Published in final edited form as:

Cancer Lett. 2017 August 01; 400: 37–46. doi:10.1016/j.canlet.2017.04.029.

GOT1-Mediated Anaplerotic Glutamine Metabolism Regulates Chronic Acidosis Stress In Pancreatic Cancer Cells

Jaime Abrego¹, Venugopal Gunda¹, Enza Vernucci¹, Surendra K. Shukla¹, Ryan J. King¹, Aneesha Dasgupta², Gennifer Goode¹, Divya Murthy¹, Fang Yu³, and Pankaj K. Singh^{1,2,4,5,*}

¹The Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, Nebraska, USA

²Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, Nebraska, USA

³Department of Biostatistics, University of Nebraska Medical Center, Omaha, Nebraska 68198, USA

⁴Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska, USA

⁵Department of Genetics Cell Biology and Anatomy, University of Nebraska Medical Center, Omaha, Nebraska, USA

Abstract

The increased rate of glycolysis and reduced oxidative metabolism are the principal biochemical phenotypes observed in pancreatic ductal adenocarcinoma (PDAC) that lead to the development of an acidic tumor microenvironment. The pH of most epithelial cell-derived tumors is reported to be lower than that of plasma. However, little is known regarding the physiology and metabolism of cancer cells enduring chronic acidosis. Here, we cultured PDAC cells in chronic acidosis (pH 6.9~7.0) and observed that cells cultured in low pH had reduced clonogenic capacity. However, our physiological and metabolomics analysis showed that cells in low pH deviate from glycolytic metabolism and rely more on oxidative metabolism. The increased expression of the transaminase enzyme GOT1 fuels oxidative metabolism of cells cultured in low pH by enhancing the non-canonical glutamine metabolic pathway. Survival in low pH is reduced upon depletion of GOT1 due to increased intracellular ROS levels. Thus, GOT1 plays an important role in energy metabolism and ROS balance in chronic acidosis stress. Our studies suggest that targeting anaplerotic glutamine metabolism may serve as an important therapeutic target in PDAC.

*Correspondence: Pankaj K. Singh, Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, 985950 Nebraska Medical Center, Omaha, NE 68198-5950., pankaj.singh@unmc.edu, (Phone) 402.559.2726, FAX: 402.559.2813.

Competing Interests: The authors have declared that no competing interests exist.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Cancer metabolism; Pancreatic cancer; Acidic microenvironment; low pH; Anaplerotic glutamine metabolism

1. Introduction

Metabolic alterations represent an important hallmark of cancer cells [1]. Metabolic reprogramming allows cancer cells to sustain uncontrolled proliferation by rapid generation of ATP, biosynthesis of macromolecules, and maintenance of redox status [2]. Cancer cells can also reprogram the major metabolic pathways (carbohydrates, proteins, lipids, and nucleic acids) to meet these basic demands for uncontrolled proliferation [3, 4]. The characteristic metabolic phenotype seen in cancer cells is the Warburg effect, which operates by enhancing glucose uptake and flux into glycolysis, while simultaneously diminishing the glucose carbon flux that enters the TCA cycle in the mitochondria, even in the presence of oxygen [5, 6]. Although ATP generation through substrate level phosphorylation is very rapid, this mechanism is far less efficient than oxidative phosphorylation in generating energy from glucose. Thus, the metabolic phenotype observed in the Warburg effect demands very high glucose uptake to meet the energetic, biosynthetic, and redox needs of cancer cells. For this reason the increased glucose uptake of cancer cells is useful for diagnosing cancer using radiolabeled glucose analog ^{18}F -fluorodeoxyglucose and positron emission tomography (FDG-PET) to image and evaluate tumor progression without the need of a biopsy [7, 8].

Because of the enhanced metabolic rate of rapidly proliferating tumor cells, the glucose that is metabolized through substrate level phosphorylation produces lactic acid as the end product. Lactic acid is a weak acid, and thus, it quickly dissociates losing a hydrogen ion [9]. Lactate is transported outside of the cell by monocarboxylate symporters along with protons resulting in decreased pH in the extracellular milieu [10, 11]. Intracellular hydrogen ions can also be removed by sodium hydrogen exchangers that import sodium ions and extrude hydrogen ions, thereby acidifying the extracellular environment [12, 13]. Similarly, vacuolar ATPases extrude hydrogen ions against their concentration gradient to the extracellular space, and hence, lower the extracellular pH [14]. *In vitro* studies have shown that rapidly growing cells, which exhibit the Warburg effect, increase the expression of these cell surface proteins to maintain an alkaline intracellular pH environment [15, 16]. Indeed, increased intracellular pH is an established permissive signal for cellular proliferation promoting survival by limiting apoptosis, a process that is associated with intracellular acidification [17, 18]. The role of low extracellular pH in carcinogenesis is thus paradoxical: on one hand, alkaline intracellular pH promotes proliferation and survival, while at the same time, extracellular pH promotes invasion and metastasis at the cost of inducing stress, senescence, and apoptosis [12, 19, 20].

In addition to glucose, glutamine metabolism is also essential for the proliferation of cancer cells. Recent studies have demonstrated that glutamate derived from glutamine is utilized by highly proliferative cells to generate non-essential amino acids (NEAAs) through the

glutamic-oxaloacetic transaminase enzymes (*GOT1* and *GOT2*), while quiescent cells metabolize glutamate through *GLUD1* (glutamate dehydrogenase 1) and subsequent decarboxylation reactions in the TCA cycle [21, 22]. Thus, glutamine can be metabolized through both anabolic (anaplerotic) and catabolic pathways.

Several oncogenes are implicated in reprogramming tumor cell metabolism. One such gene is *KRAS*, which upon accumulating activating mutations serves as a key signature oncogene that serves a prominent role in malignant transformation and tumor progression in PDAC [23, 24]. PDAC cells with oncogenic *KRAS* have reprogrammed glucose and glutamine metabolism to serve anabolic processes [25, 26]. Canonical glutamine metabolism occurs through glutamate synthase (*GLS*)-mediated conversion of cytoplasmic glutamine into glutamate. Glutamate is then metabolized in the mitochondria through *GLUD1* into alpha-ketoglutarate that enters the TCA cycle [27]. The non-canonical pathway metabolizes glutamate to aspartate and alpha-ketoglutarate through *GOT2*; aspartate is subsequently metabolized to oxaloacetate by *GOT1* in the cytosolic compartment. Aspartate is metabolized by malate dehydrogenase (*MDH*) to malate, which is then metabolized by malic enzyme (*ME*) to produce pyruvate. These anaplerotic reactions increase the NADPH/NADP ratio thereby maintaining ROS balance. PDAC cells are dependent on these reactions for maintenance of intracellular ROS levels as it is evidenced by the decrease in cell survival upon knockdown of enzymes in the pathway [26].

Due to metabolic reprogramming by oncogenic *KRAS* present in 90% of PDAC cases, extracellular acidification is highly abundant. While the regulation of pH in cancer cells has been studied thoroughly, the metabolic adaptations to chronic acidosis induced stress are not well defined. Therefore, in the current study, we investigated the metabolic basis of adaptation to chronic low pH stress in PDAC cells, which exhibit high glycolytic capacity, by subjecting them to chronic acidosis. We utilized PDAC cells with oncogenic *KRAS* to identify the metabolomic alterations in PDAC cells under chronic acidosis and identify vulnerabilities for therapy. Here, we report a pronounced increase in non-canonical anaplerotic glutamine metabolism, which serves the bioenergetic needs and maintains ROS balance in cells undergoing acidosis stress.

2. Materials and methods

2.01 Cell culture

Cell culture of PDAC cell lines S2-013 and Capan-1 have been described previously [28, 29]. Cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich D5648) containing 4.5g/L of glucose and 0.584g/L of glutamine (Hyclone); additionally, the media was supplemented with 5% FBS. Low pH of the media was set at 6.9~7.1 by adding 1g/L NaHCO₃ and control pH was set by using 3.7g/L NaHCO₃. To establish chronic low pH exposure, we cultured the cells in pH 6.9~7.0 continuously for 14 days. Cells were maintained in low pH and control pH media for all experiments.

Cell transfections for producing replication-incompetent lentivirus were performed by utilizing Turbofect followed the manufacturer's protocol [28, 30]. Stable short hairpin RNA (shRNA) constructs were obtained from Sigma-Aldrich: shGOT1 (34784;

CCGGGCGTTGGTACAATGGAACAACTCGAGTTTGGTTCCATTGTACCAACGCTTTT TG) and shGOT1 (34785; CCGGGCTAATGACAATAGCCTAAATCTCGAGATTTAGGC TATTGTCATTAGCTTTTTTG). Cells were transfected in control pH culture conditions and after puromycin selection and knockdown validation clones were plated in low pH for 14 days to establish chronic acidosis. Cells were validated by STR profiling.

2.02 Metabolomics

Polar metabolite isolation was performed as described previously [31]. In short, 0.75×10^7 cells were cultured for 24h in regular DMEM. Cells were then washed with PBS and culture medium was exchanged with fresh medium 2 hours before metabolite extraction, the pH of the media was maintained. Polar metabolites were then extracted with 80% methanol. Metabolite extracts were subjected to LC-MS/MS analysis using multiple reaction monitoring methods described previously [32]. Data acquisition was carried out utilizing AnalystTM 1.6 software (AB SCIEX), and peaks were integrated with MultiquantTM (AB SCIEX). Peak areas were normalized to the respective protein concentrations. Extraction and analysis of polar metabolites were performed three times after cells had been cultured in low pH for 14-days.

2.03 Reactive oxygen species assay

Reactive oxygen species levels were determined by using oxidation-sensitive fluorescent dye 2',7'-dichlorofluorescein diacetate (DCFDA). Control and low pH cells were seeded at 3.0×10^4 cells per well in a clear bottom black 96-well plate. After the cells had adhered, the media was replaced with fresh DMEM containing 10 μ M DCFDA, with or without respective treatments. H₂O₂ was used as a positive control and N-acetyl cysteine (NAC) was used as a negative control. Control, and treated cells were incubated at 37°C for 30 mins. The cells were washed with PBS and 100uL of PBS was added to the wells for measuring the emission of DCFDA using Biotek Cytation3 plate reader. DCFDA was measured using an excitation of 495nm and an emission of 529nm. These experiments were repeated three times with similar results.

2.04 Colony formation assay

Refer to supplemental information.

2.05 Cell cycle analysis

Refer to supplemental information.

2.06 Glucose/Glutamine Uptake

Refer to supplemental information.

2.07 Lactate Release

Refer to supplemental information.

2.08 ATP assay

Refer to supplemental information.

2.09 Cytotoxic assays

Refer to supplemental information.

2.10 Quantitative real-time PCR

Refer to supplemental information.

3. Results

3. 01 Pancreatic cancer cell growth is diminished under low pH conditions

Intracellular pH value is known to have a significant role in conveying proliferation and death signals [16]. For example, it has been observed that proliferating cells require an intracellular alkaline pH value greater than 7.2, to allow for growth-factor stimulated cells to enter the S-phase of the cell cycle at a faster rate, and proceed to the G₂ and M phases more rapidly [33, 34]. Furthermore, a higher pH is known to suppress mitotic arrest due to activated DNA damage checkpoints; therefore, maintaining an alkaline intracellular pH enhances bypassing of cell cycle checkpoints allowing cells to have unrestricted proliferation [35, 36]. While intracellular acidic pH promotes pro-apoptotic *BAX* by enhancing conformational changes that facilitate mitochondrial insertion thereby increasing pore formation allowing increased permeability to the mitochondria and release of pro-apoptotic proteins such as cytochrome-c into the cytosol [37]. PDAC cells in culture (pH 7.4) exhibit the Warburg Effect [28]. Hence, we investigated whether chronic acidosis of extracellular milieu would have an effect on cell growth. To address this question, we determined the growth kinetics of PDAC cell lines (S2-013 and Capan-1) in various acidic pH values of tumors reported in the literature and identified that the pH value between 6.9–7.0 resulted in significant growth reduction when compared to the physiological pH (Fig. 1A). To determine if reduced growth in low pH is due to reduced clonogenicity we conducted colony formation assays and identified that cells in low pH culture had reduced clonogenicity compared to the cells that are cultured in control pH (Fig. 1B). Subsequently, we synchronized growth of cells cultured in control and low pH conditions with a double thymidine block and released them for 20 hours before collecting them to be fixed and stained with propidium iodide, to determine DNA content using flow cytometry. We identified that cells in the G₁/G₀ phases are significantly increased in low pH culture; we further observed decreased percentage of cells in the S and M phases (Fig. 1C). This data indicates cell cycle arrest in the G₁/S transition in PDAC cells in the low pH environment that results in reduced rate of cell cycle progression and growth.

3. 02 Reduced glucose uptake and metabolism in low pH conditions

Rapid growth and progression through the cell cycle are associated with up-regulation of glycolysis. We thus performed H³- glucose uptake assays and determined that cells in low pH culture have a significant reduction in glucose uptake compared to cells in control pH (Fig. 2A). We also conducted a lactate release assay and found that in chronic low pH exposure there is a significant reduction in lactate release (Fig. 2B). To determine if the reduction in glucose uptake and lactate release was due to a decrease in glycolysis, we isolated polar metabolites from cells in both control and low pH culture and conducted

liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS)-based metabolomics analyses. Our LCMS analysis demonstrated that glycolysis metabolites are significantly reduced at low pH (Fig. 2C). Collectively, our data demonstrates a clear departure from the classic Warburg Effect metabolic phenotype.

3.03 Increased glutamine metabolism and oxidative phosphorylation in low pH conditions

Our metabolomics data also demonstrated that cells in low pH have an active mitochondrial metabolism (Fig. 3A). As a parallel nutrient source, glutamine is essential for oxidative metabolism because it can be metabolized to generate alpha-ketoglutarate and enter the TCA cycle [27]. To evaluate if alterations in glutamine metabolism complement for the reduced glucose metabolism, we next evaluated glutamine uptake. We supplemented cells with H³-glutamine and observed that cells in chronic acidosis stress have a significant increase in glutamine uptake (Fig. 3B). To determine if the increase in glutamine uptake had an effect on oxidative metabolism and ATP generation we collected cell lysates from cells cultured in low pH and control pH conditions, and observed that cells in low pH generate significantly more ATP than cells in control pH culture (Fig. 3C). Next, we treated both control and low pH cells with oligomycin, an inhibitor of complex V (ATP synthase) in the electron transport chain, which acts by blocking the channel formed by the F₀ complex of ATP synthase inhibiting H⁺ movement down its concentration gradient, thereby preventing ATP synthesis [38]. Treatment of cells in control and low pH with oligomycin showed that cells in low pH are much more sensitive than cells in control pH, by several orders of magnitude (Fig. 3D). These data demonstrate that chronic acidosis influences a metabolic shift that enhances glutamine metabolism over glucose metabolism resulting in a change in ATP generation from substrate level phosphorylation to oxidative phosphorylation.

The metabolomic analysis identified a significant increase in the levels of metabolites involved in non-canonical glutamine metabolism in S2-013 and Capan1 cells cultured under chronic low pH (Fig. 4A). Furthermore, the qPCR analysis demonstrated increased mRNA levels of genes involved in non-canonical anaplerotic glutamine metabolism under chronic acidosis conditions (Fig. 4B). To further validate these observations, cells in control and low pH were treated with metabolic inhibitors targeting canonical and non-canonical glutamine metabolism. Cells were treated with aminooxyacetic acid (AOA) and epigallocatechin gallate (EGCG) to inhibit the transaminase enzymes (*GOT1*, *GOT2*) and *GLUD1*, respectively. We determined that the cells in low pH were at least an order of magnitude more sensitive to AOA treatment (Fig. 4C); however, the inhibitory concentration of EGCG was only modestly different in control and low pH (Fig. 4D). Hence, our data indicate that cells with chronic acidosis develop a metabolic phenotype that is highly dependent on anaplerotic glutamine metabolism, as demonstrated by increased metabolite levels, enzyme transcription, and increased sensitivity to a transaminase inhibitor (Fig. 4E).

3.04 GOT1 counters ROS production under low pH conditions

Based on our metabolomics analysis, we predicted that the low glycolytic rate observed in chronic acidosis reduces the flow of glycolysis metabolites into the pentose phosphate pathway, thereby reducing the generation of glycolysis-derived NADPH. However, anaplerotic glutamine metabolism can provide the fuel for proliferation by generating ATP

through the TCA cycle, generation of NADPH for redox reactions, and production of NEAAs used in protein biosynthesis [22, 26, 27]. GOT1 converts aspartate into oxaloacetate that eventually gets converted into pyruvate by a reaction catalyzed by malic enzyme 1 (ME1) [26]. ME1 catalysis results in the production of NADPH that allows cells to quench ROS. Thus, we speculated that up-regulation of anaplerotic glutamine metabolism is required for maintenance of intracellular ROS during chronic acidosis-induced stress. To this end, we identified increased *GOT1* levels in low pH conditions. GOT1 is a cytosolic enzyme that is critical for connecting the mitochondria and cytosolic compartments in glutamine anaplerosis, and hence, allows this metabolic process to complete [26]. To establish the role of GOT1 in ROS maintenance under low pH conditions, we generated stable knockdowns of *GOT1* (Fig. 5A). Recent reports have shown that *GOT1* and glutamine reprogramming are increased in PDAC [39]. Of note, under control pH conditions, we observed minimal growth inhibition in *GOT1* knockdown cells, compared to control shRNA-transfected cells. However, *GOT1* knockdown cells demonstrate significant growth inhibition compared to control cells in low pH (Fig. 5B–C). The most prominent PDAC phenotype we have observed in low pH culture is the increase in oxidative metabolism, which can lead to a substantial increase in reactive oxygen species (ROS). ROS are diverse in their functions, and depending on their concentration they may have different outcomes [40]. For instance, low concentrations of ROS may induce proliferative signaling and activation of survival pathways, but at high levels, there is ROS-induced pathology due to damages in DNA, proteins, and lipids. As a result, oxidative damage may cause in growth inhibition, senescence, and cell death [41–45]. We measured ROS levels using 2,7-dichlorofluorescein diacetate (DCFDA) and found that *GOT1* knockdown cells in low pH have significantly higher levels of ROS than control cells in alkaline pH (Fig. 5D). To further validate these observations, we measured intrinsic ROS levels and ROS levels induced after hydrogen peroxide supplementation using DCFDA. We utilized the ROS-insensitive dye 5(6)-Carboxy-2',7'-dichlorofluorescein diacetate (CDCFDA) as a negative control and H₂O₂ as a positive control (Fig. 5E). Of note, the inhibition of non-canonical glutamine metabolism by *GOT1* knockdown results in increased ROS levels, which are further increased in low pH culture conditions. We conclude that cells in low pH generate more ROS that is increased upon inhibition of anaplerotic glutamine metabolism due to decreased *GOT1*.

Cancer cells are known to have increased expression of enzymes that suppress high ROS levels to prevent senescence and apoptosis [46–48]. ROS production can be increased by oxidation of nicotinamide dinucleotide phosphate (NADPH) by NADPH oxidase enzymes (*NOX*). It can also be, through mitochondrial electron leakage, generating increased superoxide levels [2]. Superoxide levels are reduced by superoxide dismutases (*SOD*) that can combine superoxide radicals with water to produce hydrogen peroxide (H₂O₂), which in turn is capable of initiating redox biology by oxidizing cysteine residues of proteins and initiate signaling events [49]. By LC-MS/MS-based quantification, we analyzed the redox status of the most abundant antioxidant molecules NADPH and GSH. Our analysis revealed that the NADP/NADPH ratio is very similar in both alkaline and acidic pH conditions, but the glutathione disulfide/glutathione (GSSG/GSH) is much higher in cells cultured in low pH (Fig. 5F). These data indicate that the antioxidative capacity of GSH is lesser in low pH. In rapidly proliferating cells, NADPH is mainly produced from glycolysis in the pentose

phosphate pathway, with smaller contributions from isocitrate dehydrogenase and malic enzyme [2]. Our qPCR analysis of the enzymes involved in anaplerotic glutamine metabolism showed that the malic enzymes *ME1* and *ME2* have a significant increase in transcription in low pH (Fig. 4B). We also performed qPCR of the enzymes involved in antioxidant metabolism and identified a very prominent increase in the expression levels of *NOX1*, *NOX2*, and *NOX3* in *GOT1* knockdown cells cultured in low pH, in comparison to control shRNA-transfected cells (Fig. 5G–I). Of note, these transcript level differences are not compensated by an increase in *SOD1/SOD2* mRNA levels (Fig. 5J–K). Therefore, cells with *GOT1* knockdown are unable to withstand low pH microenvironment due to the generation of cytotoxic ROS levels.

3.05 Oxaloacetate can rescue GOT1 knockdown cells under low pH

The reaction carried out by *GOT1* uses aspartate as a substrate and converts it into oxaloacetate (OAA) [26]. Hence, we supplemented knockdown cells in low pH with oxaloacetate to re-establish anaplerotic glutamine metabolism and allow knockdown cells to suppress ROS. As a positive control of ROS suppression, we supplemented cells with N-acetyl cysteine (NAC; ROS quencher), and aspartate and alanine, the non-essential amino acid products of this pathway, were used as negative controls. The growth of cells in low pH was compared to the cells supplemented with 3mM of NAC, 2mM of OAA, or 0.1mM of NEAA for 96 hours. Our results indicate that both NAC and OAA enhance cell growth and colony formation in low pH under *GOT1* knockdown conditions, while supplementation with NEAA had no effect (Fig. 6A–B). To determine if OAA treatment has the same effect as NAC in decreasing ROS levels we supplemented cells with OAA, NAC, and H₂O₂ to subsequently measure ROS levels using DCFDA. Our results showed that only the cells with *GOT1* knockdown cultured in low pH had decreased ROS levels when supplemented with 2mM of OAA (6C). Thus, our data indicates that inhibition of anaplerotic glutamine metabolism by removing *GOT1* results in increased ROS levels and the addition of OAA rescues cell growth by resuming this metabolic pathway.

4. Discussion

Acidification of the tumor microenvironment is a common feature of PDAC (and of most epithelial tumors). Understanding the metabolic changes that PDAC cells undergo due to acidosis stress is extremely critical in designing more effective treatments. Our metabolomic analysis shows that cells in low pH depart from Warburg effect metabolism and that they increase anaplerotic glutamine metabolism to allow cells to generate vast amounts of ATP, which in turn allows for maintaining cellular homeostasis during acidosis stress. We demonstrate for the first time that anaplerotic glutamine metabolism-mediated countering of ROS levels serves as the survival mechanism for pancreatic cancer cells under chronic acidosis.

Glucose and glutamine are the primary nutrients for cancer cells; however, only glutamine can provide both carbon and nitrogen [50]. Indeed, glutamine is an important growth signal [51]. Furthermore, glutamine can be metabolized into products such as nucleic acids, glucosamine, and NEEAs [51]. Based on our experiments to determine cell growth in low

pH and rescue of cell survival with oxaloacetate upon GOT1 depletion, we believe that utilization of glutamine under low pH stress is not meant to induce growth signals or biosynthesis of macromolecules. Instead, it is used for energy biosynthesis to maintain homeostasis through moderation of high ROS levels stressing the cells.

Recent studies have demonstrated the advantages of non-canonical glutamine metabolism over the canonical pathway. In the study conducted by Coloff *et al.*, it was demonstrated proliferating cells could take advantage of metabolism through the transaminase enzymes, whereas, quiescent epithelial cells have decreased transaminase expression; furthermore, glutamine metabolism is diverted to *GLUD* diminishing the biosynthetic potential of glutamine metabolism [22]. Similarly, in the study by Son et al. it was demonstrated that oncogenic *KRAS* plays a significant role in glutamine metabolic reprogramming in PDAC through the transcriptional upregulation of *GOT1* and inhibition of *GLUD1* expression [26]. Furthermore, this and other studies reveal the role of non-canonical anaplerotic glutamine metabolism in the generation of NADPH and possibly ROS regulation through coupling with other redox balance pathways such as glutathione synthesis [26, 52]. Here, we have shown that PDAC cells have the potential to reprogram metabolic pathways allowing them to and maintain homeostasis in acidosis stress conditions.

In our studies, the increase in ROS levels is primarily due to leakage in mitochondrial superoxides from up-regulation of oxidative metabolism during low pH stress and also from up-regulation in the transcription of NADPH oxidases. For this reason, PDAC cells develop increased ROS levels that result in reduced proliferation during low pH stress. Furthermore, we find that inhibition of anaplerotic glutamine metabolism results in an increase in ROS levels and a further reduction in proliferation (Fig 5–6). Glutamine metabolism has the capacity of generating carbon, nitrogenous sources, and NADPH for redox balance [22, 26]. Since various studies have shown that there is increased expression of the transaminase enzymes driving non-canonical glutamine metabolism in PDAC due to oncogenic *KRAS*, we predict that oncogenic *KRAS* plays a significant role in metabolic reprogramming in low pH stress. The pH of the tumor microenvironment is heterogeneous while our experiments maintained a homogeneous pH value, there may be additional differences in the metabolic phenotype that correlates with the pH value inducing cellular stress we have seen here. Our findings may have implications to future therapeutic approaches since we have discovered the metabolic pathways the extracellular pH of the tumor microenvironment can modulate in pancreatic cancer cells. Our data may provide new targets to synergize with other known therapies for pancreatic cancer and increase therapeutic effectiveness against PDAC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by funding from the National Institutes of Health grant (R01 CA163649, R01 CA210439, and R01 CA216853, NCI) to PKS, American Association for Cancer Research (AACR)—Pancreatic Cancer Action Network (PanCAN) Career Development Award (30-20-25-SING) to PKS, and the Specialized Programs of Research Excellence (SPORE, 2P50 CA127297, NCI) to PKS. We would also like to acknowledge the Fred & Pamela Buffett Cancer Center Support Grant (P30CA036727, NCI) for supporting shared resources.

References

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011; 144:646–674. [PubMed: 21376230]
2. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. *Nature reviews Cancer*. 2011; 11:85–95. [PubMed: 21258394]
3. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*. 2009; 324:1029–1033. [PubMed: 19460998]
4. Newsholme EA, Crabtree B, Ardawi MS. The role of high rates of glycolysis and glutamine utilization in rapidly dividing cells. *Bioscience reports*. 1985; 5:393–400. [PubMed: 3896338]
5. Warburg O. On respiratory impairment in cancer cells. *Science*. 1956; 124:269–270. [PubMed: 13351639]
6. Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? *Nature reviews Cancer*. 2004; 4:891–899. [PubMed: 15516961]
7. Rohren EM, Turkington TG, Coleman RE. Clinical applications of PET in oncology. *Radiology*. 2004; 231:305–332. [PubMed: 15044750]
8. Weissleder R. Molecular imaging in cancer. *Science*. 2006; 312:1168–1171. [PubMed: 16728630]
9. I. John Wiley and Sons. *Essentials of Biochemistry, Acids, Bases, and pH*. Wiley; 2004.
10. Poole RC, Halestrap AP. Transport of lactate and other monocarboxylates across mammalian plasma membranes. *The American journal of physiology*. 1993; 264:C761–782. [PubMed: 8476015]
11. Halestrap AP, Wilson MC. The monocarboxylate transporter family--role and regulation. *IUBMB life*. 2012; 64:109–119. [PubMed: 22162139]
12. Parks SK, Chiche J, Pouyssegur J. Disrupting proton dynamics and energy metabolism for cancer therapy. *Nature reviews Cancer*. 2013; 13:611–623. [PubMed: 23969692]
13. Sardet C, Franchi A, Pouyssegur J. Molecular cloning, primary structure, and expression of the human growth factor-activatable Na⁺/H⁺ antiporter. *Cell*. 1989; 56:271–280. [PubMed: 2536298]
14. Forgac M. Vacuolar ATPases: rotary proton pumps in physiology and pathophysiology. *Nature reviews Molecular cell biology*. 2007; 8:917–929. [PubMed: 17912264]
15. Semenza GL. Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. *Critical reviews in biochemistry and molecular biology*. 2000; 35:71–103. [PubMed: 10821478]
16. Webb BA, Chimenti M, Jacobson MP, Barber DL. Dysregulated pH: a perfect storm for cancer progression. *Nature reviews Cancer*. 2011; 11:671–677. [PubMed: 21833026]
17. Lagadic-Gossman D, Huc L, Lecœur V. Alterations of intracellular pH homeostasis in apoptosis: origins and roles. *Cell death and differentiation*. 2004; 11:953–961. [PubMed: 15195071]
18. Matsuyama S, Llopis J, Deveraux QL, Tsien RY, Reed JC. Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. *Nature cell biology*. 2000; 2:318–325. [PubMed: 10854321]
19. Frantz C, Karydis A, Nalbant P, Hahn KM, Barber DL. Positive feedback between Cdc42 activity and H⁺ efflux by the Na-H exchanger NHE1 for polarity of migrating cells. *The Journal of cell biology*. 2007; 179:403–410. [PubMed: 17984318]
20. Pope BJ, Zierler-Gould KM, Kuhne R, Weeds AG, Ball LJ. Solution structure of human cofilin: actin binding, pH sensitivity, and relationship to actin-depolymerizing factor. *The Journal of biological chemistry*. 2004; 279:4840–4848. [PubMed: 14627701]
21. Hensley CT, Wasti AT, DeBerardinis RJ. Glutamine and cancer: cell biology, physiology, and clinical opportunities. *The Journal of clinical investigation*. 2013; 123:3678–3684. [PubMed: 23999442]
22. Coloff JL, Murphy JP, Braun CR, Harris IS, Shelton LM, Kami K, Gygi SP, Selfors LM, Brugge JS. Differential Glutamate Metabolism in Proliferating and Quiescent Mammary Epithelial Cells. *Cell Metab*. 2016; 23:867–880. [PubMed: 27133130]
23. Ying H, Dey P, Yao W, Kimmelman AC, Draetta GF, Maitra A, DePinho RA. Genetics and biology of pancreatic ductal adenocarcinoma. *Genes & development*. 2016; 30:355–385. [PubMed: 26883357]

24. Bardeesy N, DePinho RA. Pancreatic cancer biology and genetics. *Nature reviews Cancer*. 2002; 2:897–909. [PubMed: 12459728]
25. Ying H, Kimmelman AC, Lyssiotis CA, Hua S, Chu GC, Fletcher-Sananikone E, Locasale JW, Son J, Zhang H, Coloff JL, Yan H, Wang W, Chen S, Viale A, Zheng H, Paik JH, Lim C, Guimaraes AR, Martin ES, Chang J, Hezel AF, Perry SR, Hu J, Gan B, Xiao Y, Asara JM, Weissleder R, Wang YA, Chin L, Cantley LC, DePinho RA. Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism. *Cell*. 2012; 149:656–670. [PubMed: 22541435]
26. Son J, Lyssiotis CA, Ying H, Wang X, Hua S, Ligorio M, Perera RM, Ferrone CR, Mullarky E, Shyh-Chang N, Kang Y, Fleming JB, Bardeesy N, Asara JM, Haigis MC, DePinho RA, Cantley LC, Kimmelman AC. Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. *Nature*. 2013; 496:101–105. [PubMed: 23535601]
27. Owen OE, Kalhan SC, Hanson RW. The key role of anaplerosis and cataplerosis for citric acid cycle function. *The Journal of biological chemistry*. 2002; 277:30409–30412. [PubMed: 12087111]
28. Chaika NV, Gebregiorgis T, Lewallen ME, Purohit V, Radhakrishnan P, Liu X, Zhang B, Mehla K, Brown RB, Caffrey T, Yu F, Johnson KR, Powers R, Hollingsworth MA, Singh PK. MUC1 mucin stabilizes and activates hypoxia-inducible factor 1 alpha to regulate metabolism in pancreatic cancer. *Proceedings of the National Academy of Sciences of the United States of America*. 2012; 109:13787–13792. [PubMed: 22869720]
29. Deer EL, Gonzalez-Hernandez J, Coursen JD, Shea JE, Ngatia J, Scaife CL, Firpo MA, Mulvihill SJ. Phenotype and genotype of pancreatic cancer cell lines. *Pancreas*. 2010; 39:425–435. [PubMed: 20418756]
30. T.F.S. Inc. T.F.S. Inc. Thermo Scientific TurboFect Transfection Reagent. Thermo Scientific; 2013.
31. Gunda V, Yu F, Singh PK. Validation of Metabolic Alterations in Microscale Cell Culture Lysates Using Hydrophilic Interaction Liquid Chromatography (HILIC)-Tandem Mass Spectrometry-Based Metabolomics. *PLoS One*. 2016; 11:e0154416. [PubMed: 27120458]
32. Yuan M, Breitkopf SB, Yang X, Asara JM. A positive/negative ion-switching, targeted mass spectrometry-based metabolomics platform for bodily fluids, cells, and fresh and fixed tissue. *Nature protocols*. 2012; 7:872–881. [PubMed: 22498707]
33. Pouyssegur J, Franchi A, L'Allemain G, Paris S. Cytoplasmic pH, a key determinant of growth factor-induced DNA synthesis in quiescent fibroblasts. *FEBS letters*. 1985; 190:115–119. [PubMed: 4043390]
34. Putney LK, Barber DL. Na-H exchange-dependent increase in intracellular pH times G2/M entry and transition. *The Journal of biological chemistry*. 2003; 278:44645–44649. [PubMed: 12947095]
35. Zhao R, Oxley D, Smith TS, Follows GA, Green AR, Alexander DR. DNA damage-induced Bcl-xL deamidation is mediated by NHE-1 antiport regulated intracellular pH. *PLoS biology*. 2007; 5:e1. [PubMed: 17177603]
36. Liao C, Hu B, Arno MJ, Panaretou B. Genomic screening in vivo reveals the role played by vacuolar H⁺ ATPase and cytosolic acidification in sensitivity to DNA-damaging agents such as cisplatin. *Molecular pharmacology*. 2007; 71:416–425. [PubMed: 17093137]
37. Khaled AR, Kim K, Hofmeister R, Muegge K, Durum SK. Withdrawal of IL-7 induces Bax translocation from cytosol to mitochondria through a rise in intracellular pH. *Proceedings of the National Academy of Sciences of the United States of America*. 1999; 96:14476–14481. [PubMed: 10588730]
38. Devenish RJ, Prescott M, Boyle GM, Nagley P. The oligomycin axis of mitochondrial ATP synthase: OSCP and the proton channel. *Journal of bioenergetics and biomembranes*. 2000; 32:507–515. [PubMed: 15254386]
39. Feld FM, Nagel PD, Weissinger SE, Welke C, Stenzinger A, Moller P, Lennerz JK. GOT1/AST1 expression status as a prognostic biomarker in pancreatic ductal adenocarcinoma. *Oncotarget*. 2015; 6:4516–4526. [PubMed: 25595905]
40. Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. *Current biology : CB*. 2014; 24:R453–462. [PubMed: 24845678]

41. Giannoni E, Buricchi F, Raugei G, Ramponi G, Chiarugi P. Intracellular reactive oxygen species activate Src tyrosine kinase during cell adhesion and anchorage-dependent cell growth. *Molecular and cellular biology*. 2005; 25:6391–6403. [PubMed: 16024778]
42. Rhee SG, Kang SW, Jeong W, Chang TS, Yang KS, Woo HA. Intracellular messenger function of hydrogen peroxide and its regulation by peroxiredoxins. *Current opinion in cell biology*. 2005; 17:183–189. [PubMed: 15780595]
43. Ohtani N, Yamakoshi K, Takahashi A, Hara E. The p16INK4a-RB pathway: molecular link between cellular senescence and tumor suppression. *The journal of medical investigation : JMI*. 2004; 51:146–153. [PubMed: 15460900]
44. Garrido C, Galluzzi L, Brunet M, Puig PE, Didelot C, Kroemer G. Mechanisms of cytochrome c release from mitochondria. *Cell death and differentiation*. 2006; 13:1423–1433. [PubMed: 16676004]
45. Han D, Antunes F, Canali R, Rettori D, Cadenas E. Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol. *The Journal of biological chemistry*. 2003; 278:5557–5563. [PubMed: 12482755]
46. Bae YS, Kang SW, Seo MS, Baines IC, Tekle E, Chock PB, Rhee SG. Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. *The Journal of biological chemistry*. 1997; 272:217–221. [PubMed: 8995250]
47. Sundaresan M, Yu ZX, Ferrans VJ, Irani K, Finkel T. Requirement for generation of H₂O₂ for platelet-derived growth factor signal transduction. *Science*. 1995; 270:296–299. [PubMed: 7569979]
48. Vaughn AE, Deshmukh M. Glucose metabolism inhibits apoptosis in neurons and cancer cells by redox inactivation of cytochrome c. *Nature cell biology*. 2008; 10:1477–1483. [PubMed: 19029908]
49. Fridovich I. Superoxide anion radical (O₂^{•-}), superoxide dismutases, and related matters. *The Journal of biological chemistry*. 1997; 272:18515–18517. [PubMed: 9228011]
50. Cooke MS, Evans MD, Dizdaroglu M, Lunec J. Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2003; 17:1195–1214. [PubMed: 12832285]
51. Hirschey MD, DeBerardinis RJ, Diehl AM, Drew JE, Frezza C, Green MF, Jones LW, Ko YH, Le A, Lea MA, Locasale JW, Longo VD, Lyssiotis CA, McDonnell E, Mehrmohamadi M, Michelotti G, Muralidhar V, Murphy MP, Pedersen PL, Poore B, Raffaghello L, Rathmell JC, Sivanand S, Vander Heiden MG, Wellen KE. T. Target Validation. Dysregulated metabolism contributes to oncogenesis. *Seminars in cancer biology*. 2015; 35(Suppl):S129–150. [PubMed: 26454069]
52. Morgan B, Sobotta MC, Dick TP. Measuring E(GSH) and H₂O₂ with roGFP2-based redox probes. *Free radical biology & medicine*. 2011; 51:1943–1951. [PubMed: 21964034]
53. Bostock CJ, Prescott DM, Kirkpatrick JB. An evaluation of the double thymidine block for synchronizing mammalian cells at the G1-S border. *Experimental cell research*. 1971; 68:163–168. [PubMed: 5165443]

Highlights

- Pancreatic cancer cell growth is significantly reduced under low pH conditions
- Low pH diminishes glucose uptake and metabolism
- Low pH facilitates glutamine metabolism and oxidative phosphorylation
- GOT1-mediated anaplerotic metabolism counters ROS production under low pH conditions
- Oxaloacetate can rescue GOT1 knockdown cells under low pH

Figure 1. Chronic acidosis reduces proliferation of PDAC cells

(A) Survival of S2-013 and Capan-1 PDAC cells cultured under conditions of varying pH of culture media by MTT assays. (B) 14-day Colony formation assays for cells cultured in control pH (7.4) and low pH (7.0). (C) Cell cycle analysis of S2-013 cells in control and low pH. The bar chart on the left represents percent distribution of cells in different phases of cell cycle. Data in figures 1A and 1B was normalized to that of the control pH 7.4 (physiological pH value). Error bars represent mean $\pm$ S.E.M. from at least three different samples. Two-way ANOVA with Bonferroni post-test analysis was used for figure 1A to compare growth in low pH versus physiological pH. A two-tailed Student's t-test was used to compare low pH and control pH (B and C) with p-values * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Figure 2. PDAC cells under chronic acidosis demonstrate reduced aerobic glycolysis (A) ^3H -glucose uptake and (B) lactate release in S2-013 and Capan-1 cells at control (7.4) and low pH (7.0). (C) LC-MS/MS-based metabolomics analysis of glycolysis metabolites from cells cultured at control and low pH. Data is normalized to that of the cells at control pH (7.4). Error bars represent mean $\pm$ S.E.M. from at least three different replicates. A two-tailed Student's t-test (A–C) was conducted to compare metabolites in cells at low pH relative to control pH with p-values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3. Oxidative metabolism is enhanced in low pH culture conditions

(A) LC-MS/MS-based metabolomics analysis of TCA cycle metabolites in control (7.4) and low pH (7.0) culture conditions. (B) ^3H -glutamine uptake of S2-013 and Capan-1 in control and low pH conditions. (C) Intracellular ATP levels of S2-013 and Capan-1 in control and low pH conditions. (D) Sensitivity of S2-013 and Capan-1 cells to oligomycin in control and low pH. Data in bar charts is normalized to the values for the control pH (7.4). Error bars represent mean $\pm$ S.E.M. from at least three different samples. A two-tailed Student's t-test was conducted on Figure 3A–3C with p-values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4. Non-canonical anaplerotic glutamine metabolism is enhanced in chronic acidosis
 (A) LC-MS/MS-based metabolomic analysis of non-canonical glutamine metabolism in control (7.4) and low pH (7.0) culture conditions. (B) Quantitative real-time PCR analysis of genes coding for enzymes involved in non-canonical glutamine metabolism in cells cultured under control and low pH. (C–D) Sensitivity of S2-013 and Capan-1 cells to treatment with aminooxyacetic acid (AOA; C) and to epigallocatechin gallate (EGCG; D) in control and low pH conditions. (E) A schematic illustration of potential metabolite flow under low pH. Bold arrows indicate glutamine metabolism in low pH through the non-canonical pathway. Data in bar charts is normalized to the values for the control pH (7.4). Error bars represent mean $\pm$ S.E.M. from at least three different replicates. A two-tailed Student's t-test was

conducted to compare control versus low pH in Figure 4A and 4B with p-values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5. GOT1 regulates cellular ROS levels in chronic acidosis

(A) Western blotting to confirm the knockdown levels of GOT1 in S2-013 with two independent targets by utilizing lentiviral delivery. Cell growth of *GOT1* knockdown and scrambled-control (shScr) cells in control pH (B) and low pH (C). (D) Measurement of intracellular ROS using carboxy- H_2 DCFDA in control and low pH. (E) Measurement of intracellular ROS by staining with carboxy- H_2 DCFDA (DCFDA), using ROS-insensitive carboxy-DCFDA (CDCF) dye as a control. (F) LC-MS/MS measurement of NADP/NADPH and GSSG/GSH ratio in control and low pH. (G-K) Quantitative real-time PCR analysis of

genes coding for enzymes involved in ROS regulation. Data in bar charts is normalized to the values for the control pH (7.4). Two-tailed Student's t-test was used on Figure 5F to compare control versus low pH. A two-way ANOVA analysis, followed by Bonferroni posttests, was conducted to compare different treatments represented on all the other panels with p-values * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6. Non-canonical glutamine metabolism regulates ROS levels in chronic acidosis

Growth kinetics of scrambled control (A), and *GOT1* knockdown cells (B–C) cultured in low pH supplemented with N-acetyl cysteine (NAC), non-essential amino acids (NEAA), and oxaloacetate (OAA). (B) Colony formation of control and *GOT1* knockdown cells supplemented with NAC and OAA. (C) Intracellular ROS levels in control and *GOT1* knockdown cells supplemented with OAA and NAC, using H_2O_2 as a positive control. Data in bar charts is normalized to the values for untreated scrambled control. Data normalized to control pH 7.4 (physiological pH value). Error bars represent mean $\pm$ S.E.M. from at least three different replicates. A two-way ANOVA analysis, followed by Bonferroni post-tests, was conducted to compare different treatments with p-value * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.