Effect of perturbation of PGK1 on the glycolysis

Perturbation of phosphoglycerate kinase 1 (PGK1) only marginally affects glycolysis in cancer cells

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Abbreviations: Glc, glucose; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; GA3P, glyceraldehyde 3-phosphate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; Pyr, pyruvate; HK, hexokinase; PGI, phosphohexose isomerase; PFK, Phosphofructokinase; TPI, triose phosphate isomerase, GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PK, phosphoglycerate kinase; LDH, lactate dehydrogenase; Gln, glutamine; WE, warburg effect; FCC, flux control coefficient.

Abstract
Phosphoglycerate kinase 1 (PGK1) plays important roles in glycolysis, yet its forward reaction kinetics are unknown, and its role especially in regulating cancer cell glycolysis is unclear. Here, we developed an enzyme assay to measure the kinetic parameters of the PGK1-catalyzed forward reaction. The Km values for 1,3-bisphosphoglyceric acid (1,3-BPG, the forward reaction substrate) were 4.36 μM (yeast PGK1) and 6.86 μM (human PGK1). The Km values for 3-phosphoglycerate (3-PG, the reverse reaction substrate and a serine precursor) were 146 μM (yeast PGK1) and 186 μM (human PGK1). The Vmax of the forward reaction was about 3.5- and 5.8-fold higher than that of the reverse reaction for the human and yeast enzymes, respectively. Consistently, the intracellular steady-state concentrations of 3-PG were between 180 and 550 μM in cancer cells, providing a basis for glycolysis to shuttle 3-PG to the serine synthesis pathway. Using siRNA-mediated PGK1-
Effect of perturbation of PGK1 on the glycolysis

specific knockdown in five cancer cell lines derived from different tissues, along with titration of PGK1 in a cell-free glycolysis system, we found that the perturbation of PGK1 had no or only marginal effects on the glucose consumption and lactate generation. The PGK1 knockdown increased the concentrations of fructose 1,6-bisphosphate (FBP), dihydroxyacetone phosphate (DHAP), glyceraldehyde 3-phosphate (GA3P), and 1,3-BPG in nearly equal proportions, controlled by the kinetic and thermodynamic states of glycolysis. We conclude that perturbation of PGK1 in cancer cells insignificantly affects the conversion of glucose to lactate in glycolysis.

Introduction

The aerobic glycolysis (Warburg effect, WE) is a metabolic hallmark of cancer cells. Inhibiting WE is recognized as an approach to treat cancer(1-4) (5). The rate limiting enzymes along glycolysis are recognized as targets for inhibiting WE (6-12).

Previous studies demonstrated that PGK1 was a rate-limiting enzyme in the glycolysis in cancer cells (13-19). PGK1 catalyzes a step at the middle of glycolysis and produces ATP and 3-PG (a precursor for serine). Given the high glycolytic rate, PGK1 activity accounts a large part in maintaining the energy homeostasis and serine biosynthesis. Clinically, PGK1 was overexpressed in many types of tumors (20-24). Experimentally, it is found that this enzyme was dynamically modulated in cells. This enzyme was transcriptional upregulated by HIF1 (25) but downregulated by PPAR-γ (26). Hu et al reported that PGK1 could be acetylated by PCAF and Sirtuin 7 and the acetylation of PGK1 enhanced its activity and promoted glycolysis and liver cancer tumorigenesis (13). Zhang et al demonstrated that polarized M2 macrophages secreted IL-6, which enhances PGK1 phosphorylation in tumor cells and promoted glycolysis, and this phosphorylation is associated with malignance and prognosis of human GBM (14). Li et al revealed that, apart from its canonical activity, PGK1 could translocate into mitochondria to phosphorylate and to activate PDHK1, which in turn phosphorylated and inhibited PDH, impairing TCA cycle and enhancing glycolysis (15). On the other hand, Tanner et al reported that a perturbation of PGK1 did not significantly affect glycolysis (27).

The mixed reports indicated a vague understanding of the mechanism by which PGK1 regulates glycolysis. As the rate control of glycolysis is fundamentally a question of kinetics and thermodynamics of the glycolysis, we sought to investigate the effect of perturbing PGK1 on the glycolysis with respect to thermodynamics and related kinetics.

In order to investigate the above-mentioned questions, we ought to know the kinetics of PGK1. PGK1 catalyzes a reversible reaction, its reverse-reaction kinetics is known, but its forward reaction kinetics is unknown because there is no available method. Some studies reported that the forward reaction activity of PGK1 could be measured by the production of NADH, with a reaction mixture containing GA3P, β-NAD and ADP (13,28). The forward reaction of PGK1 can promote GAPDH to produce NADH (13,28). This method may tell the difference of the NADH generation rate with or without PGK1, but cannot accurately measure the
activity of PGK1, nor the kinetic parameters. Therefore, we developed a method to accurately measure the forward reaction kinetics of PGK1.

Results
The rational to measure the forward reaction rate of PGK1

We designed a coupled-enzyme assay to measure the forward-reaction rate of PGK1.

Step 1:
In a reaction mixture containing GA3P, and NAD, and Pi, add excessive amount of GAPDH, reaction 1 would rapidly reach equilibrium

\[ \text{GA3P} + \text{NAD} + \text{Pi} \xleftrightarrow{\text{GAPDH}} 1,3\text{-BPG} + \text{NADH} \]

The equilibrium of the reactions is monitored at 340 nm, which increases at first, then remains stable (Figure 1), indicative of equilibrium state.

Step 2,
Add ADP and PGK1 into the reaction mixture to initiate reaction 2 (Figure 1)

\[ 1,3\text{-BPG} + \text{ADP} \xrightarrow{\text{PGK1}} 3\text{-PG} + \text{ATP} \]

As consumption of 1,3-BPG immediately disrupt the equilibrium state of reaction 1, driving GA3P and NAD to 1,3-BPG and NADH. The rate of NADH generation can be readily spectrophotometrically monitored. According to the reactions 1 and 2, we could derive the following equations:

1. the number of 3-PG molecules generated = the numbers of 1,3-BPG molecules consumed,
2. the number of 1,3-BPG molecules consumed = the numbers of GA3P molecules consumed,
3. the number of GA3P molecules generated = the numbers of NADH molecules generated.
4. Therefore, NADH generation rate = turnover rate of 1,3-BPG to 3-PG catalyzed by PGK1

Therefore, the initial rate of PGK1 could be accurately measured.

The kinetic parameters of PGK1

For measurement, based on the equilibrium of reaction 1, we set a serial concentrations of GA3P, which give rise to a serial concentrations of 1,3-BPG (Figure 2A). Then, we determined the \( K_m \) and \( V_{max} \) value of human and yeast PGK1 (Figure 2B &C). The \( K_m \) values for 1,3-BPG were 4.36 μM (yeast) and 6.86 μM (human), whereas the \( K_m \) values for 3-PG were 146 μM (yeast) and 186 μM (human), and the \( V_{max} \) of the forward reaction (from 1,3-BPG to 3PG) was about 3.5 (human) and 5.8 (yeast) folds higher than that of the reverse reaction (Figure 2D), indicating that the enzyme favors the forward reaction. By setting 1,3-BPG at saturating concentration (about 50 μM) and varying ADP concentrations (0.02 mM to 2 mM), we obtained \( K_m \) value for ADP (Figure 2D).

The potential physiological implications of the kinetic parameters of the PGK1 for 3-PG shuttle to serine

We sought to explore the physiological meaning of the kinetic parameters of PGK1 in glycolysis. The low \( K_m \) for 1,3-BPG and high \( K_m \) for 3-PG, and the much higher rate of the forward reaction than that of the reverse reaction is a biochemical basis to maintain a low concentration of 1,3-BPG and a high concentration of 3-PG in cells. Indeed, cellular 3-PG concentrations were kept at relatively high concentration, between 0.18 and 0.55 mM (depending on
Effect of perturbation of PGK1 on the glycolysis

3-PG is a precursor for serine synthesis. Phosphoglycerate dehydrogenase (PHGDH), which catalyzes the first step for de novo serine synthesis, has the $K_m$ value of 0.26 mM for 3-PG (29). Keeping a high cellular 3-PG concentration is very important for this molecule to shuttle to serine synthesis, because the specific activity (at saturating concentration of 3-PG) of PHGDH in cancer cell is very low. The activity was undetectable even using 0.15 mg cell lysate protein in our assay system, whereas HK activity could be accurately determined using 0.03 mg cell lysate protein.

HK2 knockdown reduced HK activity by $\sim 50\%$, glucose consumption by $\sim 40\%$, and lactate generation by $\sim 50\%$ (Figure 3A). On the other hand, HK2 knockdown did not significantly reduce the 3-PG concentration (Figure 3B) and serine synthesis, as manifested by the analysis of the serine isotopologues (Figure 3C). The m$^+0$ serine species is provided by the culture medium, m$^+3$ serine isotopologue was generated from $[^{13}C_6]$glucose through 3-PG. The consumption rate of m$^+0$ serine was comparable between control cells and HK2 knockdown cells (Figure 3C, left panel), and the percentages of extracellular and intracellular m$^+0$ and m$^+3$ serine species were also comparable between control cells and HK2 knockdown (Figure 3C, middle and right panels). A fraction of serine was further for glycine synthesis. The consumption rate of glycine (m$^+0$, which is provided by culture medium) is comparable between control and HK2 knockdown cells (Figure 3D, left panel), so were the percentages of extracellular and intracellular glycine (m$^+2$, which is derived from m$^+3$ serine) (Figure 3D, middle and right panels). These data supported that glucose carbon shuttling to serine and glycine was not significantly affected by HK2 knockdown, even HK2 knockdown reduced glycolysis rate to lactate by 50%.

Taken together, the kinetic parameters of PGK1 is associated with a stable steady-state concentration of 3-PG in cancer cells, which is around the $K_m$ value of PHGDH, underlying a sound biochemical basis for glycolysis to shuttle to de novo serine synthesis pathway.

The characteristics of cell lines

We used 5 cell lines from different tissue origins (cervical cancer cell line HeLa, gastric cancer cell line MGC80-3, colon cancer cell line RKO, lung cancer cell line A549, and liver cancer cell line SK-HEP-1). These cells were derived from different organs from different patients, representing 5 types of cancer cell lines. They exhibited widespread mutations (30-33). HeLa cells had wild type $TP53$, but p53 protein was repressed because of overexpression of human papillomavirus type 16 E6 (34,35); In MGC80-3, decreased expression of TWSG1 was detected compared to normal gastric cells (36). In RKO cells, $TP53$ was wild type while $PTEN$, $KRAS$, $BRAF$, $PIK3CA$ was mutated (30,32). Wild type $TP53$ was detected in both SK-HEP-1 and A549. $BRAF$ was mutated in SK-HEP-1, while $CDKN2A$ and $KRAS$ were mutated in A549 (31,33).

Despite the difference, they shared a same metabolic feature - Warburg effect. They exhibited similar pattern of glycolytic enzymes (Figure 4A) and they converted most incoming glucose to lactate (Figure 4B). If the glucose consumption rate is expressed by kg glucose/kg of cells per day, the number would be larger than 1.5 kg per kg cancer cells per day (Figure 4C). Thus,
Effect of perturbation of PGK1 on the glycolysis

the data demonstrated that these cells shared the feature of Warburg effect. This is consistent with the general consensus that Warburg effect is characteristic of essentially all types of cancer cells (37-39). Mechanistically, Warburg effect is programmed by a complex signaling network comprised of oncogenic activation and tumor suppressor inactivation or insufficiency, including but not limited to Ras, Raf, ERK, JNK, Myc, HIF, P53, PI3K, Akt, etc (38,40-47).

The thermodynamic state of the glycolysis and the pattern of the glycolytic intermediates

For clarity, we list the terms and abbreviations: the specific activity, the enzyme activity assayed at saturating substrate concentration; the relative enzyme concentration, as the absolute concentrations of enzymes in cells could not be determined, we used relative concentration, which is based on the specific activity, e.g., the relative PGK1 concentration of HeLa cells is 1, then the relative concentration of the PGK1-knockdown cell is the PGK1 specific activity in knockdown cells divided by the PGK1 specific activity in control cells. So we term [PGK1] the relative concentration of PGK1. In addition, the abbreviation used are: [Glc], the concentration of glucose; [G6P], the concentration of glucose 6-phosphate; [F6P], the concentration of fructose 6-phosphate; [FBP], the concentration of fructose 1,6-bisphosphate; [DHAP], the concentration of dihydroxyacetone phosphate; [GA3P], the concentration of glyceraldehyde 3-phosphate; [1,3-BPG], the concentration of 1,3-bisphosphoglycerate; [3-PG], the concentration of 3-phosphoglycerate; [2-PG], the concentration of 2-phosphoglycerate; [PEP], the concentration of phosphoenolpyruvate; [Pyr], the concentration of pyruvate.

The transient knockdown specifically reduced PGK1 activity without significantly affecting other glycolytic enzymes (Table 2, Figure 5A & B). The knockdown efficiency varied from cell to cell (Table 2), ranging from 70% (HeLa) to 52% (RKO).

We quantified the cellular concentrations of the glycolytic intermediates including ATP, ADP, NAD, and NADH (Table 1). Using these data, we calculated the mass action ratio (Q) and the actual Gibbs free energy (ΔG) of each reaction along the glycolysis (Table 3). According to Q and ΔG, it is clear that the reactions from the step catalyzed by aldolase to the step catalyzed by enolase were all at near equilibrium state (Figure 5 C-G). The reaction catalyzed by the PGI is also at near equilibrium state. PGK1 knockdown did not significantly affect the thermodynamic state of the glycolysis (Figure 5 C-G). The reactions catalyzed by HK2, PFK1, and PK were far from equilibrium, generating the driving force for glycolysis.

As the reactions catalyzed PGI, and aldolase through enolase were all at near equilibrium state in the steady-state glycolysis, we could reason out a pattern of the glycolytic intermediates, which is characterized by the [F6P]/[G6P], [DHAP]/[GA3P]/[FBP], [GA3P]/[DHAP], [2-PG]/[3-PG], and [PEP]/[2-PG]. This pattern was not significantly affected by the perturbation of PGK1 (Figure 6). In essence, this pattern is determined by the thermodynamic nature of the glycolysis in cancer cells.
The effect of PGK1 knockdown on the glycolytic intermediates

Because the reactions from aldolase to enolase are all at near equilibrium states, in theory, we could infer that when PGK1 amount is reduced, its rate would be temporarily reduced, leading to an accumulation of its substrate, 1,3-BPG. As the reactions catalyzed by aldolase, TPI, and GAPDH are all at near equilibrium, the concentrations of FBP, DHAP, GA3P, and 1,3-BPG would all accumulate in a same or similar proportion according to the thermodynamic state of these reactions. Experimentally, we observed that siRNA knockdown induced an increase of the concentrations of FBP, DHAP, and GA3P (Figure 7A). Nevertheless, we lack method to quantify the amount of 1,3-BPG. siRNA knockdown did not significantly affect the concentrations of other glycolytic intermediates, including ATP, ADP, NAD, NADH (Figure 7B).

Estimating the intracellular [1,3-BPG]

As the reactions from aldolase to enolase were at near equilibrium state, and as the PGK1 knockdown by siRNA did not significantly alter the thermodynamic state of glycolysis, we could estimate 1,3-BPG according to the mass action ratio of GAPDH, where Q is approximately equal to Keq. The estimated [1,3-BPG] was about 1.48 – 2.04 folds higher in PGK1 knockdown cells than in control cells (Figure 7C).

PGK1 knockdown does not significantly affect the glycolysis rate

According to the PGK1 kinetic curve and the \( K_m \) value of PGK1 toward 1,3-BPG (Figure 2C), intracellular [1,3-BPG] is evidently not saturating PGK1 in both control and PGK1 knockdown cells. Theoretically, the increased [1,3-BPG] can significantly increase the catalytic rate of PGK1 and cancel out or at least attenuate the effect of the siRNA-induced loss of PGK1 amount on its catalytic rate as well as on the glycolytic rate. This was validated by the experiments, which demonstrated that lactate generation and glucose consumption by cancer cells were insignificantly or marginally affected by siRNA knockdown (Figure 8A). In addition, the following lines of evidence also support that glycolysis rate in cancer cells is not sensitive to PGK1 knockdown:

The PGK1 specific activities are 3 orders of magnitude higher than the rate-limiting HK (Figure 4A). Even though the actual activities of PGK1 in PGK1 knockdown cells were lower than those in control cells, they were all significantly higher than the cellular glycolysis rate (Figure 8B).

Second, as the reactions from aldolase to enolase were all in a near equilibrium state, the reaction catalyzed by pyruvate kinase, with the large and negative \( \Delta G \) (Table 3), is the force to drive the glycolysis flux to pyruvate. Kinetically, the actual activities of pyruvate kinase in control and PGK1 knockdown cells (Supplementary table 1) were comparable and they were 1 to 2 orders of magnitude higher than the glycolytic rate, sufficiently to drive the upstream intermediates to pyruvate. The results were consistent with our previous study concerning the flux control of glycolysis in cancer cells with respect to the kinetics and thermodynamics of this enzyme (48).

Third, the Q of the reaction catalyzed by LDH in both control and PGK1-knockdown cells (Table 3) were much smaller than the Keq, generating a sufficiently large and
negative ΔG that favors the forward reaction. Kinetically, the actual LDH activities in control and PGK1-knockdown cells were 2 orders of magnitude higher than the glycolytic rate (Supplementary table 1).

Finally, we used $^{13}\text{C}_6$glucose to trace lactate generation and the results showed that glucose-derived lactate (m+3) consisted of about 95% of total lactate in control and PGK1 knockdown cells (Figure 8C). This confirmed that glucose-derived lactate in both control and PGK1-knockdown cells were not significantly different from each other; supporting the notion that glycolysis in cancer cells is not sensitive to perturbation of PGK1.

**PGK1 knockdown moderately reduces serine consumption and de nova serine synthesis**

We used $^{13}\text{C}_6$glc to trace glucose carbon incorporation into serine. The m+0 serine species is provided by the culture medium and the m+3 serine isotopologue was generated from $^{13}\text{C}_6$glucose through 3-PG. Although glucose consumption rate and lactate generation rate were comparable between HeLa-NC and HeLa-siPGK1 (Figure 9A), the consumption rate of m+0 serine was about 20% lower in HeLa-siPGK1 cells than in control cells (Figure 9B). HeLa-siPGK1 showed a higher percentage of extracellular and intracellular m+0 serine than HeLa-NC, whereas the m+3 species was the reverse (Figure 9B). Consistently, glycine consumption rate was lower by HeLa-siPGK1 than by HeLa-NC, so were the percentages of glycine isotopologues (Figure 9C).

The data indicated that PGK1 knockdown moderately reduced serine consumption and serine de nova synthesis by HeLa cells.

**The cell free system**

If the above-proposed mechanism is valid, we should observe it in a different model. Glycolysis in living cells is influenced by many factors, e.g., glycolysis is connected to subsidiary metabolic branches (pentose phosphate pathway, serine synthesis pathway, etc.), glycolytic enzymes are influenced by dynamic regulation, including compartmentation, allosteric regulation, chemical modification (phosphorylation, acetylation, etc.), all of which could influence the rate and the intermediate concentrations. In order to demonstrate the effect of perturbing PGK1 on glycolysis, we used a cell-free glycolysis system as described by us previously (49) to avoid or at least minimize the influences. We used 30-minute incubation for the subsequent experiments concerning the cell-free glycolysis.

We prepared HeLa-siPGK1 cell lysate and titrated the PGK activity in the cell lysate. The results derived from the cell-free glycolysis system were fully agreeable with those from the living cells as described below.

First, we used the PGK1-knockdown cell lysate to measure the flux control coefficient (FCC) values. The FCC, through the unperturbed point (i.e., the relative [PGK1] at 1.0) was nearly zero, based on either the rate of glucose consumption or the rate of lactate generation (Figure 10A).

Second, the [FBP], [DHAP], and [GA3P] were nearly inversely proportional to [PGK1], except the last point ([PGK1] at 1.4), and no significant change of other intermediates (G6P, F6P, 3-PG, 2-PG, PEP, pyruvate) was detected (Figure 10B). The ratios of $\frac{[F6P]}{[G6P]}$, $\frac{[DHAP][GA3P]}/[FBP]$, $\frac{[GA3P][DHAP]}{[2-PG][3-PG]}$, and $\frac{[PEP][2-PG]}{[2-PG]}$ were not significantly affected by the amount of PGK1 (Figure 10C). Accordingly, we could
estimate the concentration of 1,3-BPG (Figure 10D).

Third, the actual PGK1 activities in cell-free system with different PGK1 concentrations were all significantly higher than the glycolysis rate (Figure 10E).

Finally, as the sum of the ΔG of the reaction from aldolase to enolase was between -0.08 and 0.35 kJ/mol (Supplementary table 2), the forward flux depends on pyruvate kinase, which catalyzed a reaction with a ΔG of -26 kJ/mol (Supplementary table 2). Kinetically, its actual activity was much high than the glycolysis rate (Supplementary table 3). For LDH-catalyzed reaction, the ΔG favors the forward reaction (Supplementary table 2), and the actual LDH activities were much higher than the glycolysis rate (Supplementary table 3).

The siRNA knockdown off-target concern and data consistency

In order to exclude the possible off-target effect of siRNA knockdown on the results, we thoroughly checked the data, including (a) that the siRNA specifically knocked down the targeted enzyme, with no significant or marginal effect on other glycolytic enzymes (Table 2), (b) that the effects of the siRNA knockdown on cells were consistent between different cell lines, assessed by the lactate production, glucose consumption, glycolytic intermediates, mass action ratios, delta G (Table 1 & 3, Figure 6, Figure 7A, Figure 8A), and (c) that the effects (lactate production, glucose consumption, FCC, glycolytic intermediate pattern) of the targeted enzyme modulated by siRNA in living cells and cell-free models are consistent with each other (Figure 5-8, 10). Moreover, the data are quantitatively interrelated and consistent with the biochemical principle. In addition, we used siRNA with different sequence to repeat the experiments and obtained the consistent results (Supplementary figures 1-2, Supplementary table 4).

The glycolysis under different conditions

For these experiments, we had to confirm the quality control, i.e., the siRNA only knockdown PGK1 without significantly affecting other glycolytic enzymes. The quality controls were summarized in Supplementary figure 3A. siRNA specifically knockdown PGK1 by ~70% in HeLa cells and ~45% in RKO after 48 hours transfection. When the transfected cells were trypsinized and seeded to plates, knockdown efficiency kept almost the same (Supplementary figure 3B).

We measured glycolysis rate (glucose consumption and lactate generation) under several conditions, including different glucose concentrations, different glutamine concentrations, and different cell density. In all the above condition, we did not observe a significant difference of glucose consumption and lactate generation rate between PGK1-knockdown and control cells (Figures 11-13, and Supplementary figures 4-6).

PGK1 knockdown and cell growth

PGK1 knockdown increased HeLa cell percentage at G0&G1 phases from ~53% to ~65%, and correspondingly reduced the cell percentage at S and G2&M phases (Figure 14A&B). Consistently, PGK1 knockdown moderately reduced cell growth rate (Figure 14C). On the other hand, glucose consumption and lactate generation were not significantly different between the 2 groups of cells. However, if taking the cell number into consideration, the rate of glycolysis in cells with PGK1 knockdown
would be higher than that in control cells. (Figure 14D).

RKO cells were different from HeLa cells (Supplementary figure 7). PGK1 knockdown did not inhibit RKO cell growth and lactate generation, but induced about 20% reduction of glucose consumption.

We do not know why PGK1 knockdown exerted different effect on different cells. Presumably, the different effect is related to the different genetic background and tissue origin.

Discussion

In summary, the study investigated 3 issues. First, we developed a coupled-enzyme assay for measuring the activity of PGK1 with respect to its forward reaction and determined the forward-reaction kinetic parameters. Second, the kinetic parameters of PGK1 are potentially important for glucose carbon to shuttle to serine synthesis. Third, integration of the thermodynamic states, the concentrations of the glycolytic intermediates, and the enzyme kinetics in the system of glycolysis could well interpret the effect of the perturbation of PGK1 on the glycolysis in cancer cells.

The flux control of aerobic glycolysis is fundamentally a question of kinetics and thermodynamics of the glycolysis as a system. Regarding kinetics, we observed that the [1,3-BPG] is inversely proportional to [PGK1] when glycolysis is at steady state. According to the principle of the enzyme-substrate kinetics, the reasonable interpretation is that PGK1 activity in the glycolysis is flexible and this flexibility renders PGK1 insensitive to perturbation. Regarding thermodynamics, we observed that the ΔGs of the reactions along the glycolysis remained unchanged in response to perturbation of PGK1, reflecting the rigid thermodynamics of the glycolysis. ΔGs controls the Q values of the reactions along the glycolysis, hence determine the concentration of the glycolytic intermediates, including 1,3-BPG. Therefore, the activity of PGK1 in the glycolysis is also controlled by the overall thermodynamic state of the glycolysis. Collectively, the effect of perturbation of PGK1 on glycolysis is related to not only PGK1 itself but also the thermodynamic state of the glycolysis.

Inhibiting aerobic glycolysis is a strategy for treating cancer(5). The way to inhibit aerobic glycolysis is to define the rate-limiting enzymes and target them. The way to define the rate-limiting enzyme is to examine if inhibition of an enzyme is correlated with an inhibition of overall glycolytic rate or not. This approach is depending on ‘proof of concept’, but its drawback is also obvious, because it does not answer why the enzyme is rate-limiting. This is probably a major reason why there are many mixed reports of the enzymes including PFK1(10,50,51), GAPDH (8-12,27), PGK1(13-15,27), PGAM (27,52), PKM2 (16-19), LDH(27,53,54), etc. . Revealing the kinetic and thermodynamic aspect concerning the effect of perturbing PGK1 may help to resolve the issue.

PGK1 knockdown may increase the rate to the subsidiary branches of the glycolysis, e.g., to methylglyoxal (MG). MG is a metabolite generated from non-enzymatic degradation of DHAP and GA3P (55). MG reacts with arginine, lysine and cysteine residues in proteins, forming advanced glycation end products (AGEs) (56). PGK1 knockdown induced an increase of concentrations of DHAP and GA3P, hence
it could enhance MG generation. Bollong et al reported that PGK1 inhibitor CBR-407-1 induced an increase of concentrations of DHAP and GA3P and enhanced generation of MG, which covalently modified KEAP1, leading to a reduced ubiquitination and the accumulation of NRF2 (57). Similarly, as GAPDH knockdown could significantly increase the steady-state concentrations of DHAP and GA3P, GAPDH inhibition may also enhance MG generation and its downstream signaling.

It is important to dissect the effects of PGK1 knockdown on glycolysis and on cell growth. PGK1 knockdown induced a moderate growth inhibition of HeLa cells, but it was irrelevant with glycolysis. Presumably, the growth inhibition induced by PGK1 knockdown might be associated with its nonglycolytic activities. Li, et al (58) reported that EGFR- and ERK-activated casein kinase 2α (CK2α) phosphorylated nuclear PGK1. Phosphorylated PGK1 bound with CDC7, recruited DNA helicase to replication origins, and promoted DNA replication and cell growth. Similarly, other glycolytic enzymes have nonglycolytic functions, which have multiple biological roles, as reviewed by Lu and Wang (59).

There are 2 isotypes of PGK, PGK1 and PGK2. PGK1 is ubiquitously expressed in all cells, while PGK2 is expressed in spermatogenic cells (60). However, a few cancers displayed moderate to strong nuclear and cytoplasmic PGK2 staining, including renal cancer, breast cancer, pancreatic cancer, ovarian cancer, testis cancer (http://proteinatlas.org/ENSG00000170950-PGK2/pathology), indicating that cancer cells could express both PGK1 and PGK2. If so, when PGK1 is perturbed, PGK2 might compensate for the perturbed levels of PGK1.

Materials and Methods

Cell lines
Human gastric cancer cell line MGC80-3, cervical cancer cell line HeLa, liver cancer cell line SK-HEP-1, colon cancer cell line RKO, and lung cancer cell line A549 were obtained from Cell Bank of Type Culture Collection of the Chinese Academy of Science (Shanghai, China) and were cultured in RPMI-1640 medium with 10% FBS. Cells were maintained in a humidified incubator at 37°C with 5% CO2.

Reagents and enzymes
Reagents were from Sigma, including ATP (#A3377), ADP (#A5285), NAD (#N0632), NADH (#N8129), NADP (#N8035), NADPH (#N7505), glucose (#G8270), G6P, (#G7879), F6P (#V900924), GA3P (#G5251), 3-PG (#P8877), 2-PG (#19710), PEP (#P7001), Pyr (#V900232), lactic acid (#L1750), HK (#H4502), PGI (#P5381), PFK (#F0137), Aldolase (#A8811), TPI (#T6258), GAPDH (#G2267), PGK (#P7634), enolase (#E6126), PK (#P7768), LDH (#L2500), G6PDH (#G8404), α-GPDH (#G6751). FBP were purchased from aladdin (China, #F111301).

Knockdown of PGK by siRNA
1.5 × 10⁵ cells were seeded into each well of 6-well plates and cultured overnight. Cells were transfected using Lipofectamine 3000 (Thermo Fisher Scientific) according to manufacturer’s protocol, with either negative control siRNA (NC) or siPGK1 (Ribobio, China). The siRNA sequences were as follows: siPGK1, sense, GCAUCAAAUUCUGCUUGGA dTdT;
Effect of perturbation of PGK1 on the glycolysis

antisense, UCCAAGCAGAAUUUGAUGC dTdT; siPGK1-1, sense,
GAGTCAATCTGCCACAGAA dTdT; antisense, UUCUGUGGCAGAUUGACUC
dT dT; NC, sense,
UUCUCCGAACGUGUCACGU dTdT; antisense, ACGUGACACGUUCGGAGAA
dTdT. 48 hours after transfection, cells were
washed with PBS and 2 ml fresh complete
RMPI-1640 plus 8 mM glucose were added
to each well. Then we collected 10 μL
media at 1, 2, 3, 4, 5, 6 hours and
determined glucose & lactate afterwards.
The cells were counted and collected for
enzyme activity assay, western blot, or
intracellular intermediates determination.

Western blot
Cells were washed with cold PBS, then
lysed with M-PER™ Mammalian Protein
Extraction Reagent (Thermo Fisher
Scientific) supplemented with cocktail
(MedChemExpress) on ice for 30 minutes.
Protein concentration was determined using
BCA protein assay kit (Thermo Fisher
Scientific). The protein was boiled for 5
minutes with loading buffer and 20 μg was
subjected to 12% SDS-PAGE, transferred to
PVDF membrane, and incubated with
primary body PGK1 (proteintech, #17811-1-
AP). GAPDH (proteintech, #60004-1-lg)
was used as internal control.

Glucose and lactate determination
We determined glucose and lactate
according to the methods described
previously with some modification (61).
Briefly, 10 μL collected media were diluted
5 times by adding 40 μL water, and then we
added 10 μL mixture or standard solution of
Glucose (Glc) / Lactate (LA) to 96-well
plate, together with 190 μL reaction buffer.
Cell-free system samples were added with
190 μL reaction buffer directly without
dilution. The reaction buffer contained 200
mM HEPES (pH 7.4), 100 mM KCl, 5 mM
Na2HPO4, 5 mM MgCl2, 0.5 mM EDTA, 2
mM ATP, 0.2 mM NADP, 0.2 U/ml HK2
and 0.2 U/ml G6PDH for glucose
determination or contained 200 mM glycine,
170 mM hydrazine (pH 9.2), 2 mM NAD,
and 5 U/ml LDH for lactate determination.
340 nm absorbance was recorded using
SpectraMax i3 (Molecular Devices) after 60
minutes reaction, and glucose or lactate
concentration was calculated according to
standard curve.

Glycolytic enzyme activity assay
For determination of PGK1 activity in
forward reaction, 1mM GA3P and 1mM
NAD was added to a cuvette, mixed well
with 1ml reaction buffer (200 mM HEPES,
100 mM KCl, 5mM Na2HPO4, 0.5 mM
EDTA and 5 mM MgCl2, pH 7.4)and record
the absorbance at 340 nm. Then 1 U/ml
GAPDH was added, wait until absorbance
at 340 nm reached a plateau, add 2mM ADP
and 1 μg protein of lysate to start the
reaction. The initial slope value was used to
calculate the enzyme activity.
We determined other enzyme activity at
saturating substrate concentration according
to previously reported methods (62) with
some modification. Briefly, 1 ml reaction
buffer was added to the cuvette, and
substrates were added as below. The
reaction was started by adding cell lysate
and mixed, and then absorbance at 340 nm
was recorded using a spectrophotometer
(DU® 700, Beckman Coulter).
HK: 0.2 mM NADP, 2 mM ATP, 10 mM
glucose, 1 U/ml G6PDH, 30 μg protein of
lysate;
PGI: 2 mM F6P, 0.2 mM NADP, 1 U/ml
G6PDH, 5 μg protein of lysate;
PFK: 0.1 mM ADP, 2 mM ATP, 2 mM F6P, 1 U/ml Aldolase, 0.1 mM NADH, 1 U/ml α-GPDH, 10 μg protein of lysate;
Aldolase: 0.1 mM NADH, 1.5 mM FBP, 1 U/ml α-GPDH, 15 μg protein of lysate;
TPI: 2 mM GA3P, 0.1 mM NADH, 1 U/ml α-GPDH, 1 μg protein of lysate;
GAPDH: 2 mM NAD, 2 mM GA3P, 4 μg protein of lysate;
PGK in reverse reaction: 2 mM ATP, 2 mM 3PG, 0.1 mM NADH, 1 U/ml GAPDH, 5 μg protein of lysate;
PGAM: 2 mM ADP, 1 mM 3PG, 1 U/ml Enolase, 0.1 mM NADH, 1 U/ml PK, 1 U/ml LDH, 10 μg protein of lysate;
Enolase: 2 mM ADP, 1 mM 2-PG, 1 U/ml PK, 0.1 mM NADH, 1 U/ml LDH, 10 μg protein of lysate;
PK: 2 mM ADP, 2 mM PEP, 0.1 mM NADH, 5 U/ml LDH, 2 μg protein of lysate;
LDH: 0.1 mM NADH, 2 mM pyruvate, 2 μg protein of lysate.

**Determination of glycolytic intermediates in cell free system**

We used previously reported methods to determine the glycolytic intermediates (62). The reaction buffer in this part contained 200 mM HEPES, 100 mM KCl, 5 mM Na2HPO4, 0.5 mM EDTA and 5 mM MgCl2, with pH adjusted to 7.4.

G6P and F6P: 100μl of supernatant and 0.2 mM NADP were added to 900 μl reaction buffer, and the reaction was started by adding 1 U/ml G6PDH. The first reaction to measure G6P ended when 340 nm absorbance reached a plateau, then 1 U/ml PGI was added to measure F6P.

FBP, DHAP and GA3P: 100 μl of supernatant and 0.1 mM NADH were added to 900 μl reaction buffer, the reaction to measure DHAP was started by adding 1 U/ml TPI was added to measure GA3P. Finally, 1 U/ml Aldolase was added to measure FBP.

3PG: 50 μl of supernatant, 2 mM ATP, 0.1 mM NADH, 1 U/ml PGK were added to 950 μl reaction buffer, and the reaction was started by adding 1 U/ml GAPDH.

2-PG, PEP and Pyr: 100 μl of supernatant, 0.1 mM NADH were added to 900 μl reaction buffer, the reaction to measure Pyr was started by adding 1 U/ml LDH. When the first reaction ended, 2 mM ADP and 1 U/ml PK were added to measure PEP. Finally, 1 U/ml Enolase was added to measure 2-PG.

**Determination of intracellular glycolytic intermediates**

Negative control or siPGK1 transfected cells were washed by ice cold PBS twice, and 600 μl 1 M pre-cold HClO4 was added to every 3 wells of 6-well plate. Cells were collected by a scraper, incubated on ice for 30 min, and neutralized by 100 μl 3 M
K$_2$CO$_3$. Then supernatant was obtained by 10,000 g centrifuge at 4°C. Then 50 μl 2 M NaOH was added to the supernatant and kept at 60°C for 5 minutes, and neutralized again by adding 50 μl 2 M HCl. These two steps eliminated intracellular NAD(H) / NADP(H) possibly. Meanwhile, a same fourth well of cells was trypsinized and collected to determine the cell number and cell size by a cell counter (JIMBIO). Through the following reactions, intermediates plus NADP (for G6P, F6P and Glucose) or NADH (for FBP-Pyr) were converted to products and NADPH or NAD, termination the reaction by NaOH or HCl will conserve the NADPH or NAD, which could be measured by cycling methods (62,63). For all the reactions, 10 μl supernatant was mixed with 40 μl reaction buffer, different reactions differs in reaction buffer:

- G6P: 200 mM HEPES, 100 mM KCl, 5 mM Na$_2$HPO$_4$, 0.5 mM EDTA, 5 mM MgCl$_2$, pH 7.4, 2 mM ATP, 0.5 mM NADP, 0.5 U/ml G6PDH;
- F6P: 200 mM HEPES, 100 mM KCl, 5 mM Na$_2$HPO$_4$, 0.5 mM EDTA, 5 mM MgCl$_2$, pH 7.4, 2 mM ATP, 0.5 mM NADP, 0.5 U/ml G6PDH, plus 0.5 U/ml PGI;
- Glucose: 200 mM HEPES, 100 mM KCl, 5 mM Na$_2$HPO$_4$, 0.5 mM EDTA, 5 mM MgCl$_2$, pH 7.4, 2 mM ATP, 0.5 mM NADP, 0.5 U/ml G6PDH, plus 0.5 U/ml HK;
- DHAP: 200 mM HEPES, 100 mM KCl, 5 mM Na$_2$HPO$_4$, 0.5 mM EDTA, 5 mM MgCl$_2$, pH 7.4, 0.5 mM NADH, 0.5 U/ml α-GPDH;
- GA3P: 200 mM HEPES, 100 mM KCl, 5 mM Na$_2$HPO$_4$, 0.5 mM EDTA, 5 mM MgCl$_2$, pH 7.4, 0.5 mM NADH, 0.5 U/ml α-GPDH, plus 0.5 U/ml TPI;
- FBP: 200 mM HEPES, 100 mM KCl, 5 mM Na$_2$HPO$_4$, 0.5 mM EDTA, 5 mM MgCl$_2$, pH 7.4, 0.5 mM NADH, 0.5 U/ml α-GPDH, 0.5 U/ml TPI, plus 0.5 U/ml Aldolase;
- 3PG: 200 mM HEPES, 100 mM KCl, 0.5 mM EDTA, 5 mM MgCl$_2$, pH 7.4, 0.5 mM NADH, 1 mM ATP, 0.5 U/ml PGK, plus 0.5 U/ml GAPDH;
- Pyr: 200 mM HEPES, 100 mM KCl, 5 mM Na$_2$HPO$_4$, 0.5 mM EDTA, 5 mM MgCl$_2$, pH 7.4, 0.5 mM NADH, 1 mM ADP, 0.5 U/ml LDH;
- PEP: 200 mM HEPES, 100 mM KCl, 5 mM Na$_2$HPO$_4$, 0.5 mM EDTA, 5 mM MgCl$_2$, pH 7.4, 0.5 mM NADH, 1 mM ADP, 0.5 U/ml LDH, plus 0.5 U/ml PK;
- 2-PG: 200 mM HEPES, 100 mM KCl, 5 mM Na$_2$HPO$_4$, 0.5 mM EDTA, 5 mM MgCl$_2$, pH 7.4, 0.5 mM NADH, 1 mM ADP, 0.5 U/ml LDH, 0.5 U/ml PK, plus 0.5 U/ml Enolase.

For G6P, F6P and Glucose determination, after 30 min incubation at 37°C, reaction was terminated by adding 10 μl 2 M NaOH, mixed well and kept at 60°C for 5 min to eliminate NADP, then the mixture was neutralized by adding 20 μl 1 M HCl. For the rest of the intermediates, after 30 minutes incubation at 37°C, reaction was terminated by adding 20 μl 1 M HCl, kept at 60°C for 5 min to eliminate NADH, then the mixture was neutralized by adding 10 μl 2 M NaOH. After that, 70 μl neutralized mixture or standard solution was added to 96-well plate together with 100 μl develop buffer (0.4 M Tris·HCl, 0.2 mM G6P, 1 U/ml G6PDH, 0.1 mM MTS, 0.1 mM PES, pH 7.8) to cycling NADPH (for G6P, F6P and glucose). For the rest of the intermediates, the develop buffer contained 0.4 M Tris·HCl, pH 7.8, 5 M ethanol, 2 U/ml Alcohol dehydrogenase, 0.1 mM MTS, 0.1 mM PES. After 30 min incubation with develop buffer at 37°C, the 490 nm absorbance of 96-well plates were recorded and analyzed.
Determination of isotopic serine and glycine by LC-MS/MS

Forty eight hours after NC or siHK2 transfection, cells were washed with PBS twice, and cultured with glucose-free RPMI 1640 medium supplemented with 10% FBS and 7mM [13C6]glucose for 3, 6 and 9 hours. Medium at 0, 3, 6, 9 hours were collected for LC-MS/MS measurement. Meanwhile, cells at 0, 3, 6, 9 hours were washed with PBS three times, and intracellular amino acid were extracted by adding 80% pre-cold methanol. Cells were collected by a scraper and 20,000 g centrifuge at 4°C was performed and the supernatant was evaporated by a vacuum centrifugal concentrator and was dissolved in 100 μl water. 10 μl dissolved metabolites or collected medium or standard was mixed with 20 μl AQC (#ab145409, abcam, amino acid derivatization agent) and 120 μl borate buffer, incubate in 60 °C for 20 min and the AQC derivatized sample was used for LC-MS/MS by a Waters Acquity UPLC system coupled to Qtrap 4000 mass spectrometer with ESI probe (Applied Biosystems Inc., Foster City, CA, USA). An AccQ-Tag Ultra RP Column was used to perform the Liquid Chromatography. The mobile phase A was 25 mM ammonia formate with 1% acetonitrile, pH 3.05 and mobile phase B was 100% acetonitrile. The gradient program was as follows: 0-0.54 min, 99.9% A, 0.1% B; 0.54-5.74 min, 99.9% A-90.9% A; 5.74-7.74 min, 90.9% A- 78.8% A; 7.74-8.04 min, 78.8% A-40.4% A; 8.04-8.64 min, 40.4% A, 8.64-8.73 min, 40.4% A-99.9% A; 8.73-9.5 min, 99.9% A. 1 μl sample or standard solution was injected to perform the analysis with a flow rate at 0.7 ml/min. During the performance, the column was kept at 55°C. The following parameters were optimized and used for MASS analysis: 40 psi curtain gas, medium collision gas, 5500V ionspray voltage, temperature of the ion source 500°C, 40 psi ion source gas1 and 40 psi ion source gas2.

Cell cycle assay

Cell cycle assay was performed using a Cell cycle staining Kit (# 70-CCS012, MultiSciences, China) according to manufacturer’s protocol. Briefly, HeLa or RKO cells were transfected with NC or siPGK1 for 48 hours, trypsinized and seeded to a new 6-well plate overnight, then collected and subjected to Flow cytometer analysis.

Determination of ATP, ADP, NAD & NADH in cell

Cells in 6-well plates were washed with ice-cold PBS twice, and 0.6 ml 80% (vol/vol) pre-cold (-20°C) methanol was added per well to extract the intracellular metabolites. Then a scraper was used to collect the cells and the cell debris was discarded by 20,000 g centrifuge at 4°C. The supernatant was evaporated by a Vacuum centrifugal concentrator and was dissolved in 100μl water for following UPLC analysis. Waters ACQUITY UPLC system with an ACQUITY UPLC HSS T3 column was used to perform the Liquid Chromatography. The mobile phase A was 20 mM Triethylamine in 99%/1% water/acetonitrile (pH 6.5) and mobile phase B was 100% acetonitrile. The gradient program was as follows: 0-3 min, 100% A; 3-4 min, 100% A-98.5% A; 4-7 min, 98.5% A- 92% A; 7-7.1 min, 92% A-100% A; 7.1-10 min, 100% A. 10 μl sample or standard solution was injected to perform the analysis with a flow rate at 0.3 ml/min. During the performance, the column was kept at 40°C.

Estimation of 1,3-BPG
Assuming at steady state, the reaction calculated by GAPDH was at equilibrium. So we can estimate the concentration by

\[ [1,3\text{-BPG}]_{\text{eq}} = \frac{[\text{GA3P}][\text{NAD}][\text{Pi}]}{[\text{NADH}]} \]

Where \( K_{eq} \) is the equilibrium constant of GAPDH, \([\text{NAD}]/[\text{NADH}]\) was set 78(48), \([\text{Pi}]\) in cell was set 1.5 mM(64).

Analysis of isotopic lactate by LC-MS/MS
Isotopic lactate tracing is based on our previously reported method (54). \([^{13}\text{C}_6]\text{Glucose} \) was purchased from Sigma. 48 hours after transfection, HeLa-NC, HeLa-siPGK1 were washed with PBS twice, and cultured in glucose-free RPMI-1640 supplemented with 10% ultrafiltrated FBS and 8 mM \([^{13}\text{C}_6]\)glucose for 6 hours. Then culture medium was collected and diluted 40 times with 100% acetonitrile and centrifuged at 25,000g for 10min at 4°C. Supernatant was collected for LS-MS/MS analysis according to methods reported previously by us (54,65). Briefly, an ACQUITY BEH Amide column was used to perform liquid chromatography, kept at 50°C during analysis and the injection volume was 7.5 μl. Mobile phase A was 10 mM ammonium acetate in 85% acetonitrile, 15% water, pH 9.0, and mobile phase B was 10 mM ammonium acetate in 50% acetonitrile, 50% water, pH 9.0. The gradient program was as follows: 0–0.4 min, 100% A; 0.4–2 min, 100–30% A; 2–2.5 min, 30–15% A; 2.5–3 min, 15% A; 3–3.1 min, 15–100% A; 3.1–7.5 min, 100% A. A 4000 QTRAP mass spectrometer (AB SCIEX) equipped with an ESI ion source (Turbospray) operated in negative ion mode using GraphPad 7. Then a tangent was obtained at point of enzymes at 1.0. FCC was calculated by the following equation (73)

\[ \text{FCC}=\text{slope} \times \frac{E}{J} \]

Where the slope stands for the tangent \( \frac{\Delta J}{\Delta E} \) obtained at point of enzymes at 1.0, \( E \) stands for the enzyme concentration, which is 1.0.

Calculation of the Gibbs free energy change \( \Delta G \) of glycolytic reactions
\[ \Delta G=\Delta G'_{310} + \frac{R}{T}\ln Q \]

where \( \Delta G'_{310} \) is the standard transformed Gibbs free energy at 37°C and Q was calculated according to intermediate concentrations and was listed in Tables. For intracellular metabolites calculations, NAD/NADH was set as 78 according to our previously reported study (48). Take HK of HeLa-NC for example. Q of HK-catalyzed reaction in cell equals to \([\text{G6P}][\text{ADP}]/[\text{ATP}][\text{Glc}]\), which was 0.019, however, \( \Delta G'_{310} \) is not available. According to \( \Delta G=\Delta H-T\Delta S \), since the change of \( \Delta H \) and \( \Delta S \) is negligible between 37°C and 25°C (66,67), we deduced the equation to new form that

\[ \Delta G'_{310} = \Delta G'_{298} + (1-30^\circ_{298})\Delta H_{298}^\circ \]

\( \Delta G'_{298} \) and \( \Delta H_{298}^\circ \) are available in references (68-72). So

\[ \Delta G = \Delta G'_{310} = \Delta G'_{298} + (1-30^\circ_{298})\Delta H_{298}^\circ + \frac{R}{T}\ln Q = -16.7 \times 0.31 \times \ln(0.019) = -2.26 \text{ kJ/mol}. \]
and J stands for the Lactate generated or Glucose consumed at enzyme 1.0

Statistical analysis

All experiments were repeated at least 2 times and all data were analyzed using GraphPad Prism 7.

Data availability

All data are contained within the manuscript and supporting information.

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Author Contributions

XH conceived the conception, designed the PGK1 enzyme assay and the study, interpreted the data, and wrote the paper. CJ, XZ, HW & YW conducted the experiments.

Declaration of Interests

The authors declare no competing interests.

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### Tables

**Table 1.** Intracellular Glc and glycolytic intermediate concentration (mM) in cells with or without PGK1 knockdown. Data are mean ± SD, n=3. The results were repeated by 2 independent experiments.

| Metabolites (mM) | Glc | G6P | F6P | FBP | DHAP | GA3P | 3PG | 2PG | PEP | Pyr | ADP | ATP | NAD | NADH |
|------------------|-----|-----|-----|-----|------|------|-----|-----|-----|-----|-----|-----|-----|-----|
| SK-HEP-1 | 3.56± | 0.56± | 0.12± | 0.38± | 0.75± | 0.05± | 0.28± | 0.09± | 0.10± | 0.20± | 0.71± | ± | 0.68± | 0.33± |
| NC | 0.037 | 0.07 | 0.08 | 0.035 | 0.04 | 0.02 | 0.04 | 0.03 | 0.014 | 0.03 | 0.12 | 0.58 | 0.11 | 0.005 |
| siPGK1 | 5.52± | 0.57± | 0.14± | 0.65± | 1.28± | 0.09± | 0.28± | 0.07± | 0.11± | 0.14± | 0.61± | ± | 0.66± | 0.035± |
| MGC80-3 | 3.9± | 0.43± | 0.12± | 0.28± | 0.58± | 0.05± | 0.18± | 0.10± | 0.15± | 0.27± | 0.59± | ± | 0.58± | 0.039± |
| NC | 0.07 | 0.04 | 0.03 | 0.01 | 0.05 | 0.02 | 0.05 | 0.066 | 0.004 | 0.02 | 0.014 | 0.21 | 0.04 | 0.004 |
| MGC80-3 | 3.96± | 0.44± | 0.11± | 0.55± | 1.17± | 0.096± | 0.14± | 0.078± | 0.15± | 0.25± | 0.83± | 4.54 | 0.78± | 0.048± |
| siPGK1 | 0.11 | 0.05 | 0.02 | 0.014*** | 0.01*** | 0.04* | 0.07 | 0.04 | 0.001 | 0.02* | 0.062 | ±0.8 | ±0.12 | ±0.005 |
| RKO-NC | 5.2± | 0.31± | 0.11± | 0.37± | 0.47± | 0.043± | 0.55± | 0.066± | 0.06± | 0.56± | 6.7± | 11.3± | 1.45± | 0.16± |
| RKO- | 0.47 | 0.015 | 0.011 | 0.034 | 0.04 | 0.003 | 0.07 | 0.028 | 0.03 | 0.04 | 0.19 | 0.54 | 0.03 | 0.01 |
| siPGK1 | 5.2± | 0.29± | 0.13± | 0.50± | 0.77± | 0.073± | 0.43± | 0.085± | 0.049± | 0.48± | 6.9± | 11.5± | 1.45± | 0.16± |
| SK-HEP-1 | 3.88± | 0.29± | 0.078± | 0.24± | 0.55± | 0.045± | 0.49± | 0.13± | 0.23± | 0.51± | 0.77± | 9.3± | 0.93± | 0.025± |
| NC | 0.24 | 0.028 | 0.022 | 0.037 | 0.047 | 0.006 | 0.09 | 0.045 | 0.064 | 0.096 | 0.05 | 0.07 | 0.001 | 0.003 |
| SK-HEP-1 | 3.4± | 0.35± | 0.11± | 0.39± | 0.8± | 0.066± | 0.52± | 0.13± | 0.29± | 0.51± | 0.77± | 9.9± | 0.72± | 0.029± |
| siPGK1 | 0.18 | 0.05 | 0.032 | 0.035** | 0.045** | 0.009* | 0.11 | 0.054 | 0.022 | 0.10 | 0.05 | 0.42 | 0.55 | 0.003 |
| A549-NC | 4.2± | 0.21± | 0.038± | 0.14± | 0.28± | 0.021± | 0.22± | 0.071± | 0.084± | 0.64± | 0.84± | ± | 1.78± | ±0.28± |
| A549- | 0.04 | 0.04 | 0.003 | 0.03 | 0.03 | 0.002 | 0.044 | 0.03 | 0.04 | 0.066 | 0.057 | 0.25 | 0.033 | 0.009 |
| siPGK1 | 3.7± | 0.177 | 0.05± | 0.20± | 0.54± | 0.042± | 0.21± | 0.069± | 0.085± | 0.625± | 0.9± | 11.1± | 1.78± | 0.28± |
| * | p<0.05; ** | p<0.01; *** | p<0.001. siPGK1 transfected cells versus NC transfected cells. |
Table 2. Enzyme activity (nmol/min/mg protein) in cells with or without PGK1 knockdown. Data are mean ± SD, n=3. The results were repeated by 2 independent experiments.

|                | HK     | PGI    | PFK    | Aldolase | TPI    | GAPDH  | PGK    | PGAM   | enolase | PK    | LDH    |
|----------------|--------|--------|--------|----------|--------|--------|--------|--------|---------|-------|--------|
| HeLa-NC        | 77.4±  | 3552.5±| 390.7± | 244.6±   | 1963.8±| 3431.5±| 14789.9±| 1963.8±| 1741.2± | 3752.1±| 7036.9±|
| HeLa-siPGK1    | 79.0±  | 3562.2±| 395.2± | 259.5±   | 2072.1±| 3215.4±| 4476.4±| 1866.2±| 1714.9± | 3521.9±| 7220.3±|
|                | 2.86±  | 31.5±  | 22.2±  | 17.9±    | 442.8± | 126.1± | 227.3± |        |         |       |        |
| MGC80-3-NC     | 82.5±  | 2450.9±| 388.9± | 239.7±   | 2016.3±| 4952.7±| 1929.7±| 2282.6±| 774.6±  | 3477.9±| 7613.1±|
| MGC80-3-siPGK1 | 81.9±  | 2308.9±| 409.8± | 249.4±   | 1969.7±| 5543.6±| 2194.5±| 741.1±  |         |       |        |
| RKO-NC         | 84.6±  | 2659.9±| 231.3± | 358.6±   | 2921.6±| 2416.4±| 1026.3±| 935.4±  | 600.2±  | 3741.6±| 4521.1±|
| RKO-siPGK1     | 81.3±  | 2650.3±| 223.5± | 335.2±   | 2888.2±| 2400.9±| 4396.8±| 873.0±  |         |       |        |
| SK-HEP-1-NC    | 88.3±  | 1665.4±| 336.6± | 296.8±   | 2084.2±| 3611.2±| 10360.8±| 956.4±  | 766.7±  | 4188.3±| 5062.2±|
| SK-HEP-1-siPGK1| 84.8±  | 1632.4±| 333.9± | 288.6±   | 1947.3±| 3516.5±| 4562.1±| 906.9±  | 709.0±  | 4138.8±| 4979.8±|
| A549-NC        | 56.6±  | 4523.0±| 196.5± | 300.1±   | 1830.6±| 2508.0±| 1264.7±| 664.5±  | 6388.0± | 5894.9±| 28.6   |
| A549-siPGK1    | 53.2±  | 4242.1±| 206.8± | 267.0±   | 1886.9±| 2594.9±| 3779.5±| 1241.0±| 654.4±  | 6498.6±| 5618.5±|

***, p<0.001. siPGK1 transfected cells versus NC transfected cells.
Table 3. ΔG of glycolytic enzymes in cells with or without PGK1 knockdown calculated according to data in Table 1.

| enzyme          | HK   | PGI  | PFK  | Aldolase | TPI   | GAPDH & PGK | PGAM | Enolase | PKM | LDH       |
|-----------------|------|------|------|----------|-------|-------------|------|----------|-----|-----------|
|                  | Keq  | ΔG0(kJ/mol) | Q    | ΔG(kJ/mol) | Q    | ΔG(kJ/mol) | Q    | ΔG(kJ/mol) |     |           |
| HeLa-NC         | 850  | -16.7 | 0.5  | 0.59     | 0.000100 | 0.05 | 158.00 | 0.48 | 363000.00 | 26300.00 | -25.10 |
| HeLa-siPGK1     | 0.019| -26.66| -15.75 | -0.94 | 0.73 | 1.28 | 2.70 | 1.50 | -26.33 | -6.10 |
| MGC80-3-NC      | 0.020| -26.45| -14.62 | 0.60 | 0.91 | -0.38 | 2.08 | 2.25 | -27.64 | -4.87 |
| MGC80-3-siPGK1  | 0.017| -26.86| -16.97 | -0.90 | 1.25 | 0.57 | 4.22 | 2.22 | -26.17 | -6.99 |
| RKO-NC          | 0.035| -25.05| 1.98  | 0.000055 | 0.09 | 185.85 | 0.12 | 0.91 | 15.87 | 348.21 |
| RKO-siPGK1      | 0.033| -25.22| -12.92 | -0.64 | 1.62 | -1.55 | 4.21 | 2.78 | -27.21 | -6.12 |
| SK-HEP-1-NC     | 0.006| -29.52| -17.92 | -0.86 | 1.24 | 5.09 | 2.29 | 2.63 | -23.97 | -8.30 |
| SK-HEP-1-siPGK1| 0.008| -28.84| -17.70 | -0.16 | 1.26 | 4.40 | 2.04 | 3.23 | -24.42 | -8.51 |
| A549-NC         | 0.004| -30.71| -17.61 | -3.18 | 1.02 | 5.13 | 2.79 | 1.59 | -20.65 | -9.59 |
| A549-siPGK1     | 0.004| -30.73| -17.30 | -0.62 | 1.11 | 3.15 | 2.84 | 1.70 | -20.82 | -9.67 |
Figure 1. The rational to measure the forward reaction rate of PGK1. In a reaction mixture containing GA3P, and NAD, Pi, add excessive amount of GAPDH, bringing the reaction rapidly to equilibrium state, which is monitored at A$_{340}$. Then add ADP and PGK1 into the reaction mixture to initiate PGK1 catalyzed forward reaction and record the initial rate (see also the text).
Effect of perturbation of PGK1 on the glycolysis

**Figure 2. Measurement of the kinetic parameters of the forward reaction catalyzed by human and yeast PGK1.** (A) Set the initial concentration of GA3P and NAD in the reaction mixture, add 1U/ml GAPDH. When the reaction reaches equilibrium, the concentration of 1,3-BPG in the mixture can be readily calculated. (B) Add 2 mM ADP and 0.0025U recombinant human PGK1 or yeast PGK1 to initiate reactions with a serial concentrations of 1,3-BPG. (C) The kinetic curves of PGK1 versus 1,3-BPG. (D) The K$_m$ and V$_{max}$ of PGK1 of the forward and reverse reactions. Data are mean ± SD, n=3. All the results were repeated by 3 independent experiments.
Figure 3. The effect of HK2 knockdown on glycolysis and serine synthesis in Hela cells.
(A) The proportional relationship between HK2 activity and glycolysis rate (lactate generation and glucose consumption). (B) 3-PG concentrations in HeLa-NC and HeLa-siPGK1 cells. (C and D) Glucose carbon shuttle to serine. Cells were incubated in the presence of 6 mM [13C6]glucose. Cells and medium were collected at 3, 6, and 9 hours and serine and glycine isotopologues were analyzed by LC-MS. (C) Left panel, the consumption of m+0 serine provided by the culture medium; middle panel, the percentage of m+0 and m+3 serine in medium; right panel, the percentage of m+0 and m+3 serine in cells. (D) Left panel, the consumption of m+0 glycine provided by the culture medium; middle panel, the percentage of m+0 and m+2 glycine in medium; right panel, the percentage of m+0 and m+2 glycine in cells. Data are mean ± SD, n=3. All the results were repeated by 2 independent experiments.
Figure 4. The characteristics of cell lines-Warburg effect. (A) Glycolytic enzyme activities in 5 cancer cell lines. (B) Glycolytic rate expressed by glucose consumption or lactate generation. Data are mean ± SD, n=3. All the results were repeated by at least 5 independent experiments. (C) Estimation of glucose consumption and lactate generation by 1 kg cancer cells in 24 hours of each cell line.
Figure 5. PGK1 knockdown does not affect the thermodynamic state of the glycolysis in cancer cells. (A & B) PGK1 activity and Western blot of PGK1 in cells with or without PGK1 knockdown. (C-G) The thermodynamic state of each reaction along the glycolysis in cancer cells. All the results were repeated by 2 independent experiments.
Figure 6. The pattern of the glycolytic intermediates in cancer cells. The data are from Table 1. The unit for \([\text{DHAP}] / [\text{GA}3\text{P}] / ([\text{FBP}])\) is mM. Data are mean ± SD, n=3. All the results were repeated by 2 independent experiments.
Figure 7. The effect of PGK1 knockdown on the concentrations of the glycolytic intermediates in cancer cells. (A) PGK1 knockdown only significantly increased the concentration of FBP, DHAP, and GA3P. (B) PGK1 knockdown did not significantly affect AMP, ADP, ATP, NAD, and NADH. (C) Estimated 1,3-BPG. The calculation is described in the Materials and Methods. Data are mean ± SD, n=3. All the results were repeated by 2 independent experiments.
Figure 8. PGK1 knockdown insignificantly or marginally affects the glycolysis rate. (A) Glucose consumption rate, lactate generation rate, and PGK1 activity. (B) The actual PGK1 activity at the cellular 1,3-BPG concentrations (Figure 7C) and the cellular lactate generation rate. (C) Tracing glucose carbon to lactate. Cells were incubated in the presence of 6 mM [13C6]glucose for 6 hours. The percentages of the generated lactate isotopologues were measured by LC-MS. Data are mean ± SD, n=3. All the results were repeated by 2 independent experiments.
Figure 9. PGK1 knockdown moderately reduces serine consumption and de novo serine synthesis. (A) Glucose consumption and lactate generation by Hela-siPGK1 and Hela-NC. (B) Serine consumption and serine synthesis by Hela-siPGK1 and Hela-NC. Left panel, the consumption of m+0 serine provided by the culture medium; middle panel, the percentage of m+0 and m+3 serine in medium; right panel, the percentage of m+0 and m+3 serine in cells. (C) Glycine consumption and glycine synthesis by Hela-siPGK1 and Hela-NC. Left panel, the consumption of m+0 glycine provided by the culture medium; middle panel, the percentage of m+0 and m+2 glycine in medium; right panel, the percentage of m+0 and m+2 glycine in cells. Data are mean ± SD, n=3. All the results were repeated by 2 independent experiments.
Effect of perturbation of PGK1 on the glycolysis

Figure 10. A cell-free glycolysis model for studying the effect of PGK1 titration on glycolysis. (A) The glucose consumption rate and the lactate generation rate in the reaction mixture titrated with PGK1. PGK1 of HeLa cells were knocked down by siPGK1 and the cells were collected and cell lysate were prepared. The cell lysate was then titrated with pure PGK1. The cell lysate with different amount of PGK1 was separately added into the reaction mixture to start the reaction, which was terminated at 30 min. Glucose, lactate, and glycolytic intermediates were measured. The flux control coefficient is calculated according to the tangent of the nonlinear regression at the point [PGK1] = 1.0. (B) The concentrations of glycolytic intermediates in the reaction mixture titrated with PGK1. (C) The pattern of the glycolytic intermediates. (D) The concentration of 1,3-BPG versus PGK1. The concentration of 1,3-BPG is described in the Materials and Methods. (E) The actual PGK activity at the 1,3-BPG concentrations (panel D) and the lactate generation rate. Data are mean ± SD, n=3. Results were repeated by 2 independent experiments.
Figure 11. Glycolysis rate of HeLa-NC and HeLa-siPGK1 cells under different glucose concentrations. (A) Glucose and lactate concentrations in culture medium. (B) Glucose consumption rate under different glucose concentrations. (C) Lactate generation rate under different glucose concentrations. (D) Relative rate of glucose consumption and lactate generation. Data are mean ± SD, n=3. Results were repeated by 2 independent experiments.
Figure 12. Glycolysis rate of HeLa-NC and HeLa-siPGK1 cells under different glutamine concentrations. (A) Glucose consumption rate under different glutamine concentrations. (B) Lactate generation rate under different glutamine concentrations. (C) Relative rate of glucose consumption and lactate generation. Data are mean ± SD, n=3. Results were repeated by 2 independent experiments.
Figure 13. Glycolysis rate of HeLa-NC and HeLa-siPGK1 cells under different cell density. (A) Glucose consumption rate under different cell density. Cells were seeded in 12-well plates. (B) Lactate generation rate under different cell density. (C) Relative rate of glucose consumption and lactate generation. Data are mean ± SD, n=3. Results were repeated by 2 independent experiments.
Figure 14. Effect of PGK1 knockdown on cell growth. (A) Representative flow cytometry figures of cell cycle analysis of HeLa-NC and HeLa-siPGK1 cells. (B) Cell cycle distribution of HeLa-NC and HeLa-siPGK1 cells. (C) Cell number of HeLa-NC and HeLa-siPGK1 cells at 0, 1, 2 days after the transfected cells were seeded to new plates. (D) Glucose and lactate concentrations in culture medium of HeLa-NC or HeLa-siPGK1 cells. Data are mean ± SD, n=3. Results were repeated by 2 independent experiments.
Perturbation of phosphoglycerate kinase 1 (PGK1) only marginally affects glycolysis in cancer cells
Chengmeng Jin, Xiaobing Zhu, Hao Wu, Yuqi Wang and Xun Hu

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