Cancer Science
Japanese Cancer Association JCA
Open Access

Enhanced expression of the M2 isoform of pyruvate kinase is involved in gastric cancer development by regulating cancer-specific metabolism

Takeharu Shiroki,[1,2] Misa Yokoyama,[1] Nobuhiro Tanuma,[3] Ryuhei Maejima,[1,2] Keiichi Tamai,[1] Kazunori Yamaguchi,[4] Tomoyuki Oikawa,[5] Tetsuya Noguchi,[5] Koh Miura,[6] Tsuneaki Fujiya,[6] Hiroshi Shima,[3] Ikuro Sato,[7] Naoko Murata-Kamiya,[8] Masanori Hatakeyama,[8] Katsunori Iijima,[2] Tooru Shimosegawa[2] and Kennichi Satoh[1]

Divisions of [1]Cancer Stem Cell, Miyagi Cancer Center Research Institute, Natori; [2]Gastroenterology, Tohoku University Graduate School of Medicine, Sendai; [3]Cancer Chemotherapy; [4]Molecular and Cellular Oncology, Miyagi Cancer Center Research Institute, Natori; Departments of [5]Gastroenterology, Miyagi Cancer Center, Natori; [6]Gastroenterological Surgery; [7]Pathology, Miyagi Cancer Center, Natori; [8]Microbiology, Graduate School of Medicine and Faculty of Medicine, The University of Tokyo, Tokyo, Japan

Key words

Aerobic glycolysis, CagA, cancer development, gastric cancer, pyruvate kinase

Correspondence

Kennichi Satoh, Division of Cancer Stem Cell, Miyagi Cancer Center Research Institute, 47-1 Nodayama, MedeshimaSiote, Natori, Miyagi 981-1293, Japan.
Tel: +81-22-384-3151; Fax: +81-22-381-1174;
E-mail: ksatoh-gi@umin.ac.jp

Funding Information

Grant-in Aid for Scientific Research (KAKENHI) (24591022, 15K19080); Japan Society for the Promotion of Science; Naito Foundation.

Received December 25, 2016; Revised February 14, 2017; Accepted February 18, 2017

Cancer Sci **108** (2017) 931–940

doi: 10.1111/cas.13211

Recent studies have indicated that increased expression of the M2 isoform of pyruvate kinase (PKM2) is involved in glycolysis and tumor development. However, little is known about the role of PKM2 in gastric cancer (GC). Therefore, we examined the expression and function of PKM2 in human GC. We evaluated PKM1 and PKM2 expression by quantitative RT-PCR in gastric tissues from 10 patients who underwent gastric endoscopic submucosal dissection, 80 patients who underwent gastrectomy, and seven healthy volunteers, and analyzed the correlation with clinicopathological variables. To assess the function of PKM2, we generated PKM2-knockdown GC cells, and investigated the phenotypic changes. Furthermore, we examined the induction of PKM2 expression by cytotoxin-associated gene A (CagA), a pathogenic factor of *Helicobacter pylori*, using CagA-inducible GC cells. We found that PKM2 was predominantly expressed not only in GC lesions but also in the normal gastric regions of GC patients and in the gastric mucosa of healthy volunteers. The PKM2 expression was significantly higher in carcinoma compared to non-cancerous tissue and was associated with venous invasion. Knockdown of PKM2 in GC cells caused significant decreases in cellular proliferation, migration, anchorage-independent growth, and sphere formation *in vitro*, and in tumor growth and liver metastasis *in vivo*. The serine concentration-dependent cell proliferation was also inhibited by PKM2 silencing. Furthermore, we found that PKM2 expression was upregulated by CagA by way of the Erk pathway. These results suggested that enhanced PKM2 expression plays a pivotal role in the carcinogenesis and development of GC in part by regulating cancer-specific metabolism.

Cancer cells produce an increased amount of lactate by glycolysis even in the presence of oxygen, a phenomenon termed "aerobic glycolysis" by Otto Warburg.[1] This "Warburg effect" was thought not only to give cancer cells a great advantage under hypoxic conditions, but also to reduce reactive oxygen species.[2,3] Recent studies showed that the M2 type of pyruvate kinase (PK) is a key glycolytic enzyme for the Warburg effect.[4,5] Pyruvate kinase regulates the final rate-limiting step of glycolysis by transferring the high-energy phosphate from phosphoenolpyruvate to ADP to generate ATP and pyruvate. Mammals have four isoforms of PK (L, R, M1, and M2), which are present in different cell types. Both PKL and PKR are encoded by the *PKLR* gene, and are expressed in the liver and red blood cells, respectively.[6,7] The PK in other tissues is produced from the *PKM* gene, through a pre-mRNA that is alternatively spliced to yield the PKM1 or PKM2 isoform, which contains exon 9 or 10, respectively.[8,9] Pyruvate kinase M1 is expressed in most differentiated tissues, whereas PKM2 is found primarily in embryonic tissues and tumor cells.[10] The PKL, R, and M1 PK types are present as highly enzymatically active tetrameric forms. In contrast, PKM2 can exist as either an active tetramer or inactive dimer, and in tumor cells it is reported to be present as a dimer with low activity.[4–7,9] As a result of this low activity of PKM2, glycolysis slows, and the subsequent pools of glycolytic intermediates are used to generate the nucleotides and amino acids that are essential for cellular growth.[11–13] In some cancer cells, PKM2's knockdown and replacement with PKM1 reverses the Warburg effect and reduces the cells' ability to form tumors in nude mice.[4] In addition, elevated PKM2 expression is found in various human cancers, suggesting that a switch of the PK (M) isoform from PKM1 to PKM2 is a pivotal event in carcinogenesis and/or cancer development.

Although the prevalence rate of gastric cancer (GC) has decreased,[14] globally, it is the fourth most common cancer in men and the fifth in women, and its death rate is second only

to lung cancer.[15] Expression of PKM2 is associated with a poor prognosis in GC patients,[16,17] and PKM2 was shown to promote GC cell growth through the regulation of Bcl-xL expression[17] or epidermal growth factor/epidermal growth factor receptor signaling.[18] However, little is known about the shift from PKM1 to PKM2 or the role of PKM2 in GC carcinogenesis and development through Warburg-effect regulation.

In this study, we show that PKM2 is upregulated in human GC tissues without a switch in isoform expression and that PKM2 knockdown in GC cells reduced their sphere-formation ability, tumorigenicity, and metastasis by altering the Warburg effect. Furthermore, we found that PKM2 was induced by cytotoxin-associated gene A (CagA) through the MAPK signaling pathway, suggesting that increased PKM2 expression is also involved in the early stage of gastric tumorigenesis.

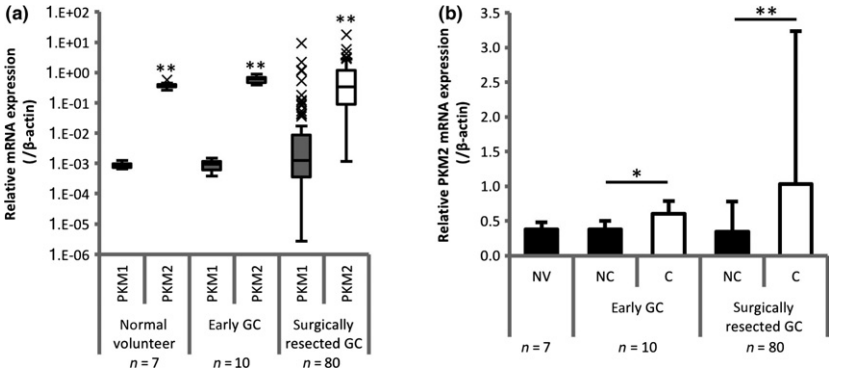

Fig. 1. Pyruvate kinase isoform M1 (PKM1) and PKM2 mRNA in normal gastric mucosa and gastric cancer (GC) tissues. (a) Expression levels of PKM1 and PKM2 were analyzed by quantitative RT-PCR in the normal gastric mucosa of healthy volunteers, and in early GC and advanced GC tissues. A significantly higher expression of PKM2 than PKM1 was found in all examined samples. (b) PKM2 mRNA levels were confirmed by quantitative RT-PCR in gastric mucosa from normal volunteers (NV), and in cancerous lesions (C) and non-cancerous tissue (NC) from early and surgically resected advanced GCs. PKM2 expression was significantly higher in GC lesions than in the corresponding non-cancerous tissues. $*P < 0.05$; $**P < 0.01$.

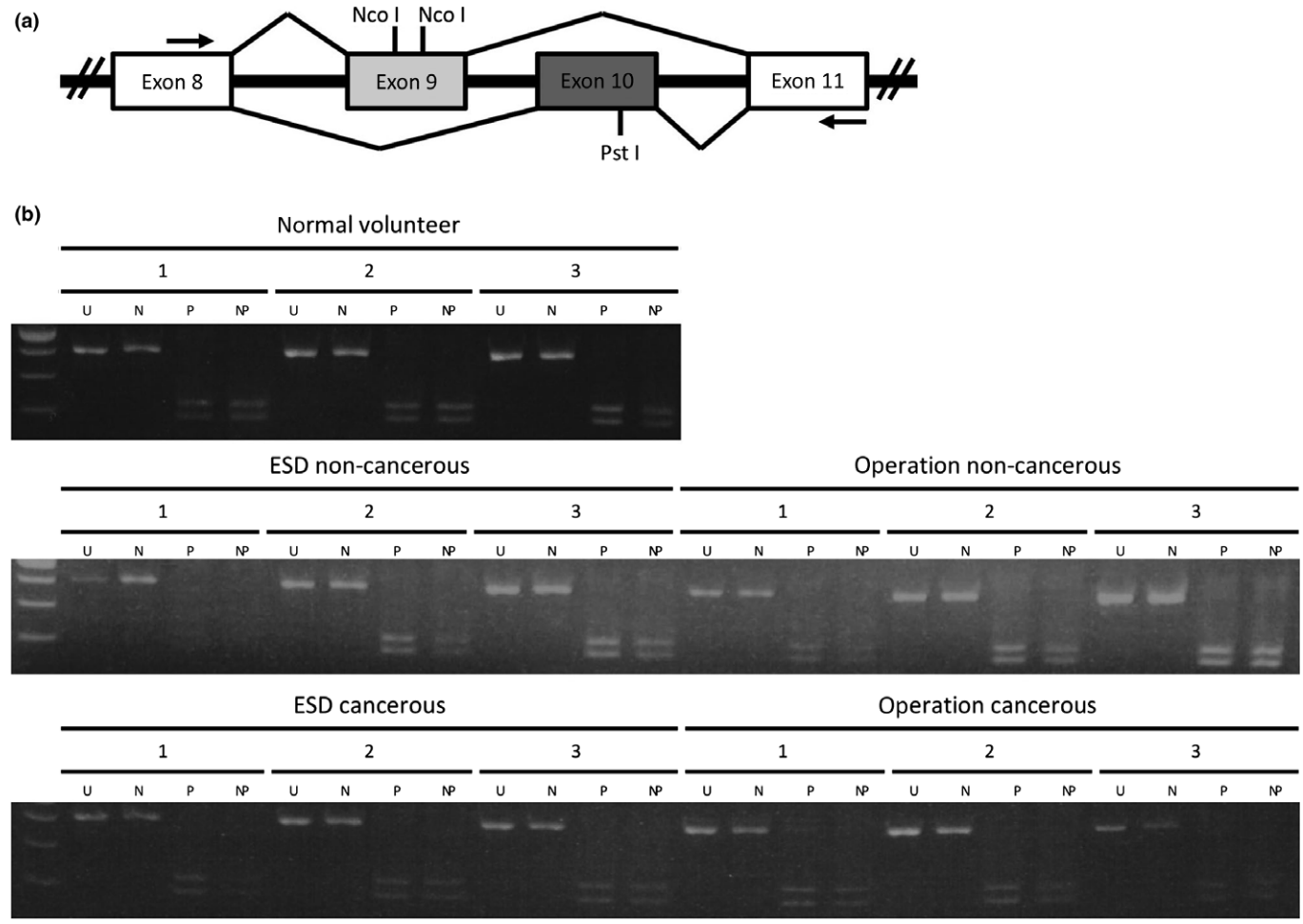

Fig. 2. Dominant pyruvate kinase M2 (PKM2) expression in gastric tissues. (a) cDNAs from normal gastric mucosa and from cancerous and non-cancerous tissues of gastric cancer patients who underwent endoscopic submucosal dissection or surgery were amplified by PCR using primers that anneal to exons 8 and 11, respectively. The alternative exons that encode the distinctive segments of PKM1 and PKM2 are exons 9 and 10, respectively. To distinguish between the PKM1 (exon 9 included) and PKM2 (exon 10 included) isoforms, the PCR products were cleaved with *Nco*I, *Pst*I, or both. (b) After amplification, the products were separated into four aliquots and digested with *Nco*I (N), *Pst*I (P), both enzymes (NP), or neither (uncut control, U). The products were separated by electrophoresis and visualized under UV light. The cDNA amplicons were completely cleaved with *Pst*I but little with *Nco*I, indicating that PKM2 was the dominant isoform in both normal and cancerous gastric tissue.

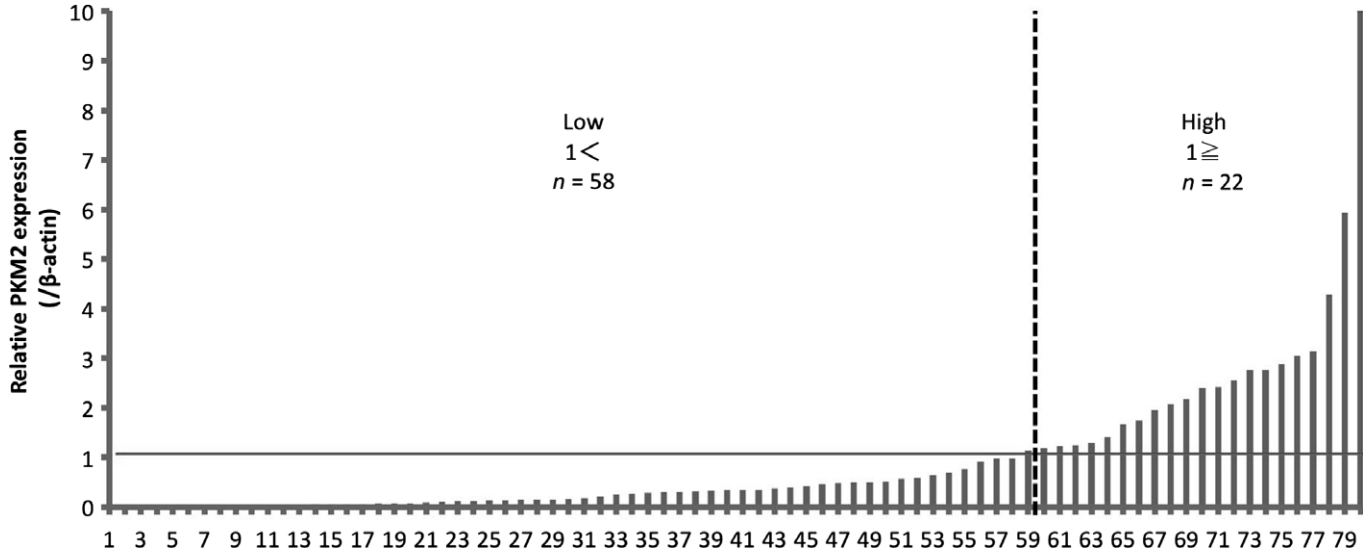

Fig. 3. Relative pyruvate kinase M2 (PKM2) mRNA expression in 80 surgically resected gastric cancer tissues. The tissues were classified according to their PKM2 expression level into a High-PKM2 group (PKM2/β-actin ≥1) and a Low-PKM2 group (PKM2/β-actin <1).

Table 1. Association of pyruvate kinase M2 (PKM2) expression with clinicopathological features of 80 resected gastric cancer specimens

	Low-PKM2 (n = 58)	High-PKM2 (n = 22)	P-value†
Age, years			
<70	32	9	0.254
≥70	26	13	
Gender			
Male	38	16	0.539
Female	20	6	
Differentiation			
Intestinal	32	14	0.494
Diffuse	26	8	
Primary tumor (T)			
T1	7	5	0.227
T2–4	51	17	
Regional lymph nodes (N)			
N0	26	6	0.152
N1–3	36	26	
Distant metastasis (M)			
M0	48	21	0.141
M1	10	1	
Stage			
I	19	8	0.761
II–IV	39	14	
Lymphatic invasion			
ly0	24	6	0.245
ly1–3	34	16	
Venous invasion			
v0	35	4	0.001
v1–2	23	18	

†Analyzed by χ^2-test. The pathologic TNM status of gastric cancers was histopathologically classified according to *TNM Classification of Malignant Tumours*, 6th edition (Union for International Cancer Control, 2002).

Materials and Methods

Tissues. A total of 187 gastric tissues, including specimens from seven healthy volunteers (biopsy) and 90 matched normal and carcinoma pairs, were evaluated in this study. The GC tissues and their corresponding normal gastric tissues were obtained from patients who underwent surgery (n = 80) or endoscopic submucosal dissection (n = 10) at Miyagi Cancer Center (Natori, Japan), between 2007 and 2013. The samples obtained surgically or endoscopically were immediately frozen or immersed in RNA*later* Stabilization Solution (Life Technologies, Carlsbad, CA, USA), respectively, and stored at −80°C. From the seven healthy volunteers, normal gastric mucosa biopsy specimens were obtained and immediately placed into RNA*later* Stabilization Solution and stored at −80°C. The pathologic TNM status of these GCs was histopathologically classified according to the *TNM Classification of Malignant Tumours*, 6th edition of the Union for International Cancer Control (2002). No patient received chemotherapy or radiotherapy before the endoscopic resection or surgery. The healthy volunteers were confirmed to have no *Helicobacter pylori* infection by ^{13}C-urea breath test (Otsuka Pharmaceuticals, Tokyo, Japan) or/and the measurement of serum *H. pylori* IgG antibody. This study was approved by the local ethics committee of Miyagi Cancer Center, and informed consent was obtained from each patient.

RNA preparation, reverse transcription, and quantitative real-time PCR. Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Tokyo, Japan) for surgically resected GC tissues, or the mirVana miRNA Isolation Kit (Life Technologies) for endoscopically resected GC tissues, normal gastric biopsy specimens from healthy volunteers, and cultured cell lines, according to the manufacturers' protocols. First-strand cDNA was generated from 0.5–1 μg total RNA using the PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa Bio, Shiga, Japan) following the manufacturer's protocol. PKM1 and PKM2 expressions were quantified by the LightCycler 480 Probes Master qRT-PCR kit (Roche Applied Science, Indianapolis, IN, USA) following the manufacturer's protocol with probes (Universal Probe Library; Roche Applied Science). The primer sets used were: PKM1, forward 5′-cagccaaaggggactatcct-3′ and reverse 5′-gaggctcgcacaagttcttc-3′; PKM2, forward 5′-ctatcctctggaggctgtgc-3′ and reverse 5′-gtggggtcgctggtaatg-3′; and β-actin, forward 5′-ccaaccgcgagaagatga-3′ and reverse 5′-

tccatcacgatgccagtg-3′. The PKM1 and PKM2 expression levels were normalized to that of β-actin.

Polymerase chain reaction–RFLP. Analysis with PCR-RFLP was carried out to accurately evaluate the relative levels of PK isoforms as described previously.[9] Complementary DNAs synthesized as described above were amplified by PCR using primer sets as described previously.[9] The primer sets annealed to exons 8 and 11, and had the following sequences: hPKMF, 5′-AGAAACAGCCAAAGGGGACT-3′; and hPKMR, 5′-CATTCATGGCAAAGTTCACC-3′. After amplification, the reactions were separated into four aliquots for digestion with: (i) *Nco*I (Roche Applied Science); (ii) *Pst*I (Roche Applied Science); (iii) *Nco*I and *Pst*I; or (iv) neither. The products were separated by electrophoresis on a 3% agarose gel with 0.5 μg/mL ethidium bromide, and visualized by UV light.

Cell lines. The GC cell lines MKN7, MKN74, MKN45, and KATO III were obtained from the Riken BioResource Center (Tsukuba, Japan) in 2011. These cell lines were not tested or authenticated after they were obtained. All cell lines were maintained in RPMI-1640 (Wako Pure Chemical Industries, Osaka, Japan) containing 10% inactivated FBS (EuroClone, Milano, Italy) with 100 U/mL penicillin and 100 μg/mL streptomycin (Nacalai Tesque, Kyoto, Japan) and cultured in a humidified 5% CO_2 incubator at 37°C.

RNA interference. To knock down PKM2 in GC cell lines, we used the Knockout RNAi Systems (Clontech

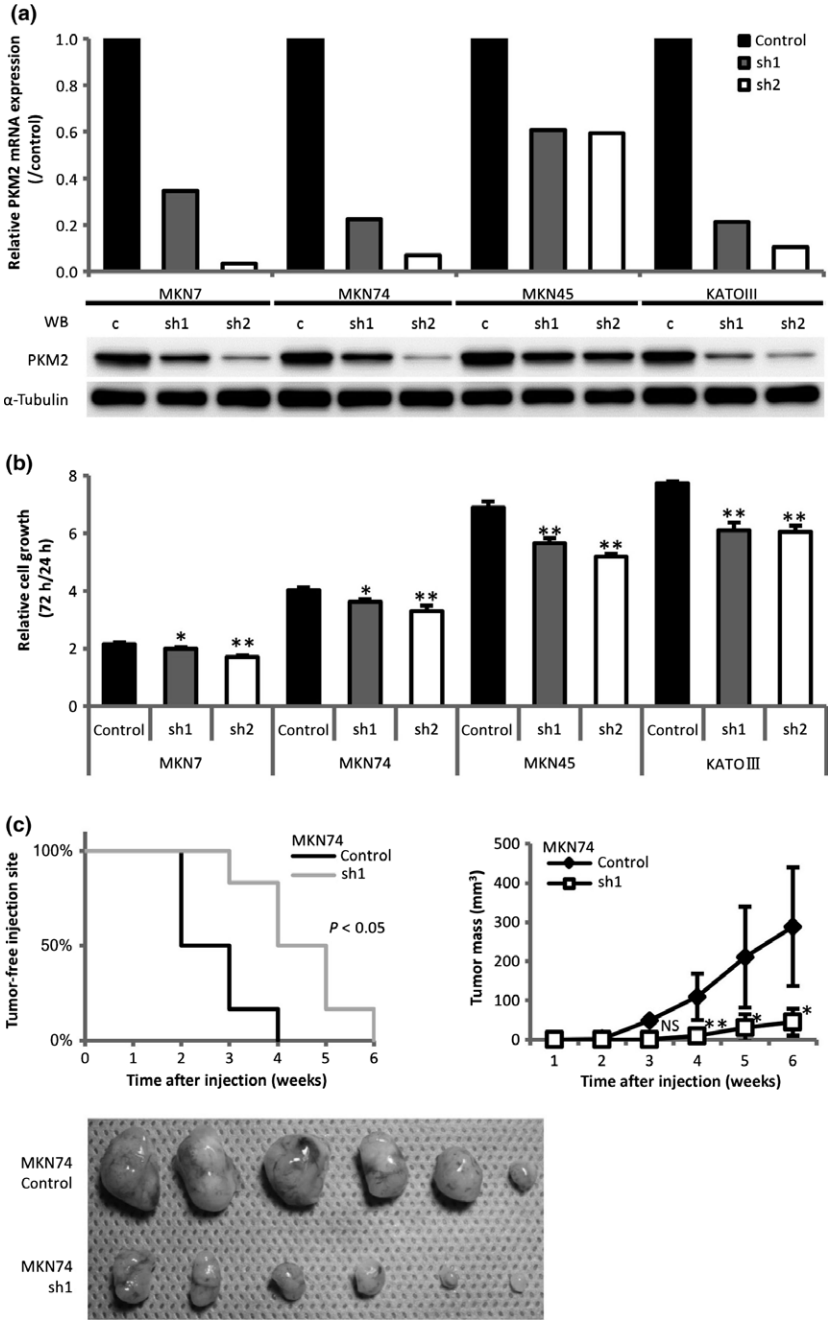

Fig. 4. Effect of pyruvate kinase M2 (PKM2) expression on tumor cell growth *in vitro* and *in vivo*. (a) PKM2 expression level was measured by quantitative RT-PCR and Western blot (WB) analyses in stably shPKM2-expressing gastric cancer (GC) cells (MKN7, MKN74, MKN45, and KATOIII). PKM2 expression was effectively reduced in the shPKM2-expressing GC cells. The quantitative RT-PCR results are shown in the bar graph, and Western blot results are shown below. (b) MTT assays showed that cell growth was significantly inhibited in all of the PKM2 knockdown (sh1 and sh2) GC cell lines. MTT assays were carried out in triplicate, and the data were analyzed by Dunnett's test. $*P < 0.05$; $**P < 0.01$. (c) MKN74-sh1 ($n = 6$) and MKN-control ($n = 6$) cells were injected s.c. into NOG mice (1×10^5 cells/injection), and then the volume of s.c. tumors was measured once a week for 6 weeks. Tumor formation was significantly delayed (left panel), and tumor growth significantly suppressed (right panel) in the mice treated with PKM2-knockdown MKN74 cells (sh1) compared with control cells. In addition, smaller tumors were formed by the PKM2-knockdown MKN 74 cells compared to control cells. Tumors were resected from NOG mice (lower panel). Tumor formation ability was analyzed by Kaplan–Meier methods and evaluated by the log–rank test (left panel). Tumor volume was calculated by the formula: ([long diameter] × [short diameter]^2) × 1/2 (middle panel). $*P < 0.05$; $**P < 0.01$. NS, not significant.

Laboratories, Mountain View, CA, USA) according to the manufacturer's protocol. We designed five shRNA sequences that targeted PKM2 according to a previous study.[19] After annealing the complementary shRNA oligonucleotides, the oligonucleotides were ligated into a pSIREN vector (sh1 and 2). The Platinum-A packaging cell lines (provided by Professor Kitamura, Division of Cellular Therapy/Division of Stem Cell Signaling, The Institute of Medical Sciences, The University of Tokyo) were then transfected with shPKM2 or pSIREN Vector (control) to produce recombinant retroviruses. Gastric cancer cells were then infected with the recombinant retroviruses, and stably infected cell lines were selected with puromycin. Cells transduced with the sh1 and sh2 vector showed an effective reduction of PKM2, and were selected for further study. The sequences of the shRNAs that targeted PKM2 were: sh1, sense 5′-gaggcagaggctgccatct-3′ and anti-sense 5′-ctccgtctccgacggtaga-3′; and sh2, sense 5′-aggcagaggctgccatcta-3′ and anti-sense 5′-tccgtctccgacggtagat-3′. All of the experiments *in vitro* were carried out in triplicate and repeated three times, and representative data are shown. Supplementary methods are included in Documents S1–S11.

Results

Pyruvate kinase M2 is dominantly expressed in normal gastric mucosa, and its expression is enhanced in tumor tissues. The PKM1 and PKM2 mRNA expression in normal mucosa (n = 7), early gastric cancer (n = 10), and resected gastric tumor (n = 80) was investigated by quantitative (q)RT-PCR,

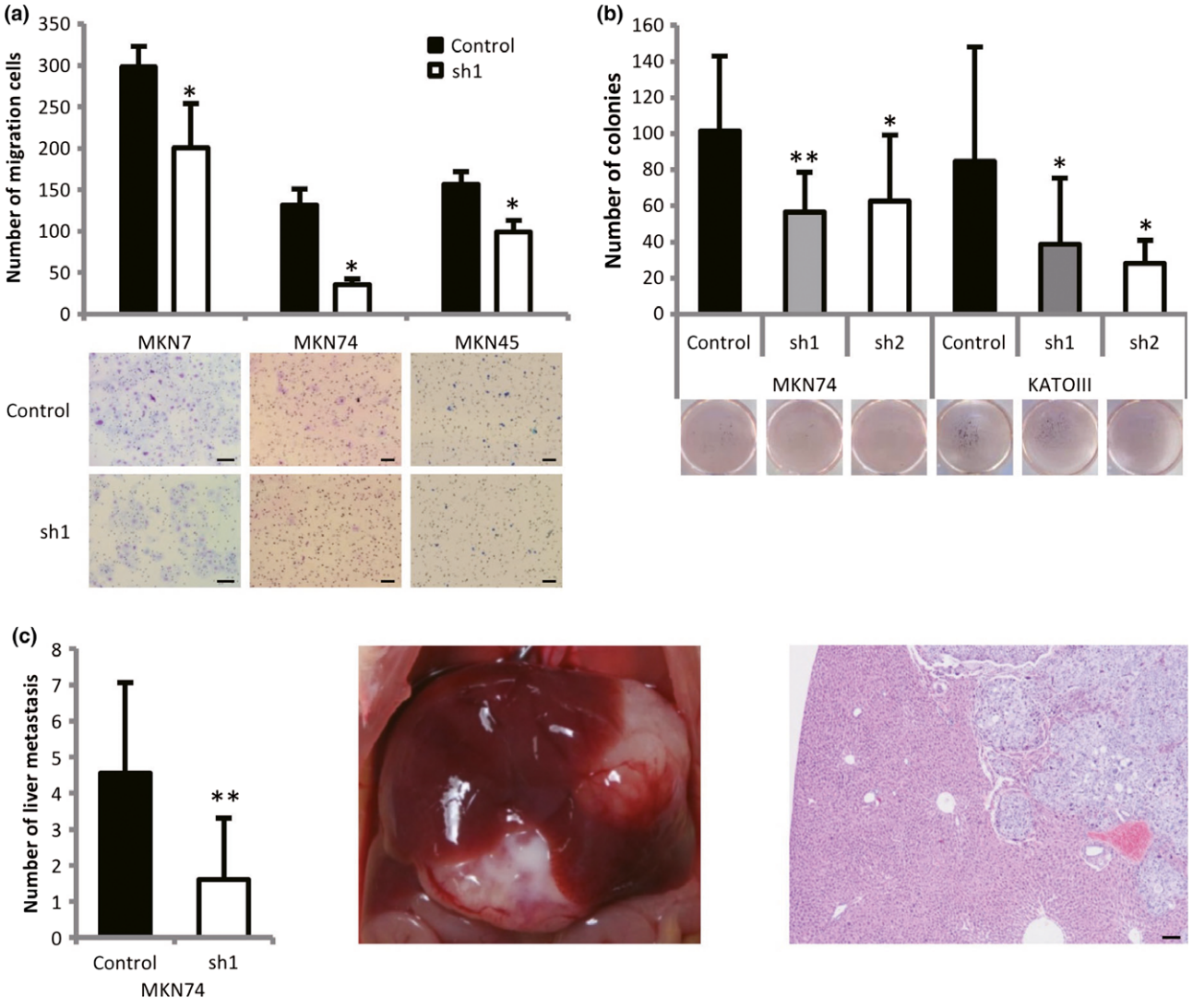

Fig. 5. Invasion capacity and anchorage-independent cell growth of pyruvate kinase M2 (PKM2)-downregulated gastric cancer (GC) cells. (a) Invasion capacity was evaluated by a two-chamber assay in which 1×10^5 or 5×10^5 cells were plated in each insert in triplicate. After 72 h, the cells on the bottom of the insert were stained and counted. Significantly fewer PKM2 downregulated migrated cells were observed compared to control cells (MKN7: ×100, bar = 100 μm; MKN74 and MKN45: ×40, bar = 100 μm). *P < 0.05. (b) Anchorage-independent growth potential of GC cells was evaluated by soft agar assay in which 1×10^4 cells in 1× media and 0.3% low-melting-point agarose were plated in triplicate. Three weeks later, the cells were stained with MTT and colonies were counted. Representative wells are shown. Significantly fewer colonies were observed with the PKM2-knockdown GC cells (sh1 and sh2) compared with control GC cells. *P < 0.05; **P < 0.01. (c) To examine metastasis, 1.0×10^5 cells of MKN74-sh1 (n = 10) and MKN-control (n = 9) cells were injected into the tail vein of mice. After 8 weeks, the mice were killed and the number of liver metastases was counted macroscopically. The number of metastases to the liver was significantly decreased for sh1-MKN74 cells compared to control cells. *P < 0.05 (left panel). Metastatic tumors could be recognized macroscopically (middle panel), and formed tumor glands in the liver (right panel) (HE; original magnification, ×40, bar = 100 μm).

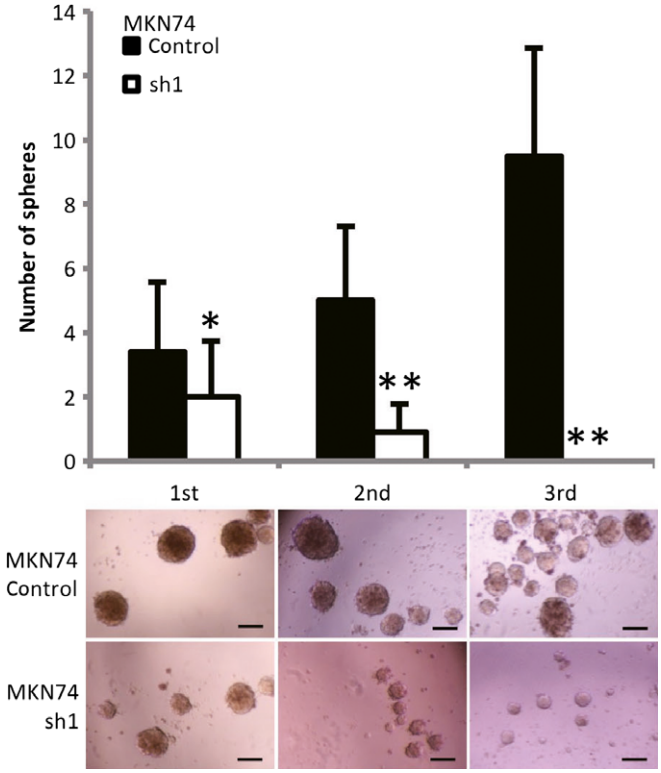

Fig. 6. Sphere formation ability of pyruvate kinase M2 (PKM2)-knockdown gastric cancer cells. Cells in serum-free DMEM/F12 containing B27 supplement, epidermal growth factor, and fibroblast growth factor were seeded into 10 wells (1×10^3 cells/well) in an ultra-low attachment 96-well plate. Two weeks later, the number of colonies was counted. The colonies were then collected and suspended. The suspended cells were then seeded in the same way. This passage was repeated three times, and the number of colonies was counted 2 weeks later. As the number of passages increased, the number of spheres decreased for the PKM2 downregulated gastric cancer cells (MKN74 sh1) but increased for the MKN control cells. Representative spheres are shown in the lower panel. Bar = 200 μm. $^*P < 0.05$; $^{**}P < 0.01$.

with normalization to the β-actin expression. As shown in Figure 1(a), PKM2 was expressed at a much higher level than PKM1 in the gastric mucosa of healthy volunteers as well as in GC tissues. To confirm the dominant expression of PKM2 against PKM1, we undertook PCR-RFLP analysis, which measures the relative level of PKM alternatively spliced mRNAs more accurately than qRT-PCR.[9] Using cDNAs as templates, PCR was carried out using a single pair of primers corresponding to constitutive exons 8 and 10 (Fig. 2a). As exons 9 and 10 are identical in length (167 bp), the resulting PCR products were digested with restriction enzymes that cleave either exon 9 (*Nco*I) or 10 (*Pst*I) to distinguish the two isoforms (Fig. 2b). These analyses showed that PKM2 (containing exon 10) is the predominant isoform in both normal mucosa and carcinoma of the stomach. In contrast, PKM1 was under the detection limit in all the tissues examined.

Furthermore, the expression level of PKM2 was significantly higher in cancerous lesions than in non-cancerous tissue (Fig. 1b). We next assessed the association between PKM2 expression and clinicopathological factors for the 80 surgically resected GC specimens. For this analysis, we classified GC tissues into a High-PKM2 group (PKM2/β-actin ≥1.0) and a Low-PKM2 group (PKM2/β-actin <1) according to the PKM2 expression level (Fig. 3). The association of PKM2 expression with the clinicopathological features of 80 resected GC tissues is summarized in Table 1. A significant relationship was found between PKM2 expression and venous invasion ($P < 0.01$).

Knockdown of PKM2 in GC cells significantly decreased cellular proliferation *in vitro* and *in vivo*. To assess the functional role of PKM2 in GC development, we generated stably shPKM2-expressing GC cells (MKN7, MKN74, MKN45, and KATOIII). Quantitative RT-PCR and Western blot analyses revealed that the PKM2 expression levels were effectively reduced in the knockdown compared to control cells (Fig. 4a). The MTT assays showed that all of the PKM2-knockdown GC cell lines had significantly impaired growth (Fig. 4b). Similarly, the s.c. tumor formation was significantly delayed (Fig. 4c), and the tumor growth was significantly reduced (Fig. 4c) in NOG mice treated with PKM2-knockdown *versus* control GC cells.

Decreased PKM2 expression inhibits GC cell migration and anchorage-independent cell growth *in vitro* and the metastatic capacity of GC cells *in vivo*. We next evaluated the role of PKM2 in GC cell migration using a two-chamber assay. The number of migrated cells was significantly decreased in PKM2-downregulated compared to control GC cells (Fig. 5a). To examine the function of PKM2 in the anchorage-independent growth of GC cells, we undertook a soft agar assay. The PKM2-downregulated MKN74 and KATOIII cells formed fewer colonies than their respective control cells (Fig. 5b).

As cell migration and anchorage-independent cell growth are generally correlated with the metastatic ability of tumor cells, we examined the metastatic capacity of PKM2-knockdown GC cells by a tail vein assay. As expected, the MKN74-sh1 cells formed fewer macroscopic liver metastases in NOG mice compared with MKN74-control cells (Fig. 5c, $P < 0.01$).

Involvement of PKM2 in sphere formation. Morfouace *et al.*[20] reported that PKM2 plays a role in glioma spheroid differentiation by interacting with Oct4, a major regulator of the self-renewal and differentiation of stem cells. To examine PKM2's role in the self-renewal of GC cells, we carried out a sphere formation assay. The PMK2-knockdown cells formed fewer spheres compared to control cells (Fig. 6). In addition, as the number of passages increased, control cells formed more spheres while the PKM2-knockdown cells formed fewer spheres.

Involvement of PKM2 in serine metabolism and the Warburg effect in GC cells. Many studies have reported that PKM2 plays a role in cancer metabolism, but little is known about its function in GC. To address the global regulatory functions of PKM2 in GC cell metabolism, we undertook metabolomic analyses of PKM2-knockdown (MKN74-sh2) and control GC cells. As shown in Figure 7(a), glycolytic intermediates were increased, and lactate production was reduced ($P < 0.01$) in MKN74-sh2 compared to control cells. Intriguingly, although most of the amino acids were decreased by PKM2 silencing, the cellular serine concentration was significantly increased (Fig. 7b). Serine is reported to promote the proliferation of various cancer cells, including those of the colon, lung, and breast.[21] Serine was also shown to regulate PKM2 activity by direct binding.[22] Collectively, these results suggest that the high concentration of cellular serine in PKM2-downregulated GC cells might be due to their inability to use serine for growth. To test this hypothesis, we treated MKN74-sh2 and MKN74-control cells with various concentrations of serine. Whereas the proliferation of MKN74-control cells increased in a serine concentration-dependent manner, this effect was not

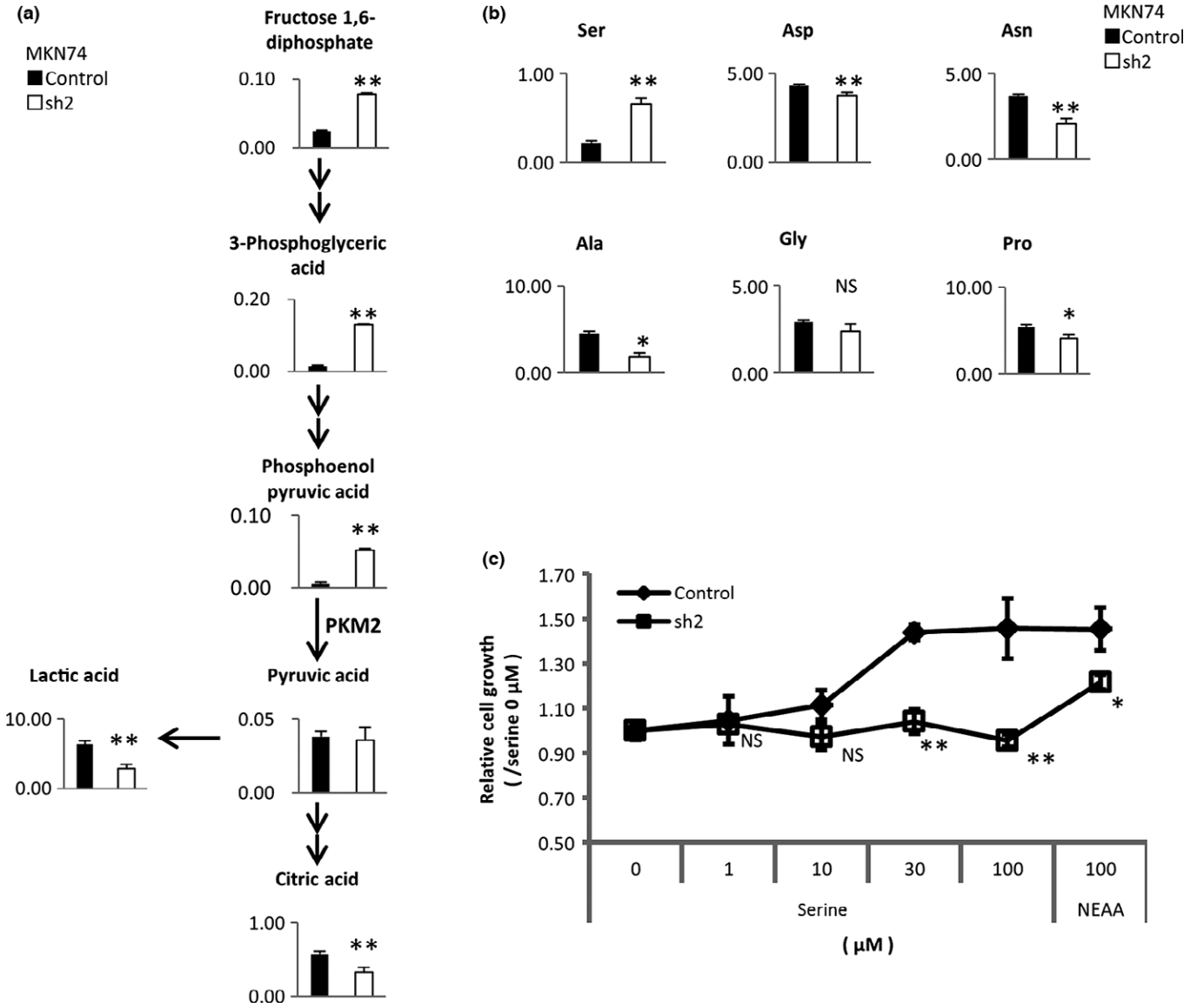

Fig. 7. Metabolome analysis of pyruvate kinase M2 (PKM2)-knockdown gastric carcinoma (GC) cells. MKN74-sh2 or MKN74-control cells were seeded in dishes (1×10^6 cells/dish) in triplicate. After 24 h, the culture medium was changed. After another 24 h, the intracellular metabolites were extracted and analyzed by capillary electrophoresis–time of flight mass spectrometry. (a) Measurement of glycolysis intermediates. The concentration of glycolysis intermediates was significantly increased in the PKM2-downregulated GC cells. (b) Cellular concentration of non-essential amino acids (NEAAs). The intracellular concentration of all NEAAs but serine (Ser) was decreased in the PKM2-knockdown cells (sh2). Ala, alanine; Asn, asparagine; Asp, aspartic acid; Gly, glycine; Pro, proline. (c) GC cells were plated in 96-well plates (5×10^3 cells/well) in the presence of varying concentrations of Ser or 100 μM NEAA in triplicate, and the cell growth was assessed by MTT assay after 72 h of incubation. The cell growth level was normalized to the value at 0 μM Ser and analyzed by Student's *t*-test. Cell growth was stimulated by Ser in control cells but not in PKM2-knockdown MKN74 cells, although treatment with NEAAs restored the impaired cell growth in these cells. $^*P < 0.05$; $^{**}P < 0.01$. NS, not significant.

observed in the PKM2-sh2 cells, although NEAA treatment induced the proliferation of these cells (Fig. 7c). These findings suggest that PKM2 is involved in GC cell growth, at least partly, by regulating serine metabolism.

Pyruvate kinase M2 expression is induced by CagA through the Erk signaling pathway. As PKM2 was upregulated in early GC tissues, as described above, we speculated that it is involved in carcinogenesis of the stomach. To address this possibility, the relationship between PKM2 and CagA, an important pathogenic protein of *H. pylori*, was examined using a CagA-inducible GC cell line (MKN28-WT-A10). As shown in Figure 8(a), both the protein and mRNA levels of PKM2 were enhanced by the induction of CagA. To explore the mechanism of the CagA-induced PKM2 expression, we investigated the effect of U0126, specific inhibitor of the Erk pathway, because CagA was previously reported to activate this pathway.[23,24] Under U0126 treatment, CagA could not upregulate PKM2 expression, suggesting that CagA induces PKM2 expression through the Erk signaling pathway (Fig. 8b).

Discussion

In this study we examined the involvement of PKM2 in gastric carcinogenesis, development, and metabolism. We clearly revealed that: (i) PKM2 was predominantly expressed not only in GC tissues but also in normal gastric mucosa; (ii) PKM2

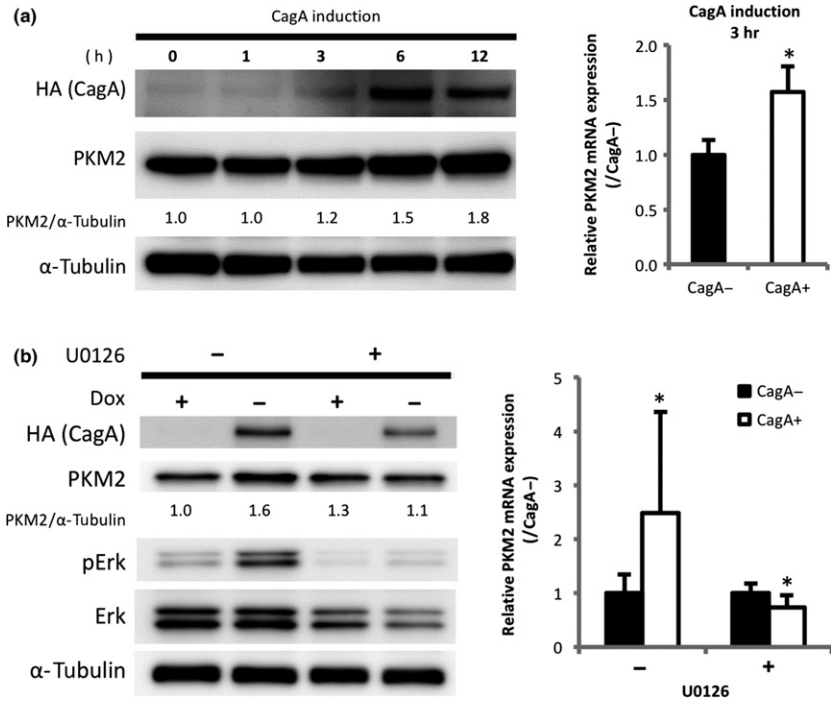

Fig. 8. Pyruvate kinase M2 (PKM2) expression is induced by cytotoxin-associated gene A (CagA) through the Erk pathway. (a) CagA expression was induced in MKN28-TetOFF-CagA cells by doxycycline (Dox) removal. After CagA induction, the cells were harvested, and RNA or protein was extracted at the times indicated. Western blot analysis showed that PKM2 was upregulated 6 h after Dox removal. The intensity of the PKM2 bands was normalized to that of α-tubulin, and values are expressed relative to the expression at 0 h (left panel). PKM2 mRNA expression was examined 3 h after CagA induction, by quantitative RT-PCR analysis in triplicate (right panel). A significant induction of PKM2 mRNA was observed (right panel). (b) To examine the role of Erk signaling in CagA-induced PKM2, the CagA-inducible cells were treated with 1 μM U0126, a MAPK inhibitor, 1 h prior to the removal of Dox. The treated cells were collected 6 h after CagA induction, and RNA or protein was extracted. The band densities for HA (CagA), PKM2, pErk, and Erk were normalized to that for α-tubulin. The PKM2 level was expressed relative to that of U0126− and Dox+ cells. The CagA-induced PKM2 expression disappeared when the MAPK inhibitor U0126 was added prior to CagA induction (left panel). PKM2 mRNA was also examined after CagA induction under Erk signaling blockade, by quantitative RT-PCR analysis in triplicate. Upregulation of PKM2 mRNA by CagA was abrogated when the cells were treated with U0126 (right). $^*P < 0.05$.

expression in GC tissues was higher than in normal gastric tissues, tended to increase with GC progression, and was significantly associated with venous invasion; (iii) PKM2 knockdown in GC cells attenuated their proliferation, migration, metastatic ability, and cancer stem cell-like properties; (iv) PKM2 suppression impaired the serine metabolism and aerobic glycolysis; and (v) CagA induced PKM2 expression through the Erk signaling pathway.

Pyruvate kinase M2 is the predominant PKM isoform in proliferative cells and cancer tissues, and a switch of PKM expression from PKM1 to PKM2 is thought to be a pivotal event in cancer formation.[10,25] However, recent studies have revealed no evidence for the exchange of PKM1 to PKM2 expression in various kinds of cancers, except glioblastoma.[26,27] Little is known about this isoform shift in gastric tissues during carcinogenesis. In the current study, we clarified that PKM2 is the principal isoform in stomach tissues and found no evidence of an isoform switch in gastric carcinogenesis. Instead, PKM2 was the major isoform in both normal gastric mucosa and the normal tissue surrounding early and advanced GC, but, importantly, its expression level was significantly enhanced in GC tissue. In addition, the expression level of PKM2 was elevated during GC progression (early to advanced stage). Similarly, the PKM2 expression is upregulated in a grade-specific manner in human glioma[28] and in Barrett's esophagus.[29] These findings suggest that an enhancement of PKM2 expression, but not a switch of the PKM isoform, plays a crucial role in the development of GC.

Previous studies indicated a correlation between PKM2 overexpression and a poor prognosis in GC patients,[16, 17] but we did not find this association. This difference might have been due to the short observation period and small number of cases in this study. However, our current findings that PKM2 expression was associated with venous infiltration and that PKM2 knockdown attenuated the aggressive behaviors of GC cells highlight the contribution of PKM2 to the acquisition of malignant potential.

The involvement of PKM2 in GC cell proliferation and anchorage-independent growth was reported previously.[17] In addition to confirming these findings, we revealed a relationship between PKM2 expression and sphere formation. Sphere formation ability is associated with cancer stem cell activity and self-renewal of cells.[30,31] Cancer stem cells are thought to cause cancer recurrence, metastasis, and resistance to chemo- or radiotherapy, and an increase in cancer stem cell-like cells is correlated with a poor prognosis.[32,33] Hence, enhanced PKM2 expression might promote GC development by helping GC cells to gain cancer stem-like properties and self-renewal ability.

Pyruvate kinase M2 is a critical determinant of the metabolic phenotype characterized by increased glycolysis with lactate generation, regardless of oxygen availability, and is known to contribute to tumor progression by regulating this

so-called Warburg effect. Here we showed that PKM2 knockdown increased the glycolytic intermediates and reduced the lactic acid production in GC cells, indicating that PKM2 suppression reversed the Warburg effect. We also found that, among amino acids, serine was specifically increased in the PKM2-downregulated GC cells. Moreover, adding serine increased the cell growth of GC cells but not of PKM2-knockdown GC cells, suggesting that GC cells with impaired PKM2 function cannot utilize serine for cell proliferation in a normal environment. This possibility is compatible with recent reports that serine activates PKM2 through direct interaction.[22,34] Collectively, these findings suggest that PKM2 promotes GC cell proliferation by regulating serine metabolism in addition to the Warburg effect.

In this study, PKM2 was found to be overexpressed at the early stage of GC compared with adjacent non-tumorous tissue. We therefore hypothesized that PKM2 is involved in gastric carcinogenesis, and investigated the correlation between *H. pylori,* one of the most important risk factors of GC, and PKM2. We revealed that CagA, a major pathogenic protein of *H. pylori*, increased PKM2 expression through the Erk signaling pathway. However, we did not observe a higher expression of PKM2 in non-cancerous lesions of GC tissues that were infected with *H. pylori* compared to the normal gastric mucosa of healthy volunteers. The reason for this discrepancy is not clear. In human gastric mucosa, CagA is degraded by autophagy shortly after *H. pylori* infection, but it is specifically accumulated in gastric cells expressing the variant form of CD44 (CD44v9), a cell-surface marker associated with cancer stem cells.[35] The amount of PKM2 induced by CagA in such *H. pylori*-infected mucosa might be below the detection level, as the proportion of cancer stem cells among cancer cells is very low. This situation may partially explain our discrepant findings. Although further examination is required to address this issue, the CD44v9-positive stem-like cells where CagA accumulates may promote gastric carcinogenesis at least partly through CagA's induction of PKM2.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research (KAKENHI grant nos. 24591022 and 15K19080) and a grant from the Naito Foundation (to K.S).

Disclosure Statement

The authors have no conflict of interest.

References

1 Warburg O. On the origin of cancer cells. *Science* 1956; **123**: 309–14.
2 Anastasiou D, Poulogiannis G, Asara JM *et al.* Inhibition of pyruvate kinase M2 by reactive oxygen species contributes to cellular antioxidant responses. *Science* 2011; **334**: 1278–83.
3 Read JA, Winter VJ, Eszes CM, Sessions RB, Brady RL. Structural basis for altered activity of M- and H-isozyme forms of human lactate dehydrogenase. *Proteins* 2001; **43**: 175–85.
4 Christofk HR, Vander Heiden MG, Harris MH *et al.* The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature* 2008; **452**: 230–3.
5 Christofk HR, Vander Heiden MG, Wu N, Asara JM, Cantley LC. Pyruvate kinase M2 is a phosphotyrosine-binding protein. *Nature* 2008; **452**: 181–6.
6 Mazurek S. Pyruvate kinase type M2: a key regulator of the metabolic budget system in tumor cells. *Int J Biochem Cell Biol* 2011; **43**: 969–80.
7 Noguchi T, Yamada K, Inoue H, Matsuda T, Tanaka T. The L- and R-type isozymes of rat pyruvate kinase are produced from a single gene by use of different promoters. *J Biol Chem* 1987; **262**: 14366–71.
8 Noguchi T, Inoue H, Tanaka T. The M1- and M2-type isozymes of rat pyruvate kinase are produced from the same gene by alternative RNA splicing. *J Biol Chem* 1986; **261**: 13807–12.
9 Clower CV, Chatterjee D, Wang Z, Cantley LC, Vander Heiden MG, Krainer AR. The alternative splicing repressors hnRNP A1/A2 and PTB influence pyruvate kinase isoform expression and cell metabolism. *Proc Natl Acad Sci USA* 2010; **107**: 1894–9.
10 Mazurek S, Boschek CB, Hugo F, Eigenbrodt E. Pyruvate kinase type M2 and its role in tumor growth and spreading. *Semin Cancer Biol* 2005; **15**: 300–8.
11 Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 2009; **324**: 1029–33.
12 Deberardinis RJ, Sayed N, Ditsworth D, Thompson CB. Brick by brick: metabolism and tumor cell growth. *Curr Opin Genet Dev* 2008; **18**: 54–61.
13 Tong X, Zhao F, Thompson CB. The molecular determinants of de novo nucleotide biosynthesis in cancer cells. *Curr Opin Genet Dev* 2009; **19**: 32–7.
14 Nagini S. Carcinoma of the stomach: a review of epidemiology, pathogenesis, molecular genetics and chemoprevention. *World J Gastrointest Oncol* 2012; **4**: 156–69.
15 Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69–90.
16 Lim JY, Yoon SO, Seol SY *et al.* Overexpression of the M2 isoform of pyruvate kinase is an adverse prognostic factor for signet ring cell gastric cancer. *World J Gastroenterol* 2012; **18**: 4037–43.
17 Kwon OH, Kang TW, Kim JH *et al.* Pyruvate kinase M2 promotes the growth of gastric cancer cells via regulation of Bcl-xL expression at transcriptional level. *Biochem Biophys Res Commun* 2012; **423**: 38–44.
18 Wang LY, Liu YP, Chen LG *et al.* Pyruvate kinase M2 plays a dual role on regulation of the EGF/EGFR signaling via E-cadherin-dependent manner in gastric cancer cells. *PLoS ONE* 2013; **8**: e67542.
19 Goldberg MS, Sharp PA. Pyruvate kinase M2-specific siRNA induces apoptosis and tumor regression. *J Exp Med* 2012; **209**: 217–24.
20 Morfouace M, Lalier L, Oliver L *et al.* Control of glioma cell death and differentiation by PKM2-Oct4 interaction. *Cell Death Dis* 2014; **5**: e1036.
21 Labuschagne Christiaan F, van den Broek Niels JF, Mackay Gillian M, Vousden Karen H, Maddocks Oliver DK. Serine, but not glycine, supports one-carbon metabolism and proliferation of cancer cells. *Cell Rep* 2016; **7**: 1248–58.
22 Chaneton B, Hillmann P, Zheng L *et al.* Serine is a natural ligand and allosteric activator of pyruvate kinase M2. *Nature* 2012; **491**: 458–62.
23 Zhou J, Xie Y, Zhao Y, Wang S, Li Y. Human gastrin mRNA expression up-regulated by *Helicobacter pylori* CagA through MEK/ERK and JAK2-signaling pathways in gastric cancer cells. *Gastric Cancer* 2011; **14**: 322–31.
24 Lee IO, Kim JH, Choi YJ *et al. Helicobacter pylori* CagA phosphorylation status determines the gp130-activated SHP2/ERK and JAK/STAT signal transduction pathways in gastric epithelial cells. *J Biol Chem* 2010; **285**: 16042–50.
25 Vander Heiden MG, Christofk HR, Schuman E *et al.* Identification of small molecule inhibitors of pyruvate kinase M2. *Biochem Pharmacol* 2010; **79**: 1118–24.
26 Desai S, Ding M, Wang B *et al.* Tissue-specific isoform switch and DNA hypomethylation of the pyruvate kinase PKM gene in human cancers. *Oncotarget* 2014; **5**: 8202–10.
27 Bluemlein K, Gruning NM, Feichtinger RG, Lehrach H, Kofler B, Ralser M. No evidence for a shift in pyruvate kinase PKM1 to PKM2 expression during tumorigenesis. *Oncotarget* 2011; **2**: 393–400.
28 Mukherjee J, Phillips JJ, Zheng S, Wiencke J, Ronen SM, Pieper RO. Pyruvate kinase M2 expression, but not pyruvate kinase activity, is up-regulated in a grade-specific manner in human glioma. *PLoS ONE* 2013; **8**: e57610.
29 Koss K, Harrison RF, Gregory J, Darnton SJ, Anderson MR, Jankowski JA. The metabolic marker tumour pyruvate kinase type M2 (tumour M2-PK) shows increased expression along the metaplasia-dysplasia-adenocarcinoma sequence in Barrett's oesophagus. *J Clin Pathol* 2004; **57**: 1156–9.
30 Ponti D, Costa A, Zaffaroni N *et al.* Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res* 2005; **65**: 5506–11.
31 Reynolds BA, Rietze RL. Neural stem cells and neurospheres–re-evaluating the relationship. *Nat Methods* 2005; **2**: 333–6.
32 Ren F, Sheng WQ, Du X. CD133: a cancer stem cells marker, is used in colorectal cancers. *World J Gastroenterol* 2013; **19**: 2603–11.

33 Chen J, Zhou J, Lu J, Xiong H, Shi X, Gong L. Significance of CD44 expression in head and neck cancer: a systemic review and meta-analysis. *BMC Cancer* 2014; **14**: 15.
34 Ye J, Mancuso A, Tong X *et al.* Pyruvate kinase M2 promotes de novo serine synthesis to sustain mTORC1 activity and cell proliferation. *Proc Natl Acad Sci USA* 2012; **109**: 6904–9.
35 Tsugawa H, Suzuki H, Saya H *et al.* Reactive oxygen species-induced autophagic degradation of *Helicobacter pylori* CagA is specifically suppressed in cancer stem-like cells. *Cell Host Microbe* 2012; **12**: 764–77.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Doc. S1. Antibodies.

Doc. S2. Western blot analysis.

Doc. S3. Cell growth assay.

Doc. S4. Cell migration assay.

Doc. S5. Soft agar colony formation assay.

Doc. S6. Sphere formation assay.

Doc. S7. Metabolome analysis.

Doc. S8. Confirmation of the serine requirement for gastric cancer cell growth.

Doc. S9. Animal experiments.

Doc. S10. Association between cytotoxin-associated gene A (CagA) and pyruvate kinase M2 (PKM2) expression.

Doc. S11. Statistical analysis.