation medium. **F.** S2-VP10 cells transfected with the control siRNA and *PHGDH* siRNA (#1 or #2) were plated into 96-well plates in serine starvation medium, and a proliferation assay was conducted using IncuCyte technology. N.S., not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

(Qiagen). Pyrosequencing reactions were carried out with two primer sets as follows: primer set 1—forward primer 5′-GAAGGTTTTTAGGAGGTAGATT-3′, reverse primer [biotin]5′-AAAACCCAACCAATCTAATAACTACTA-3′, sequencing primer 5′-GAAAGTAAGGAGTAG TTTTGTAT-3’; primer set 2—forward primer 5′-GAAGGTTTTTAGGAGGTAGATT-3′, reverse primer [biotin]5′-AAAAACTAACCAAAACCCAACCAATCTAA-3′, sequencing primer 5′-TTAGGAGGTAGAGGG-3’. PCR was carried out using a PyroMark PCR Kit (Qiagen) with an annealing temperature of 56 °C (as a result of setting the conditions for determining the optimum annealing temperature). A total of 6 CpG sites were evaluated for their methylation status (Sup. Fig. 3A).

2.6. Measurement of PGAM enzymatic activity

PGAM enzymatic activity was measured [14]. In brief, cell lysates were incubated in reaction buffer (100 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.5 mM EDTA, 2 mM $MgCl_2$, 0.2 mM NADH, 3 mM ADP, and 10 μM 2,3-diphosphate glycerate) supplemented with an enzyme mixture (0.6 U lactate dehydrogenase, 0.5 U pyruvate kinase, and 0.1 U enolase). The reactants were incubated at 37 °C, and the enzymatic reaction was started by the addition of 1 mM 3-PG. PGAM enzymatic activity was measured as NAD + release.

2.7. Xenograft experiments

Tumor development was assessed in two groups of mice fed different diets: 1) Control group mice were fed a control diet (a modified AIN-93G diet with mixed amino acid substitution; Oriental Yeast Co., Ltd., Japan) throughout the experiments. 2) SG(−) group mice were fed a serine- and glycine-free (SG(−)) diet (the control diet without serine and glycine but with all other ingredients, including other amino acids, exactly the same [Oriental Yeast Co., Ltd.]) throughout the experiments. After the mice were on the indicated diet for two weeks, 1.0×10^6 S2-VP10 (shControl), PK-59 (shControl), S2-VP10 (shPHGDH), or S2-VP10 cells (shPGAM1) were implanted subcutaneously into the mouse flank. The mice were sacrificed 4 weeks after tumor cell inoculation, and the tumor and body weights of these mice were evaluated. The protocol is described in Figs. 4A and 6C.

2.8. Statistical analysis

A database was created using Microsoft Excel (Microsoft® Excel for Mac, Microsoft, Redmond, WA, USA) and analyzed using commercial software (JMP version 13® ; SAS Institute, Cary, NC, USA). Survival curves were constructed using the Kaplan-Meier method, and the log-rank test was used to evaluate the statistical significance of differences. All experiments were carried out in triplicate, and the data shown are representative of consistently observed results. The data are presented as the mean ± standard error of the mean (SE) values. The Mann-Whitney *U* test was used to compare continuous variables between the two groups. Categorical variables were compared using the χ^2-test. P-values less than 0.05 were considered statistically significant.

3. Results

3.1. PHGDH expression is significantly correlated with the serum serine concentration and poor prognosis in PDAC patients

We first compared amino acid concentrations between 20 healthy controls and 20 PDAC patients to investigate the amino acid composition in blood. Although the concentrations of essential amino acids were relatively uniform in the healthy controls, they were decreased in PDAC patients, indicating amino acid malnutrition (Fig. 1A and B). However, the concentrations of some nonessential amino acids, i.e., serine, glycine, citrulline and ornithine, were maintained in the plasma of PDAC patients (Fig. 1C). Among these nonessential amino acids, we focused on serine and glycine due to their function in the energy metabolism of cancer cells. Serine is derived mainly from three independent sources—dietary intake, biosynthesis and conversion from glycine—and supports the nucleotide synthesis and proliferation of cancer cells [15,16]. To investigate whether the serine biosynthesis pathway is involved in maintaining the plasma serine concentration in PDAC patients, we examined the expression of PHGDH, a key enzyme in serine biosynthesis, by IHC staining in resected tissues from 213 PDAC patients (Fig. 1D, left panel, and Sup. Table 1). Notably, the plasma serine concentration in PDAC patients with PHGDH-high tumors was significantly higher than that in those with PHGDH-low tumors (Fig. 1D, right panel). This result suggests that de novo serine biosynthesis in tumors is related to the plasma serine concentration in PDAC patients.

We subsequently assessed the relationship between PHGDH expression and PDAC patient survival by Kaplan-Meier analysis. PDAC patients with PHGDH-high tumors exhibited significantly shorter disease-free survival ($P = 0.02$) and overall survival ($P = 0.019$) times than those with PHGDH-low tumors (Fig. 1E). These findings suggest that serine biosynthesis through PHGDH expression in PDAC cells is involved in cancer progression and results in the poor prognosis of PDAC patients.

3.2. Maintenance of the intracellular serine concentration is essential for PDAC cell growth under serine deprivation

To examine the importance of PHGDH expression in PDAC cell growth, we conducted a PHGDH loss-of-function study using two small interfering RNAs (siRNAs). Although PHGDH mRNA and protein expression were markedly decreased in PHGDH siRNA-transfected S2-VP10 and S2-013 cells (Fig. 2A), the growth of these PDAC cells was not affected by PHGDH knockdown (Fig. 2B). Therefore, we next conducted a growth assay under serine deprivation to examine the effect of serine uptake on PDAC cell growth. The growth of four of the five tested PDAC cell lines were not affected by serine deprivation, whereas the growth of PK-59 cells was significantly decreased by serine deprivation (Fig. 2C).

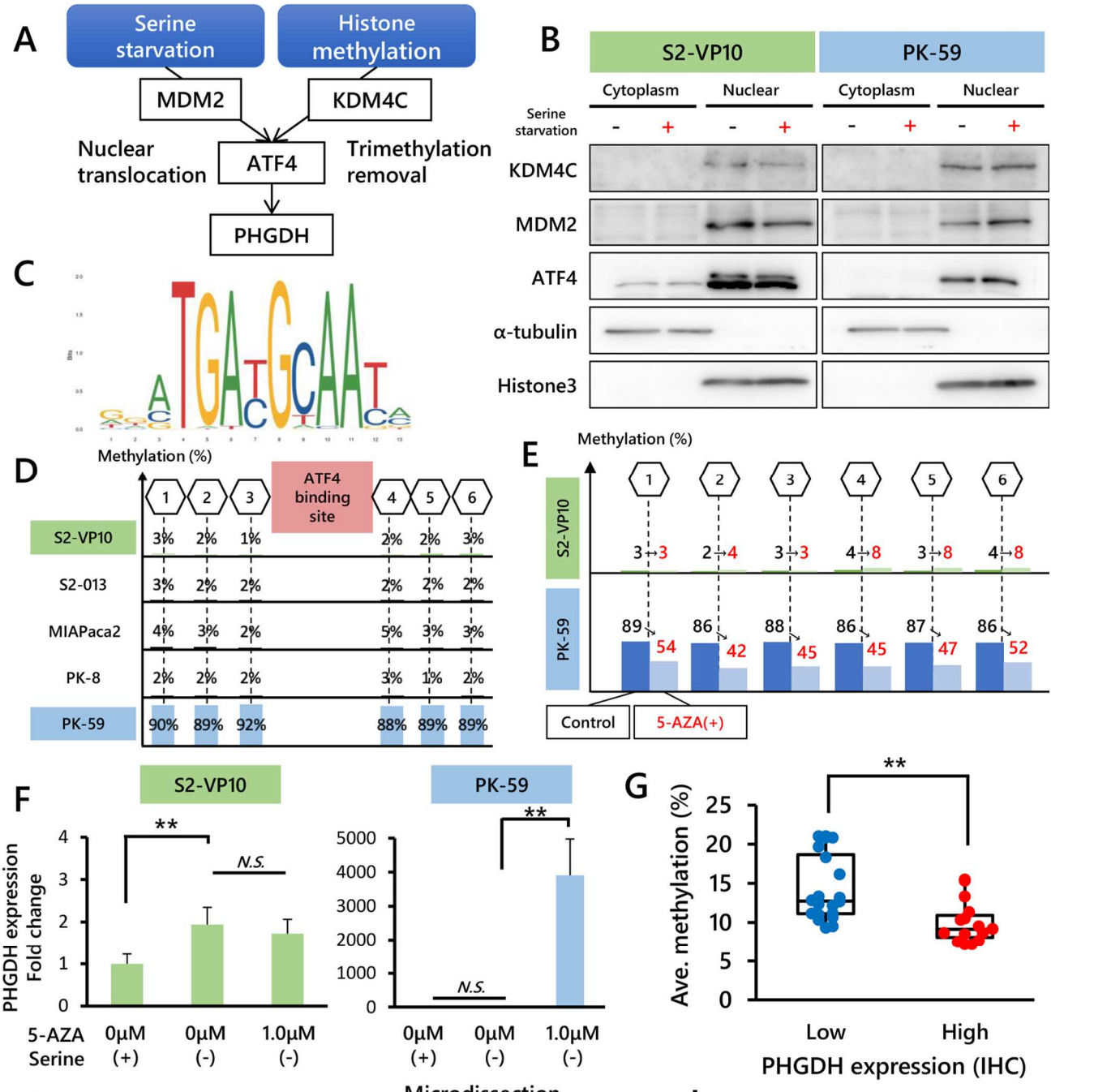

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Fig. 3. Epigenetic mechanism for the induction of PHGDH expression under serine starvation.
A. Previously reported mechanisms involved in PHGDH expression. **B.** Verification of changes in the expression level and localization of these key regulators of PHGDH expression under serine starvation via cell fractionation. Protein extraction was performed 48 h after replacement of the culture medium with serine starvation medium. **C.** The binding motif in the transcription factor ATF4 was searched in JASPAR, a public database. **D.** Methylation levels of 6 CpG sites surrounding the ATF4 binding site in the promoter region of PHGDH were measured in 5 PDAC cell lines by pyrosequencing. **E.** Each cell line was treated with 5-AZA for 96 h, and the methylation levels of the same 6 CpG sites were measured by pyrosequencing. **F.** The changes in PHGDH expression in 2 cell lines cultured in serine starvation medium after 5-AZA treatment were evaluated by qRT-PCR. **G.** IHC staining of PHGDH was performed on resected PDAC specimens (n = 35), and the specimens were divided the into PHGDH-high (n = 15) and PHGDH-low (n = 20) groups. DNA was extracted from the same resected specimens by macrodissection, and the methylation levels of 6 CpG sites were measured as previously described. The average methylation level was compared between the PHGDH-high and PHGDH-low groups. **H.** One sample per group was selected from the PHGDH-high and PHGDH-low groups, and DNA was extracted by microdissection. Scale bar, 200 μm. **I.** The methylation levels of the 6 CpG sites were measured as previously described and compared to the results for DNA extracted by macrodissection. N.S., not significant; *, $p < 0.05$; **, $p < 0.01$.

Serine starvation induces strong p53-independent upregulation of PHGDH [17]. Thus, we examined PHGDH expression changes in these PDAC cells after serine deprivation. Consistent with the cell growth patterns, PHGDH expression was increased in a time-dependent manner in all PDAC cell lines except PK-59 cells, in which it was not affected by serine deprivation (Fig. 2D). Notably, MS analysis revealed that under serine deprivation, the intracellular serine concentration was maintained in S2-VP10 cells but gradually decreased in PK-59 cells (Fig. 2E). Moreover, the growth of S2-VP10 cells transfected with PHGDH siRNA was significantly decreased under serine deprivation (Fig. 2F). These findings suggest that PHGDH induction is vital for intracellular serine maintenance and PDAC cell growth under serine deprivation.

3.3. DNA hypomethylation in CpG islands has a strong impact on PHGDH expression in PDAC cells

PHGDH has been reported to be amplified or overexpressed in various types of cancer [18]. However, induction of PHGDH under serine deprivation is caused by the nutrient microenvironment and thus cannot be explained by gene amplification. ATF4 is a master transcriptional regulator of amino acid metabolism by mediating the transcription of serine pathway genes, including PHGDH. In addition, MDM2 and KDM4C regulate the transcriptional activity of ATF4 through direct binding to ATF4 [19,20] (Fig. 3A). Therefore, we examined MDM2 nuclear translocation and KDM4C nuclear expression but found that serine deprivation did not affect these ATF4 mediators in either S2-VP10 or PK-59 cells (Fig. 3B). In addition, we confirmed that none of the PDAC cell lines had genetic mutations in the ATF4 binding site (Sup. Fig. 3B). Based on these findings, we next examined the methylation status of CpG sites surrounding the ATF4 binding motif in the promoter region of PHGDH (Fig. 3C). Notably, the four PDAC cell lines showing PHGDH induction by serine deprivation exhibited hypomethylation of the indicated CpG sites, whereas these CpG sites were highly methylated in PK-59 cells (Fig. 3D). To address the importance of the DNA methylation status in PHGDH induction, S2-VP10 and PK-59 cells were treated with the DNA-demethylating agent 5-azacytidine (5-AZA). S2-VP10 cells were not influenced by 5-AZA treatment; however, in PK-59 cells treated with 5-AZA, the methylation level was markedly decreased, and PHGDH induction by serine deprivation was apparent (Fig. 3E and F). These results suggest that the methylation status of the PHGDH promoter region is critical for PHGDH induction by serine deprivation in PDAC cells.

To next investigate the relationship between the methylation status and PHGDH expression in human PDAC tissues, we examined the methylation status by pyrosequencing and PHGDH expression by IHC staining in 35 resected PDAC tissues. The methylation level in PHGDH-high cases (n = 15) was significantly lower than that in PHGDH-low cases (n = 20) (Fig. 3G and Sup. Fig. 3C). However, PDAC tissues contain an abundance of stroma, and this pyrosequencing was performed using DNA from macrodissected samples. To exclude the influence of the tumor stroma on the DNA methylation status, we conducted microdissection to enrich DNA from PDAC cells and consequently showed the concordance between the macrodissected and microdissected samples, indicating the validity of the methylation levels determined using DNA from macrodissected PDAC samples (Fig. 3H).

3.4. Serine biosynthesis via PHGDH induction is critical for maintaining tumor growth in mice under serine starvation

Next, to investigate the importance of serine biosynthesis via PHGDH induction *in vivo*, we established a xenograft mouse model using S2-VP10 and PK-59 cells. According to the scheme shown in Fig. 4A, nude mice transplanted with S2-VP10 or PK-59 cells were fed a diet with or without serine and glycine. S2-VP10 cells rapidly formed tumors in mice fed the SG(−) diet and in mice fed the control diet, whereas PK-59 tumor formation was significantly reduced in mice fed the SG(−) diet compared with mice fed the control diet (Fig. 4B). The number of Ki67-positive proliferating cells in S2-VP10 tumors in mice fed the SG(−) diet was maintained compared with that in mice fed the control diet, whereas the number of Ki67-positive cells in PK-59 tumors was significantly reduced in mice fed the SG(−) diet compared with mice fed the control diet (Fig. 4C). Moreover, the proportion of PHGDH-expressing cells in S2-VP10 tumors in mice fed the SG(−) diet was significantly increased compared with that in mice fed the control diet, and PHGDH-expressing cells were hardly detected in PK-59 tumors regardless of diet type (Fig. 4D).

To evaluate serine biosynthesis in tumor cells *in vivo*, we examined serine concentrations in tumor tissues. Serine concentrations in S2-VP10 tumors in mice fed the SG(−) diet were maintained compared with those in mice fed the control diet, whereas intratumoral serine in PK-59 tumors was significantly reduced in mice fed the SG(−) diet compared with mice fed the control diet (Fig. 4E). In addition, the serine concentration in plasma showed a trend similar to that of intratumoral serine (Fig. 4F), suggesting that serine biosynthesis in tumor tissues influences the plasma serine concentration in mice under serine starvation.

3.5. PGAM1 deficiency leads to 3-PG accumulation and subsequently enhances serine biosynthesis under serine deprivation

Phosphoglycerate mutase 1 (PGAM1) is a glycolytic enzyme that catalyzes the conversion of 3-phosphoglycerate (3-PG) to 2-phosphoglycerate (2-PG) [21]. 3-PG is not only a glycolytic intermediate but also a substrate for serine biosynthesis at a branch point (Fig. 5A). Although PGAM1 has been shown to coordinate glycolysis and serine

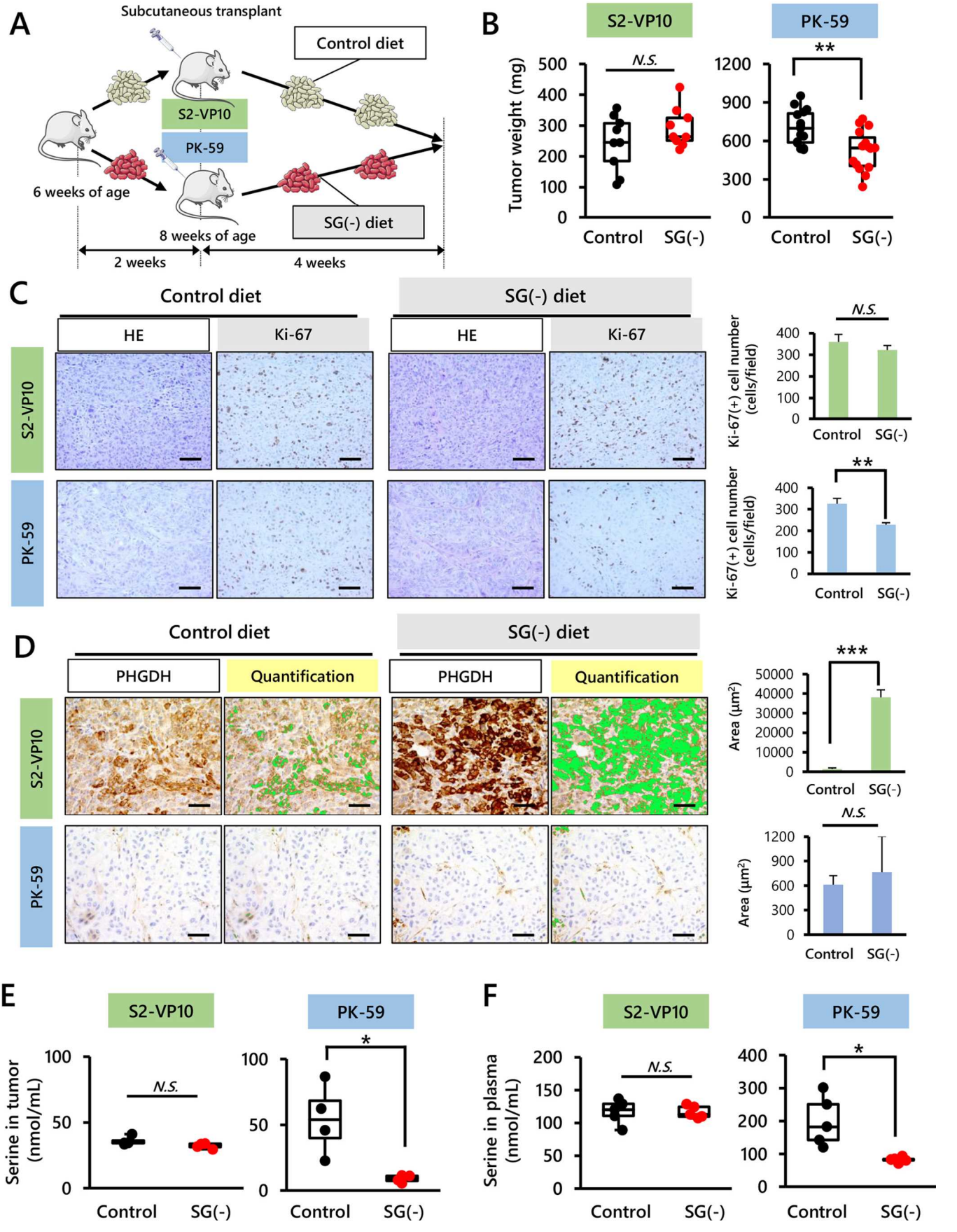

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Fig. 4. Tumorigenic activity of two PDAC cell lines with different methylation statuses of the PHGDH promoter region under serine starvation.
A. *In vivo* experimental design. Six-week-old mice were divided into 2 groups: the control diet group or the serine- and glycine-free (SG(−)) diet group. Two weeks after starting the special diet, it was confirmed that there was no difference in body weight between the groups. Then, 1.0×10^6 cells of each cell line were subcutaneously transplanted, and mice were fed each diet as indicated for 4 weeks. **B.** Comparison of tumor weight between the control diet and SG(−) diet groups. **C.** Representative HE staining and IHC staining for Ki-67. The bar charts show the quantification of Ki-67-positive cells. Scale bar, 100 μm. **D.** Representative IHC staining for PHGDH and quantification of PHGDH-positive cells. The bar charts show the quantification of stained cells. Scale bar, 50 μm. **E.** Comparison of the serine concentration in tumor tissue between the control diet and SG(−) diet groups. Four samples per group were selected and measured. **F.** Comparison of the plasma serine concentration between the control diet and SG(−) diet groups. Five samples per group were selected and measured. N.S., not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

biosynthesis, the importance of PGAM1 activity in serine biosynthesis is still controversial [22,23]. To address the prognostic impact of PGAM1 expression in PDAC tissues, we examined PGAM1 expression by IHC staining in resected tissues from 192 PDAC patients. However, Kaplan-Meier analysis revealed no difference in prognosis between the PGAM1-high and PGAM1-low groups (Sup. Fig. 4C and Table 1). Moreover, little is known about the role of PGAM activity in serine biosynthesis in cancer cells under serine starvation. We first silenced PGAM1 using two siRNAs to examine the effect of PGAM1 expression on the growth of S2-VP10 cells. PGAM1 mRNA and protein expression were markedly decreased in S2-VP10 cells transfected with PGAM1 siRNA (Fig. 5B), and this depletion markedly decreased PGAM1 activity (Fig. 5C). We also confirmed that PHGDH expression was not changed in S2-VP10 cells transfected with PGAM1 siRNA (Sup. Fig. 5). Although we observed no significant difference in cell growth between S2-VP10 cells transfected with control siRNA and those transfected with PGAM1 siRNA (Fig. 5D), notably, under serine deprivation, cell growth was significantly increased in S2-VP10 cells transfected with PGAM1 siRNA compared with those transfected with control siRNA (Fig. 5E). Moreover, under serine deprivation, the extracellular serine concentration in the culture medium of S2-VP10 cells transfected with PGAM1 siRNA was significantly higher than that in the culture medium of cells transfected with control siRNA (Fig. 5F). Given this finding, we next examined the intracellular concentrations of glycolytic intermediates and serine by metabolomic analysis. As expected, the intracellular serine concentration was markedly increased in S2-VP10 cells transfected with PGAM1 siRNA compared with those transfected with control siRNA under serine deprivation. Importantly, the concentrations of upstream intermediates of PGAM1 mediating the catabolic step in glycolysis tended to be increased under serine deprivation; 3-PG accumulation was especially apparent in S2-VP10 cells transfected with PGAM1 siRNA compared with S2-VP10 cells transfected with control siRNA (Fig. 5G). These findings suggest that 3-PG accumulation caused by PGAM1 deficiency synergistically promotes serine biosynthesis by inducing PHGDH in a serine-deprived microenvironment.

3.6. PHGDH deficiency suppresses but PGAM1 deficiency enhances tumor growth in mice under serine starvation

To further investigate the importance of PHGDH and PGAM1 expression in tumor development under serine starvation, we established a xenograft mouse model using S2-VP10 cells stably transfected with control short hairpin RNA (shRNA; shControl), shPHGDH or shPGAM1. We first confirmed that the mRNA and protein expression levels of PHGDH and PGAM1 were markedly decreased in the corresponding shRNA-transfected S2-VP10 cells (Fig. 6A). Although the growth of these PDAC cells was not affected by PHGDH or PGAM1 knockdown on a standard diet (Fig. 6B left panels), we observed the clear difference under conditions of serine deprivation; S2-VP10 cell growth was significantly decreased by PHGDH knockdown and significantly increased by PGAM1 knockdown compared with the control (Fig. 6B, right panels). According to the scheme shown in Fig. 6C, nude mice transplanted with these S2-VP10 cells were fed a diet with or without serine and glycine. Although the size of tumors formed from S2-VP10 cells transfected with shPHGDH or shPGAM1 tended to be slightly decreased compared with that of tumors formed from S2-VP10 cells transfected with shControl in mice fed the control diet, the difference among these three groups was not significant (Fig. 6D). However, in mice fed the SG(−) diet, tumor growth was significantly decreased by PHGDH knockdown and increased by PGAM1 knockdown compared with the control (Fig. 6E). Moreover, the number of Ki67-positive proliferating cells in S2-VP10 tumors was comparable among the shControl, shPHGDH and shPGAM1 groups of mice fed the control diet (Fig. 6F). However, in mice fed the SG(−) diet, the number of Ki67-positive cells was significantly decreased in the shPHGDH group and significantly increased in the shPGAM1 group compared with the shControl group (Fig. 6G).

4. Discussion

Metabolic adaptation is a cunning strategy by which cancer cells survive in a tumor microenvironment with deprivation of multiple nutrients and oxygen during tumor growth and metastasis [24]. Increased serine biosynthesis is one of the major metabolic adaptations that has been reported in cancer cells, and serine is a central node in the biosynthesis of many molecules [16,25]. To address the importance of serine biosynthesis in cancer growth, a previous compelling study showed that serum serine and glycine can be significantly depleted by dietary manipulation in mice and that deprivation of these nonessential amino acids can inhibit cancer growth without detrimental effects [17]. Moreover, serine deprivation enhanced the antitumor activity of biguanides as antineoplastic agents targeting cancer cell energy metabolism [26]. Therefore, deprivation of serine and/or glycine is a potentially attractive therapeutic intervention for cancer.

However, activated Kras confers resistance to the anticancer effects of deprivation of serine and glycine, reflecting the ability of activated Kras to upregulate the expression of serine biosynthesis pathway enzymes [13]. PHGDH is an important enzyme in the first step of serine biosynthesis from glycolysis. PHGDH inhibition reduces glycolytic serine synthesis, regulating one-carbon unit availability in nucleotide synthesis and suppressing the growth of PHGDH-dependent cancer cells in culture and in orthotopic xenograft tumors [27]. PHGDH is frequently amplified in several types of cancer [18,28,29]. Although gene amplification of the PHGDH locus has not been found in PDAC, PHGDH was highly expressed in almost half of the PDAC tissues, and high PHGDH expression was significantly associated with poor prognosis of PDAC patients in our cohort. Supporting this finding, PHGDH expression was an independent prognostic indicator for pancreatic cancer patients in

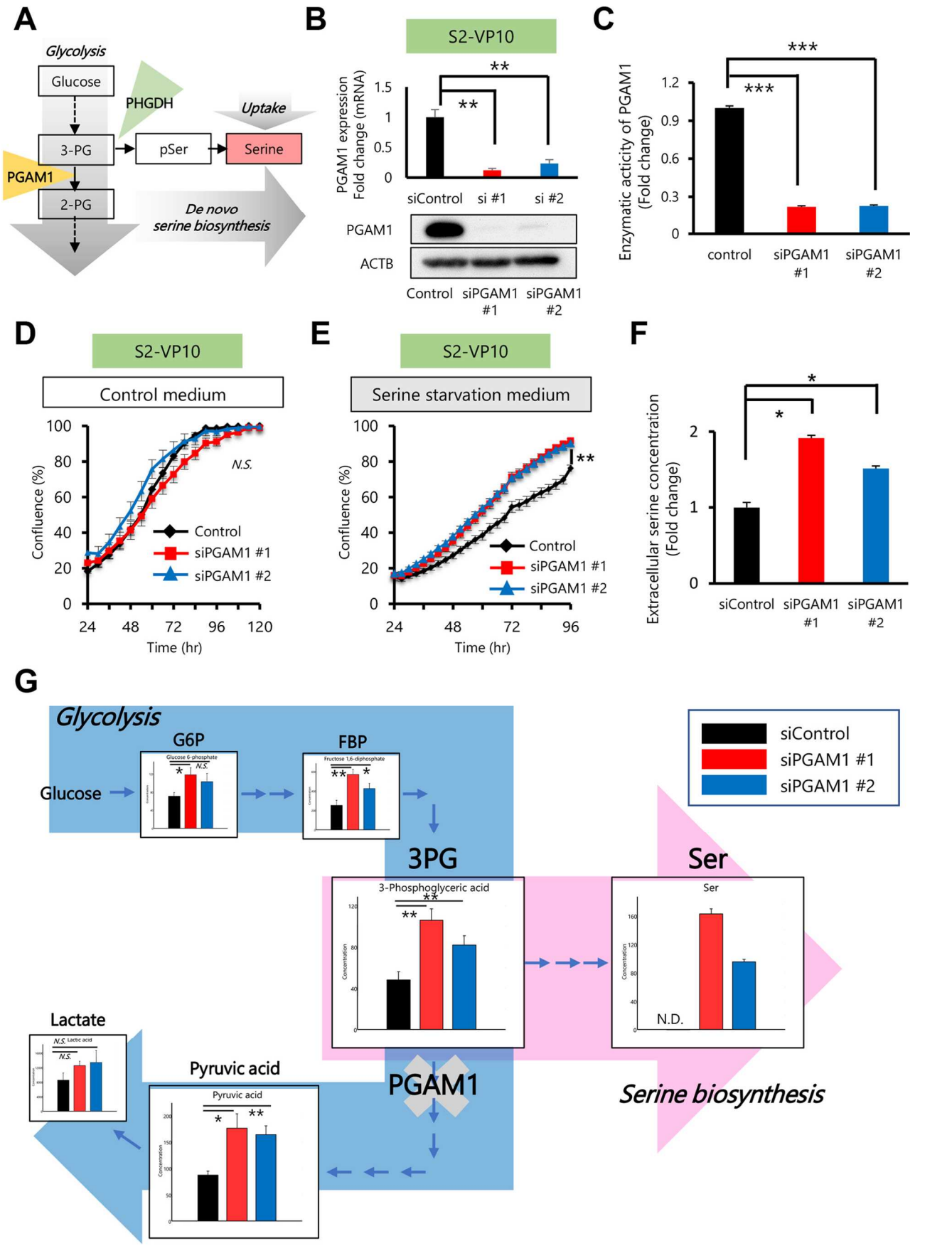

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Fig. 5. Inhibiting PGAM1 expression and subsequent 3-PG accumulation enhances serine biosynthesis and PDAC cell growth under serine starvation.
A. Serine biosynthesis is a branch of glycolysis. PGAM1 is one of the glycolytic enzymes that converts 3-PG to 2-PG. 3-PG is the glycolytic intermediates from which serine biosynthesis starts. **B.** The expression of PGAM1 in S2-VP10 cells transfected with the *PGAM1* siRNA (#1 or #2) relative to that in S2-VP10 cells transfected with the control siRNA was evaluated by qRT-PCR and Western blot analysis. **C.** The enzymatic activity of PGAM1 in S2-VP10 cells transfected with the *PGAM1* siRNA (#1 or #2) relative to that in S2-VP10 cells transfected with the control siRNA was evaluated. **D.** S2-VP10 cells transfected with the control siRNA and *PGAM1* siRNA (#1 or #2) were plated into 96-well plates in normal medium, and a proliferation assay was conducted using IncuCyte technology. **E.** S2-VP10 cells transfected with the control siRNA and *PGAM1* siRNA (#1 or #2) were plated into 96-well plates in serine starvation medium, and a proliferation assay was performed using IncuCyte technology. **F.** S2-VP10 cells transfected with the control siRNA and *PGAM1* siRNA (#1 or #2) were plated into 6-well plates in serine starvation medium and cultured for 48 h. Then, serine concentrations in the culture supernatants were measured. **G.** Comprehensive analysis of glycolysis and serine biosynthetic metabolites in S2-VP10 cells transfected with the control siRNA and *PGAM1* siRNA (#1 or #2). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

another cohort [30,31].

Notably, the growth of PK-59 cells, a PDAC cell line harboring activated Kras [32], was inhibited *in vitro* and *in vivo* in response to serine and glycine deprivation. This observation indicates that Kras activation is not sufficient to induce resistance to the anticancer effects of serine and glycine deprivation and suggests that another factor affects tolerance to serine starvation. In the current study, the growth of PDAC cells transfected with PHGDH siRNA in normal medium was slightly, but not significantly, decreased, whereas the growth of S2-VP10 cells transfected with PHGDH siRNA was significantly decreased under serine deprivation. These findings indicate that the significance of PHGDH in PDAC growth is strengthened under serine deprivation. In addition, we demonstrated that the methylation status of CpG sites surrounding the ATF4 binding motif in the promoter region is critical for PHGDH induction by serine and glycine deprivation in PDAC cells. In the current study, we could not address the regulation of methylation of the PHGDH promoter region, and thus, further investigations are required to prove the mechanism.

PGAM has been identified to be required for overcoming cellular senescence and for immortalization of mouse embryonic fibroblasts [33]. This finding suggests an oncogenic function of PGAM activity during cellular transformation and cancer initiation. However, the role of PGAM1 in cancer cells is more complicated and controversial than previously expected due to the metabolic shift from glycolysis to serine biosynthesis mediated by PGAM activity. PGAM1 expression is frequently upregulated and associated with poor prognosis in several types of cancer [34,35]. Inhibition of PGAM1 by knockdown or treatment with the small molecule inhibitor PGMI-004A resulted in a reduction in cancer cell proliferation and tumor growth [22]. Moreover, treatment with an allosteric PGAM1 inhibitor effectively suppressed PDAC cell growth in an orthotopic xenograft model using a PDAC cell line and in patient-derived xenograft models [36]. Bisphosphoglycerate mutase (BPGM) deletion decreases both histidine phosphorylation and the enzymatic activity of PGAM1, followed by the restoration of cell growth through the upregulation of de novo serine synthesis accompanied by 3-PG accumulation [23]. In the current study, although the size of tumors formed from PGAM1-deficient S2-VP10 cells tended to be reduced compared with that of tumors formed from control S2-VP10 cells in mice fed the control diet, PHGDH induction mediated by serine starvation and 3-PG accumulation mediated by PGAM1 deficiency synergistically promoted the growth of S2-VP10 cells under serine and glycine deprivation both *in vitro* and *in vivo*. Based on the observation of amino acid malnutrition in PDAC patients, our present findings imply that PGAM1 inhibition may result in the opposite effect on PDAC growth under serine starvation.

In conclusion, we identified amino acid malnutrition in blood samples from PDAC patients and demonstrated the synergistic effect of PHGDH induction and 3-PG accumulation mediated by serine starvation and by PGAM1 deficiency, respectively, on adaptive serine biosynthesis in PDAC cells. Moreover, we revealed the importance of the methylation status of CpG sites surrounding the ATF4 binding motif in the promoter region in PHGDH induction in PDAC cells under serine and glycine deprivation. Given our findings in the current study, the status of nutrients the PDAC microenvironment, such as glucose and amino acids, should be considered when applying therapy targeted toward metabolic enzymes. Taken together, our findings provide evidence that PHGDH expression in PDAC tissues could be a representative biomarker of adaptive serine biosynthesis and that targeting the serine biosynthesis pathway by inhibiting PHGDH is a potent therapeutic approach to eliminate PDAC cells in nutrient-deprived microenvironments.

Author contributions

R. Itoyama: Resources, data curation, software, formal analysis, validation, investigation, visualization, methodology, and writing (original draft). **N. Yasuda**: Resources, investigation, methodology, and writing (review and editing). **F. Kitamura**: Resources, investigation, methodology, and writing (review and editing). **T. Yasuda**: Resources, investigation, methodology, and writing (review and editing). **L. Bu**: Investigation and methodology. **X. Hu**: Investigation and methodology. **T. Uchihara**: Investigation and methodology. **K. Arima**: Investigation and methodology. **A. Yonemura**: Resources, formal analysis and methodology. **K. Miyake**: Resources, investigation and methodology. **Y. Okamoto**: Investigation, visualization and methodology. **T. Akiyama**: Resources and methodology. **K. Yamashita**: Formal analysis and validation. **Y. Nakao**: Formal analysis and validation. **T. Yusa**: Resources. **Y. Kitano**: Resources. **T. Higashi**: Resources. **T. Miyata**: Resources. **K. Imai**: Resources. **H. Hayashi**: Resources. **Y. Yamashita**: Resources. **T. Mikawa**: Investigation and methodology. **H. Kondoh**: Supervision and writing (original draft, review and editing). **H. Baba**: Supervision and writing (original draft, review and editing). **T. Ishimoto**: Conceptualization, supervision, funding acquisition, validation, project administration, and writing (original draft, review and editing).

Declaration of competing interest

No potential conflicts of interest are disclosed.

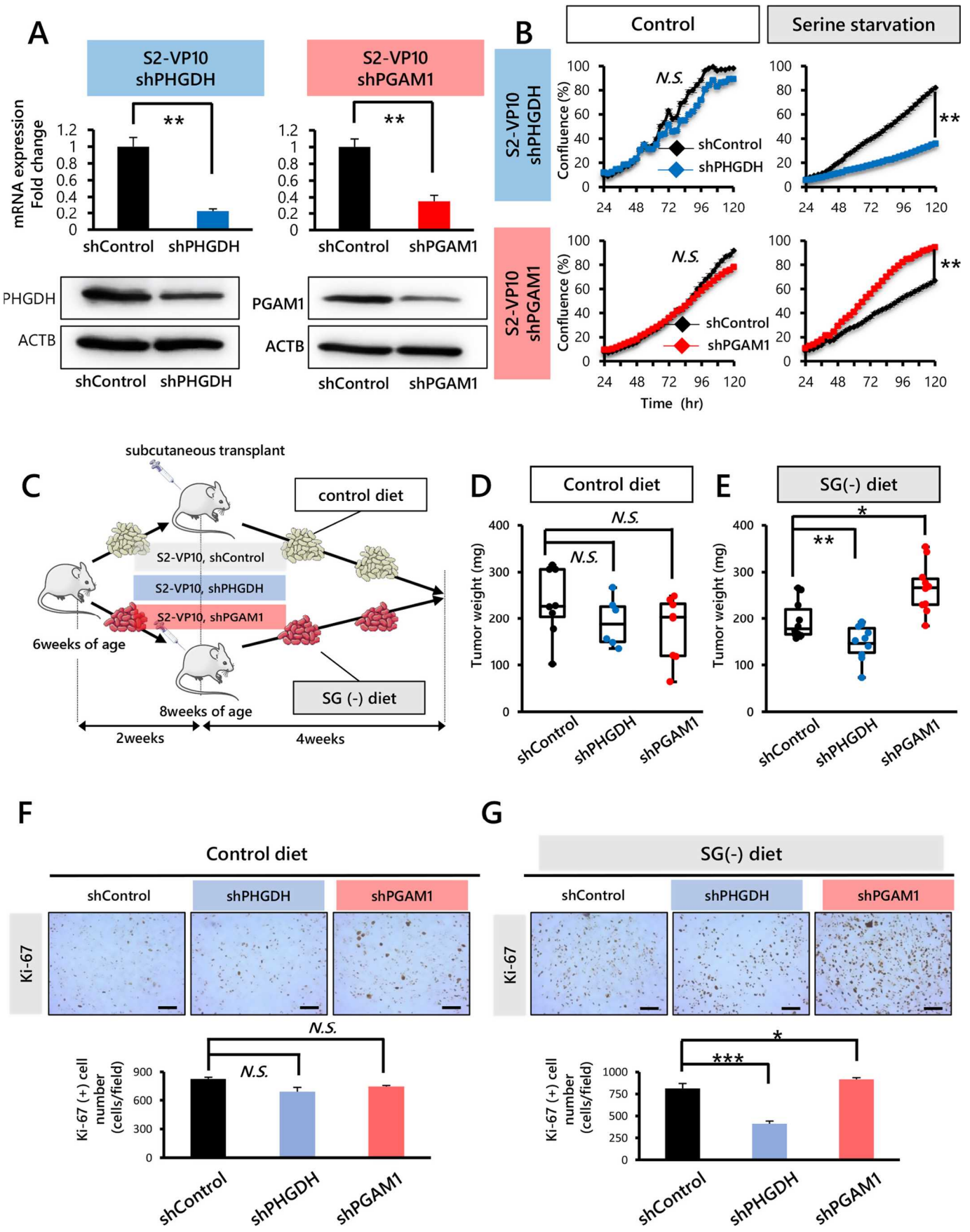

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Fig. 6. The importance of PHGDH and PGAM1 expression in tumor growth under serine starvation.
A. The expression of PHGDH in S2-VP10 cells transfected with *PHGDH* shRNA relative to that in S2-VP10 cells transfected with control shRNA was evaluated by qRT-PCR and Western blot analysis. The same experiment was performed with shRNA targeting PGAM1. **B.** S2-VP10 cells transfected with control shRNA and *PHGDH* shRNA were plated into 96-well plates in normal medium or serine starvation medium, and a proliferation assay was conducted using IncuCyte technology. The same experiment was performed using S2-VP10 cells transfected with *PGAM1* shRNA. **C.** *In vivo* experimental design. Six-week-old mice were divided into 2 groups: the control diet group and the SG(−) diet group. Two weeks after starting the special diet, it was confirmed that there was no difference in body weight between the groups. Then, 1.0×10^6 cells of each cell line (S2-VP10 cells transfected with control shRNA, *PHGDH* shRNA or *PGAM1* shRNA) were subcutaneously transplanted, and mice were fed each diet as indicated for 4 weeks. **D.** Comparison of tumor weight in the control diet group. **E.** Comparison of tumor weight in the SG(−) diet group. **F.** Representative IHC staining for Ki-67 in the control diet group. The bar charts show the quantification of Ki-67-positive cells. Scale bar, 100 μm. **G.** Representative IHC staining for Ki-67 in the SG(−) diet group. The bar charts show the quantification of Ki-67-positive cells. Scale bar, 100 μm. N.S., not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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Appendix A. Supplementary data

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