ROS/KRAS/AMPK Signaling Contributes to Gemcitabine-Induced Stem-like Cell Properties in Pancreatic Cancer

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Poor prognosis in pancreatic cancer (PanCa) is partially due to chemoresistance to gemcitabine (GEM). Glucose metabolism has been revealed to contribute to the therapeutic resistance and pluriopotent state of PanCa cells. However, few studies have focused on the effects of GEM on cancer cell metabolism, stemness of tumor cells, and molecular mechanisms that critically influence PanCa treatment. We demonstrate that GEM treatment induces metabolic reprogramming, reducing mitochondrial oxidation and upregulating aerobic glycolysis, and promotes stem-like behaviors in cancer cells. Inhibiting aerobic glycolysis suppresses cancer cell stemness and strengthens GEM’s cytotoxicity. GEM-induced metabolic reprogramming is KRAS dependent, as knockdown of KRAS reverses the metabolic shift. GEM-induced metabolic reprogramming also activates AMP-activated protein kinase (AMPK), which promotes glycolytic flux and cancer stemness. In addition, GEM-induced reactive oxygen species (ROS) activate the KRAS/AMPK pathway. This effect was validated by introducing exogenous hydrogen peroxide (H2O2). Taken together, these findings reveal a counterproductive GEM effect during PanCa treatment. Regulating cellular redox, targeting KRAS/AMPK signaling, or reversing metabolic reprogramming might be effective approaches to eliminate cancer stem cells (CSCs) and enhance chemosensitivity to GEM to improve the prognosis of PanCa patients.

INTRODUCTION
Cytotoxic chemotherapy with gemcitabine (GEM) continues to be the first-line treatment for pancreatic cancer (PanCa). However, GEM treatment has been minimally effective at the improving the prognosis of PanCa patients.1 One of the speculative causes of this limited effectiveness has been the postulated existence of relatively rare but highly chemoresistant cancer stem cells (CSCs).2,3 CSCs have been reported to possess inherently high tumor-initiating potential, which is implicated in tumor relapse, as well as in establishment of metastases.4,5

Accumulating evidence has shown that resistance of some cancers to chemotherapy may result from dysregulation of glucose metabolism.6 Tumor cells rewire their metabolic pathways to ensure an energy supply, and as a part of survival programs activated in response to environmental threats.7 In addition, ionizing radiation has been found to induce metabolic changes that correlate with induction of the CSC phenotype,8 thus promoting expansion of the CSC-like population and enhancing resistance to anticancer drugs.7 Elucidation of the effects of GEM on cancer cell metabolism and stemness is expected to contribute to our understanding of the underlying mechanism(s) of chemoresistance in PanCa cells.

Accumulating evidence suggests that PanCa cells’ metabolic switch ability is regulated by various oncogenic signals.9 Oncogenic KRAS mutations function as a key driver in initiation and maintenance of approximately 90% of PanCa cases.10 The KRAS oncogene encodes a small GTPase (21 kDa), which is active in its GTP-bound form and inactive when bound to GDP.11 Aberrant KRAS activation can cause dysfunction in oxidative phosphorylation (OXPHOS). Compensatory elevated aerobic glycolysis can drive tumor development.12–14 With KRAS ablation, surviving tumor cells experience impaired aerobic glycolysis, have increased mitochondrial activity, and rely largely on OXPHOS for energy. At the same time, AMP-activated protein kinase (AMPK) phosphorylation is lower in KRAS-ablated tumor cells than in KRAS-expressing cells.15 The transition from OXPHOS to aerobic glycolysis is a prerequisite to the reprogramming of differentiated cells to a pluripotent state.16 Such a transition in cellular metabolism could cause an energy crisis. To restore an adequate energy supply, AMPK, a key regulator of cellular energy...
metabolism, stimulates ATP generation by promoting glucose uptake and transport. In addition to acting as a stress-response molecule, AMPK is associated with drug resistance and enrichment of CSCs. Increased levels of reactive oxygen species (ROS) upon GEM treatment have been reported previously. Multiple studies have shown that high ROS levels are cytotoxic and low ROS levels may be tumor promoting. In this study, we demonstrated that low-dose GEM can induce metabolic reprogramming toward aerobic glycolysis, promoting PanCa cell stem-like properties and chemoresistance. Mechanistically, GEM-induced metabolic reprogramming and cancer stemness are regulated by ROS-mediated activation of the KRAS/AMPK pathway. These findings may shed light on how GEM induces therapeutic resistance and provide novel potential approaches for better management of PanCa.

RESULTS

GEM Treatment Induces Metabolic Reprogramming of PanCa Cells toward Dependence on Aerobic Glycolysis

Recently, we demonstrated that GEM-resistant PanCa cells underwent a metabolic shift, becoming more glycolytic. However, it remains unclear whether this metabolic reprogramming is induced by GEM treatment. We first examined the effect of GEM on expression of glycolytic genes. GEM treatment increased the mRNA expression in PanCa cells of many glycolytic enzymes reported to contribute to tumor aggressiveness or drug resistance in cancer cells; these include GLUT1, HKII, PFKP, ALDOA, TPI1, PKG1, PGAM1, PKM2, and LDHA (Figure S1A).

We utilized 2-(N-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyoxyglucose (2-NBDG), a fluorescent deoxyglucose analog, to monitor glucose uptake and found that GEM treatment increased 2-NBDG uptake in PanCa cells (Figure 1A). In addition, after GEM treatment, the lactate concentration in the culture medium increased dramatically, indicating increased conversion of pyruvate to lactate (Figure 1B). Importantly, when 2-deoxy-D-glucose (2-DG)—a glucose analog that competes with glucose for uptake via GLUT1, is phosphorylated by HKII, and allosterically inhibits HKII, thus functioning as a glycolysis inhibitor—was introduced, GEM-induced elevation in lactate production was significantly reduced (Figure 1C). Similarly, GEM dose-dependently upregulated protein and mRNA levels of glucose transporter 1 (GLUT1), which is responsible for uptake of extracellular glucose and lactate dehydrogenase A (LDHA), an enzyme responsible for conversion of pyruvate into lactate (Figure 1D). Taken together, high glucose uptake and lactate production suggest that glycolysis was upregulated upon GEM treatment.

At the intersection of aerobic glycolysis and respiration, pyruvate dehydrogenase kinase (PDK)—which inhibits pyruvate dehydrogenase (PDH) and prevents entry of pyruvate into OXPHOS-based metabolism—plays a key role in cellular glucose metabolism. Interestingly, we detected four genes encoding kinases (PDK1, PDK2, PDK3, and PDK4) that inhibit PDH enzyme complex activity and found that PDK2 was significantly induced at the mRNA level in all three cell lines treated with GEM (Figure S1B). We therefore examined protein levels of PDK2. As expected, GEM dose-dependently upregulated expression of PDK2 protein (Figure 1E). Similarly, GEM significantly increased levels of phospho-PDHE1-α (Ser 293) (p-PDHE1-α) in a dose-dependent manner (Figure 1E). PDK phosphorylates the PDHE1-α subunit, and inactivates the PDH enzyme complex that converts pyruvate to acetyl-coenzyme A, thereby inhibiting pyruvate metabolism via the tricarboxylic acid (TCA) cycle. In addition, mitochondrial membrane potential (ΔΨm) is generated by OXPHOS activity. Thus, a decrease in ΔΨm is indicative of decreased electron transport and OXPHOS activity. We observed that GEM treatment decreased ΔΨm (Figure 1F) without affecting cell viability (Figure S2A). These findings suggest that GEM treatment induces metabolic reprogramming.

Inhibition of Aerobic Glycolysis Abrogates GEM-Induced Enhancement of Cancer Stemness

We first investigated the cancer stem-like properties of GEM-treated PanCa cells. CSCs are defined by their ability to form tumor spheres in culture, under non-adherent conditions. PanCa cells treated with a sub-lethal dose of GEM (Figure S2A) exhibited an enhanced ability to form tumor spheres in ultra-low adhesion plates (Figure S2B). We also found that the mRNA and protein expression levels of the pluripotent cell markers Nanog and Sox2 were increased in cells treated with GEM in a dose-dependent manner (Figure S2C). We further found that GEM significantly increased expression of ALDH, OCT4, KLF4, CXCR4, and CD24, which have previously been reported to be CSC markers in all three cell lines, and EPCAM, which has been reported to be a CSC marker in SW1990 cells (Figure S2D). GEM-mediated upregulation of ABCG2 and MDR1 levels has been associated with drug resistance (Figure S2E). In addition, ID-1, MUC-1, and MUC-4 expression is upregulated upon GEM treatment, indicating decreased differentiation (Figure S2F). Furthermore, GEM dose-dependently increased expression of epithelial-mesenchymal transition (EMT)-associated genes, such as N-cadherin, Vimentin, Snail1, and Zeb1, in PanCa cells (Figure S2G).

We further investigated whether inhibition of aerobic glycolysis could abrogate GEM-induced PanCa cell stemness. We found that 2-DG, a glycolytic inhibitor, partially abrogated GEM-induced expression of Nanog and Sox2 at protein and mRNA levels (Figures 2A and 2B). To confirm that the enhanced cancer cell stemness induced by GEM was glycolysis-dependent, we examined the expression of CD133, a CSC surface marker on PanCa cells, which has previously been reported to be a CSC marker in SW1990 cells (Figure S2D). Treatment with 2-DG significantly decreased GEM-induced CD133 expression (Figures 2C and 2D). Treatment of PanCa cells with a nontoxic low dose of 2-DG (5 mM, 24 h) suppressed sphere formation (Figures S2A and S2H). 2-DG significantly reinforced GEM cytotoxicity in PanCa cells, significantly decreasing its lethal dose 50% (LD50) in PanCa cells (Figures 2E and 2F). Thus, inhibition of glycolysis suppressed GEM-induced stemness and enhanced the therapeutic efficacy of GEM in PanCa cells.

We further detected the involvement of glycolysis in tumorigenesis of pancreatic cancer cells treated with GEM (Figure 3A). As shown in
Figures 3B and 3C, pretreatment of pancreatic cancer cells (SW1990) with a sub-lethal dose of GEM increased the tumor growth rate and cell size of SW1990 cell xenografts in mouse models compared with controls. Remarkably, 2-DG inhibited the growth-promoting effects of GEM pretreatment on pancreatic cancer cells. These results are in agreement with the abrogation of GEM-induced stemness by inhibiting glycolysis. To verify whether tumorigenesis in different treated groups correlated with stemness, we performed CD133, Nanog, and Sox2 immunostaining. As shown in Figure 3E, levels of stemness markers, including CD133, Nanog, and Sox2, increased in the GEM treated group but were abrogated in cells also treated with 2-DG.

Oncogenic KRAS Is Involved in GEM-Induced Metabolic Reprogramming and Cancer Cell Stemness

It has already been reported that KRAS (G12V) transformation leads to mitochondrial dysfunction and metabolic switching.12 We therefore investigated the role of KRAS in GEM-induced metabolic reprogramming. We first evaluated the effect of GEM on KRAS activation. As revealed in Figure 4A, GEM exposure effectively activated KRAS,
producing higher levels of GTP-bound KRAS compared with those in the control group. We assessed the effect of KRAS knockdown on metabolic reprogramming. Knockdown of KRAS decreased expression of most glycolytic genes (Figures S3A and S3B). Importantly, knockdown of KRAS reduced lactate production (Figure 4B), suppressed expression of PDK2, and consequently decreased p-PDHE1-α levels (Figure 4C), indicating restoration of OXPHOS. In addition, DJm was not decreased upon KRAS knockdown, as revealed by fluorescence microscopy and flow cytometry analyses (Figure 4D). Maintenance of DJm indicates functional OXPHOS. These results suggest that activation of KRAS can inhibit mitochondrial function and drive the metabolic switch to aerobic glycolysis.

We further assessed the role of KRAS in maintenance of cancer stemness and chemoresistance. KRAS knockdown decreased sphere formation ability (Figure 4E) and expression of pluripotent markers (Figures 4F and 4G). In addition, KRAS knockdown significantly enhanced the cytotoxicity of GEM, 2-DG, or both, as demonstrated by decreased LD50 (Figure 4H). Taken together, these results establish that the metabolic switch from aerobic glycolysis to OXPHOS, induced by KRAS knockdown, inhibited cancer stemness and sensitized PanCa cells to chemotherapeutics.

The role of KRAS in GEM-induced metabolic reprogramming and cancer cell stemness was further assessed in vivo (Figure 5A). A pancreatic cancer xenograft model was established by subcutaneous injection of pancreatic cancer cells into the right flank of nude mice. Treatments were initiated after comparable tumor volumes were reached in tumor-bearing mice (approximately 100 mm³) as described in the Supplemental Materials and Methods. The role of KRAS was assessed by local injection of cholesterol-conjugated KRAS small interfering RNA (siRNA) into the tumor mass. The efficacy of KRAS siRNA transfection in vivo was evaluated by immunohistochemically analyzing KRAS expression in tumor tissue. While the growth rate and final size of tumors in the GEM chemotherapy group were slightly, but not dramatically, reduced compared with that in the NC siRNA group, KRAS siRNA alone substantially decreased tumor growth. GEM combined with KRAS siRNA significantly decreased the tumor’s growth compared with GEM chemotherapy alone (Figures 5B–5D). KRAS expression was significantly downregulated in GEM-treated and -untreated groups after KRAS siRNA transfection (Figure 5E). Immunohistochemical staining of glycolytic enzymes, including GLUT1, LDHA, HKII, and PDK2, showed enhanced glycolysis in the group treated with gemcitabine, which could be abrogated by in vivo silencing of KRAS (Figure 5F). Similarly, GEM-induced enhancement of cancer stemness was reduced by KRAS knockdown in vivo (Figure 5G).

GEM-Induced Metabolic Shift Activates AMPK
As observed above, GEM induces a metabolic shift from OXPHOS toward aerobic glycolysis, which is a less efficient pathway to supply cellular energy. AMPK is an evolutionarily conserved energy sensor, modulating cellular energy flux in response to energy crises. We therefore examined the effects of GEM on AMPK activation. As expected, GEM dose-dependently enhanced phosphorylation of AMPKα (Thr172) (p-AMPKα) (Figure 6A), which has been
speculated to correlate with enzyme activity.35 To further examine whether the rewired cellular metabolism activates AMPK, we examined the effect of KRAS knockdown on AMPK activation. As expected, knockdown of KRAS reduced levels of p-AMPKα (Figure 6B), suggesting a regulatory role of KRAS-mediated cellular metabolism in AMPK activation.

AMPK’s involvement in the regulation of glycolytic flux is well documented.36 We therefore investigated whether AMPK is involved in GEM-induced metabolic reprogramming. We pretreated cancer cells with compound C, an inhibitor of AMPK, followed by exposure to GEM. GEM-induced p-AMPKα expression was abrogated by compound C, which showed marginal effect on p-PDHE1-α generation (Figure 6C). Compound C also significantly inhibited GEM-induced lactate production (Figure 6D), suggesting that glycolysis was inhibited. We also observed that PanCa cells treated with compound C exhibited reduced sphere formation (Figure 6E), indicating diminished expression of pluripotency markers (Figure 6F). In addition, compound C strengthened the cytotoxicity of GEM toward PanCa cells, resulting in a decreased LD50 (Figures 6G and 2F).

To further investigate the potential role of AMPK in glycolytic flux, we introduced a selective AMPK activator, A-769662. Treatment with A-769662 led to AMPK activation but did not exhibit a significant effect on p-PDHE1-α levels (Figure S4A). We did not observe a significant difference in lactate production after A-769662 treatment (data not shown). However, A-769662 treatment promoted expression of glycolytic genes, such as GLUT1 and LDHA (Figure S4B). Unlike compound C, A-769662 promoted sphere formation, pluripotent cell marker expression, and tumor cell viability at appropriate concentrations (Figures S4C–S4E). Taken together, these findings demonstrate that AMPK mediates GEM-induced upregulation of aerobic glycolysis and promotes reacquisition of cancer cell stemness.

GEM-Induced ROS Participates in Activation of the KRAS/AMPK Pathway, Metabolic Reprogramming, and Cancer Stemness

Induction of ROS upon GEM treatment has been previously reported.19,20 To examine whether GEM-induced ROS activates the KRAS/AMPK pathway, we first examined ROS production upon GEM treatment. Consistent with previous findings, GEM treatment induced a dose-dependent increase in ROS levels, which was abrogated by introduction of N-acetyl-cysteine (NAC) (a free-radical scavenger) (Figure 7A). Importantly, GEM-induced upregulation of both KRAS-GTP and p-AMPKα was abrogated by pretreatment with NAC (Figure 7B). Next, we found that GEM-induced lactate production was reduced (Figure 7C) and p-PDHE1-α expression...
was diminished by NAC treatment (Figure 7D), indicating that GEM-induced activation of KRAS/AMPK signaling was ROS-dependent.

We further found that GEM-induced sphere formation (Figure 7E), CD133 expression (Figure 7F), and pluripotency markers (Figures 7G and 7H) were all inhibited by pretreatment with NAC. To avoid ROS-induced oxidative damage, cancer cells often upregulate their antioxidant production capacity. We observed a significant increase in the expression of antioxidant genes, such as superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), catalase (CAT), glutathione peroxidase 1 (GPX1), and nuclear factor (erythroid-derived 2)-like 2 (Nrf2), in PanCa cells (Figure S5), suggesting that
GEM-induced oxidative stress is accompanied by an increase in antioxidant reactions.

To further establish the role of oxidative stress in activation of the KRAS/AMPK pathway, we introduced exogenous hydrogen peroxide ($H_2O_2$) to increase ROS levels to a comparable extent to levels induced by GEM in PanCa cells (Figure 8A). Similar to GEM treatment, $H_2O_2$ induced KRAS/AMPK activation, which was also inhibited by NAC (Figure 8B). In addition, $H_2O_2$ increased glycolytic gene expression (Figure S6) and lactate production (Figure 8C).
indicating that glycolytic flux was elevated. Importantly, H$_2$O$_2$-induced lactate and p-PDHE1-α generation was suppressed following NAC treatment (Figures 8C and 8D). Moreover, H$_2$O$_2$-induced sphere formation, CD133 expression, and expression of pluripotency markers were all decreased after treatment with NAC (Figures 8E–8H). Taken together, these findings establish that GEM induces metabolic reprogramming and cancer stemness via ROS-dependent activation of the KRAS/AMPK pathway (Figure 9).

**DISCUSSION**

Chemoresistance is one of the main causes of poor prognosis in pancreatic cancer. Accumulating evidence suggests that CSCs are resistant to conventional chemotherapy and radiotherapy and may be one of the major causes of cancer recurrence after chemotherapy. Therefore, elucidation of the mechanisms underlying chemoresistance and reacquisition of CSC-like properties could shed light on potential therapeutic approaches to improve PanCa...
treatment. GEM has been the standard chemotherapy regimen for PanCa patients but has yielded unsatisfactory treatment outcomes. In the present study, to the best of our knowledge, we have demonstrated for the first time that GEM treatment induces metabolic reprogramming toward aerobic glycolysis via ROS-mediated activation of the KRAS/AMPK pathway, enhancing stemness in PanCa cells.

Pluripotent stem cells and embryonic stem cells have been reported to undergo metabolic transition, including upregulation of glycolysis and downregulation of OXPHOS. Similar to these phenomena, we found that GEM induces metabolic reprogramming that enhances aerobic glycolysis and decreases mitochondrial oxidation. This metabolic reprogramming is accompanied by enhanced cancer cell stemness. Enhanced aerobic glycolysis induced by GEM promotes acidification of the microenvironment, partly due to the extrusion of lactic acid, decreasing the cytotoxic efficiency of GEM on PanCa cells. Therefore, safe and efficient inhibition of glycolysis is a promising anticancer therapy, either by itself or in combination with chemotherapy. In our study, inhibition of glycolysis using 2-DG suppresses cancer stemness and strengthens the cytotoxicity of GEM, suggesting a rationale for combining glycolytic inhibitors with conventional chemotherapy for PanCa treatment.

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Figure 8. ROS Involvement in Activation of the KRAS/AMPK Pathway

(A) ROS levels in PANC-1 cells pretreated with NAC (5 mM, 1 h), followed by treatment with H2O2 (200 μM) for 12 h were assessed using flow cytometry. (B) Activation of the KRAS/AMPK pathway was assessed by western blot. PANC-1 and SW1990 cells were pretreated with NAC (5 mM, 1 h) followed by treatment with H2O2 (200 μM) for 24 h. (C) Lactate production in PANC-1 cells pretreated with NAC (5 mM, 1 h) followed by treatment with H2O2 (200 μM) for 36 h. (D) Levels of p-PDHE1-α in cells treated as in (B) were examined by western blotting. (E–H) H2O2-induced ROS were involved in inducing cancer cell stemness. Cells were treated as in (B). (E) Sphere formation assay of PANC-1 cells is shown. (F and G) CD133 and pluripotency markers Nanog and Sox2 were examined using flow cytometry (F) or western blot (G) in PANC-1 and SW1990 cells treated as in (B). (H) mRNA expression of Nanog and Sox2 in PANC-1 cells treated as in (B). β-actin served as an internal control. Ratios were obtained as previously described. All experiments were repeated at least three times. Error bars represent means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.
II and III clinical trials.\textsuperscript{43} Considering the fact that 2-DG can also be taken up by highly metabolic normal tissues, including heart and brain, we must be careful when 2-DG is applied in patients.\textsuperscript{39} Metabolic reprogramming has been recognized as a core hallmark of cancer cells.\textsuperscript{44} Oncogenic KRAS can produce several phenotypic hallmarks of cancer including altered cellular metabolism.\textsuperscript{11} KRAS transformation can promote glycolytic activity and decrease oxidative flux.\textsuperscript{45} Oncogenic KRAS knockdown decreased glucose uptake and lactate production, which demonstrated that oncogenic KRAS promotes glycolytic flux in PanCa.\textsuperscript{14} Ectopic KRAS expression inhibits mitochondrial respiratory chain activity, suppressing mitochondrial respiration.\textsuperscript{12} Viale et al. found that surviving cells cultured in semi-solid medium with KRAS ablation exhibited decreased aerobic glycolysis and relied on mitochondrial oxidation for survival.\textsuperscript{15} Our studies showed that GEM induces KRAS activation, which, in turn, regulates metabolic reprogramming, because knockdown of KRAS can inhibit aerobic glycolysis flux and restore mitochondrial respiration. Restoration of cellular metabolism by KRAS knockdown suppresses cancer cell stemness and sensitizes PanCa cells to GEM, 2-DG, or both, suggesting a tumorigenic and chemoresistant role for KRAS. Similarly, activation of mitochondrial oxidation following PDK2 inhibition could activate mitochondrial apoptotic signaling, causing the death of chemoresistant cancer cells.\textsuperscript{60} Mitochondrial OXPHOS is essential for efficient apoptosis.\textsuperscript{47,48} The oncogenic role of mutant KRAS prompts intensive efforts to explore pharmacological approaches. The association between oncogenic KRAS activity and metabolic reprogramming might provide promising directions for the development of novel therapeutic approaches.\textsuperscript{11} It has been reported that GEM-induced ROS is generated by activation of NADPH oxidase (NOX) via nuclear factor \(\kappa B\) (NF-\(\kappa B\)) activation, as siRNA-mediated depletion of p22\(^{\text{phox}}\) (a catalytic subunit of the NOX complex) alleviated GEM-induced ROS production.\textsuperscript{58} We also found that GEM-induced ROS was NOX-dependent, because inhibition of NOX using Apocynin (a NOX inhibitor) reduced ROS production.\textsuperscript{19} ROS-induced activation of NF-\(\kappa B\) can also promote HIF-1\(\alpha\) and CXCR4 expression, which play a role in GEM resistance in PanCa cells.\textsuperscript{21} Our findings showed that GEM-induced ROS participates in activating the KRAS/AMPK pathway, and this effect can be reversed by pre-treatment with the free-radical scavenger NAC. CSCs reportedly possess mechanisms to fine-tune ROS levels by tightly regulating metabolic pathways such as aerobic glycolysis, rather than by oxidative phosphorylation, to reduce oxidative stress.\textsuperscript{50} Nomura et al. found that CD133\(^+\) pancreatic CSCs showed increased glycolytic flux and decreased mitochondrial activity.\textsuperscript{33} This metabolic switch decreased ROS production upon GEM treatment and favored CSC survival. Thus, the metabolic switch induced by GEM is likely a cellular response to oxidative stress, and a prerequisite for induction of CSCs. In addition, GEM-induced expression of antioxidant genes may also play a role in maintenance of intracellular redox homeostasis. GEM treatment increased Nrf2 expression, which regulates production of glutathione (GSH), a major cellular antioxidant that maintains ROS levels within safe parameters to prevent oxidative damage.\textsuperscript{20} In addition, ROS-dependent regulation of energy metabolism can drive carbohydrate flux to the pentose phosphate pathway, thereby increasing NADPH levels to counteract intracellular ROS.\textsuperscript{31} Cancer cells can rewire metabolic pathways in response to changes in cellular energy and nutrient status, suggesting the complexity of targeting cellular metabolism.

AMPK is a conserved energy sensor and master regulator of cellular metabolism.\textsuperscript{34} We found that GEM treatment activates AMPK, probably resulting from the energy crisis induced by GEM treatment, given that restoration of mitochondrial oxidation by KRAS...
knockdown inhibits AMPK activation. Interestingly, a specific AMPK activator promotes glycolysis, while an AMPK inhibitor inhibits glycolytic flux, suggesting a direct regulatory role of AMPK in glycolysis, consistent with recent findings. AMPK activation may also promote intracellular NADPH production in response to oxidative stress under energy stress conditions. AMPK is also critical in modulating self-renewal and chemoresistance of CSCs. We found that AMPK activation promoted glycolysis, cancer cell stemness, and cell survival, suggesting that AMPK inhibition could be a potential means of targeting the malignant behavior of PanCa.

In conclusion, we demonstrated a counterproductive effect of GEM in PanCa treatment. GEM induces KRAS activation, which contributes to metabolic reprogramming and enhances cancer cell stemness. The metabolic shift activates AMPK, which further promotes glycolysis and enhances cancer cell pluripotency. Furthermore, GEM-induced ROS participates in activating KRAS/AMPK signaling, metabolic reprogramming, and maintenance of cancer cell stemness. Considering the implication of metabolic reprogramming in stimulating pluripotency and the contributory role of aerobic glycolysis in chemoresistance, interventions targeting cellular metabolism might provide new therapeutic approaches against PanCa.

MATERIALS AND METHODS

Cell Lines
The human PanCa cell lines PANC-1 and SW1990 were obtained from the American Type Culture Collection (ATCC) (Manassas, USA). Patu8988 cells were obtained from Keygen (KeyGen Biotech, China). All three cell lines harbor a point mutation at codon 12 of the KRAS gene (GGT→GAT in PANC-1 and SW1990, and GGT→GTT in Patu8988). Cells were maintained in RPMI Medium 1640 basic (GIBCO by Thermo Fisher Scientific, #8117284) supplemented with 1% penicillin/streptomycin, and 10% fetal bovine serum (GIBCO Invitrogen, Grand Island, NY, USA).

Cell Viability Assay
The effects of various treatments on PanCa cell viability were measured by 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay as previously described. Pancreatic cancer cell viability was analyzed using an MTT assay (Sigma-Aldrich, #M2128 and #A9251). Cells (PANC-1, SW1900) (6,000 per well) were seeded in 96-well plates overnight. Cells were then treated with different concentrations of gemcitabine (1 to 80 μM) with or without 2-DG (5 mM) for 48 h at 37°C. PANC-1 cells (3 × 10^5) were seeded and transfected with siRNA-targeting KRAS (siKRAS) or a negative control siRNA (ncsiKRAS) for 48 h and then treated with GEM (5 μM), 2-DG (5 mM), or their combination for another 36 h. Next, 20 μL of MTT (5 mg/mL in PBS) was added, and cells were incubated for another 4 h. After that, the membranes of each well were discarded, and DMSO (Sigma-Aldrich, #D2650) (150 μL) was added. Following agitation for 10 min in the dark on an Eppendorf shaker, absorbance was read at 490 nm in a microplate spectrophotometer. Each concentration was investigated in five replicates. Data were expressed relative to the untreated group.

Western Blot Analysis
Western blots were performed as previously described. Cells were lysed with radioimmunoprecipitation assay lysis buffer (Beyotime Biotechnology, Shanghai, China, #P0013C) at 4°C. Equal amounts of protein were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA, #IPVH00010), which were then blocked with 5% non-fat milk for 1 h and incubated with primary antibodies overnight at 4°C. Membranes were washed and incubated with horseradish peroxidase-coupled secondary antibodies (Aspen, Wuhan, China, #AS1107), and visualized using ECL substrate (Thermo Fisher, Waltham, MA, USA, #32109). Details of various primary antibodies used are provided in the Supplemental Materials and Methods.

Quantitative Real-Time PCR Assay
Total RNA was extracted using TRizol (Invitrogen, #A33254). Equal amounts of RNA (0.5 μg) were reverse-transcribed to cDNA using PrimeScript RT Master Mix (Takara Bio, #RR036A). Quantitative real-time PCR analysis was performed using a quantitative SYBR Green PCR Kit (Takara Bio, #RR430A), according to the manufacturer’s instructions. The data were interpreted with the 2^(-ΔΔCT) method, and gene-expression levels were normalized against β-actin levels.

Flow Cytometric Analysis
Mitochondrial membrane potential (∆Ψm) of PanCa cells was measured using a mitochondrial membrane potential potential assay kit with JC-1 (Beyotime, #C2006), according to the manufacturer’s instructions. Fluorescence was measured by flow cytometry or fluorescent microscopy. Analysis of the cell surface marker CD133 was performed as previously described.

siRNA-Mediated Knockdown of KRAS In Vitro and In Vivo
A siRNA sequence targeting the coding region of KRAS (siKRAS: 5′-GGAAGCAAGTAATTGAG-3′) and a negative control not matching any human gene sequence were obtained (RiboBio, Guangzhou, China). KRAS knockdown was performed by transfecting cells with Lipofectamine 2000 (Invitrogen, #11668019) according to the manufacturer’s instructions. Cholesterol-conjugated KRAS siRNA for in vivo RNA interference and its negative control were obtained from RiboBio.

Sphere Formation, Glucose Uptake, and Lactate Production Assays
Sphere formation, glucose uptake, and lactate production assays were performed as previously described. Cells (5–10 × 10^5) subjected to different treatments were cultured in serum-free DMEM–Ham’s nutrient mixture (F12) (1:1) medium (Invitrogen) supplemented with 20 ng/mL of epithelial growth factor and 10 ng/mL of basic fibroblast growth factor (PeproTech, #GMP100-15 and #100-25) for 2 weeks. Number of spheres larger than 50 μm was counted using a microscope (Olympus, Tokyo, Japan). For determination of glucose uptake, complete medium was replaced with a glucose-free medium, and cells were incubated for 2 h. Cells were then incubated with the fluorescence-labeled glucose analog 2-NBDG (Cayman Chemical, Ann Arbor, MI, USA) at a final concentration of 10 μM for 30 min at 37°C. Uptake
of 2-NBDG was analyzed using flow cytometry and fluorescence microscopy. To assess lactate production, we cultured cells (3 × 10^5) overnight. After different treatments, supernatants were collected and centrifuged. Lactate production was assessed using a lactic acid assay kit (Nanjing Jiancheng Bio, Nanjing, China, #A019-2-1) according to the manufacturer’s protocol. Lactic acid levels were normalized to total protein in each sample.

**Determination of Intracellular ROS Levels**

Cells were treated as previously indicated, and incubated with dichlorodihydro-fluorescein diacetate (DCHF-DA) (Beyotime, #S0033) at a final concentration of 10 μM in serum-free medium for 20 min at 37°C.

**KRAS Activation Assay**

The level of active KRAS was assessed using a RAS activation assay kit (NewEast Biosciences, Malvern, PA, #81101), according to the manufacturer’s protocol. Cells were cultured in 10 cm plates to 80%–90% confluence before different treatments. Total cell lysates were collected and incubated with anti-active RAS monoclonal antibody plus protein A/G agarose bead slurry at 4°C overnight. After different treatments, supernatants were collected and centrifuged. Agarose beads were resuspended in 20 μl of 2x reducing SDS-PAGE sample buffer and boiled. Precipitated active RAS was detected by immunoblot analysis using anti-KRAS monoclonal antibody, according to the manufacturer’s instructions.

**Tumor Xenografts**

All of the procedures involving animals in this study were in accordance with the ethical standards of the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology. Male BALB/c nude mice (5 weeks old) were purchased from HFK Bioscience (Beijing, China). For assessment of glycolysis inhibition on gemcitabine-induced cancer cell stemness in vivo, SW1990 cells were pretreated with 5 μM GEM (https://www.selleckcn.com; Shanghai, China, #S1714) or 5 mM 2-DG (Sigma-Aldrich, St. Louis, MO, USA, #D8375) or a combination of both for 24 h.

The significance of KRAS inhibition in gemcitabine-induced aerobic glycolysis and cancer stemness in pancreatic cancer in vivo was studied by subcutaneous inoculation of cancer cells into nude mice. SW1990 cells (3 × 10^6/100 μl/mouse) were subcutaneously injected into the right flank of nude mice (n = 5 for each group) followed by treatment with NC siRNA, GEM + NC siRNA, KRAS siRNA, or GEM + KRAS siRNA. Details are provided in Supplemental Materials and Methods.

**Immunohistochemistry**

Paraffin-embedded xenograft samples were used for immunohistochemical staining of KRAS, cancer stemness markers, including Nanog, Sox2, and CD133, and markers of the cells’ glycolysis state, including GLUT1, LDHA, PDK2, and HKII. Details are provided in Supplemental Materials and Methods.

**Statistical Analysis**

All statistical analyses were performed using GraphPad Prism 5.0 software (San Diego, CA). Unpaired t tests, one-way ANOVA with Bonferroni post-tests, or two-way ANOVA were used to calculate statistical significance. Results were presented as means ± SD. p values < 0.05 were considered statistically significant.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.omto.2019.07.005.

**AUTHOR CONTRIBUTIONS**

T.Y. conceived the study, T.Y., H.Z., S.W., and Z.Z. designed the study and wrote the manuscript. H.Z., S.W., H.L., and Q.D. performed the experiments. C.W. and Q.S. provided conceptual advice. Q.S. revised the manuscript.

**CONFLICTS OF INTEREST**

The authors declare no competing interests.

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