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ORIGINAL ARTICLE

Basic Study

Glutathione metabolism is essential for self-renewal and chemoresistance of pancreatic cancer stem cells

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Author contributions: Jagust P and Sancho P performed the experiments, acquired and analyzed data; Alcalá S and Sainz Jr B compiled and validated the samples for RNAseq analysis; Heeschen C and Sancho P interpreted data, designed the study and wrote the manuscript; all authors approved the final version of the manuscript.

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Abstract

BACKGROUND

Cellular metabolism regulates stemness in health and disease. A reduced redox state is essential for self-renewal of normal and cancer stem cells (CSCs). However, while stem cells rely on glycolysis, different CSCs, including pancreatic CSCs, favor mitochondrial metabolism as their dominant energy-producing pathway. This suggests that powerful antioxidant networks must be in place to detoxify mitochondrial reactive oxygen species (ROS) and maintain stemness in oxidative CSCs. Since glutathione metabolism is critical for normal stem cell function and CSCs from breast, liver and gastric cancer show increased glutathione content, we hypothesized that pancreatic CSCs also rely on this pathway for ROS detoxification.

AIM

To investigate the role of glutathione metabolism in pancreatic CSCs.

METHODS

Primary pancreatic cancer cells of patient-derived xenografts (PDXs) were cultured in adherent or CSC-enriching sphere conditions to determine the role of glutathione metabolism in stemness. Real-time polymerase chain reaction (PCR) was used to validate RNAseq results involving glutathione metabolism genes in adherent *vs* spheres, as well as the expression of pluripotency-related genes following treatment. Public TCGA and GTEx RNAseq data from pancreatic cancer

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vs normal tissue samples were analyzed using the webserver GEPIA2. The glutathione-sensitive fluorescent probe monochlorobimane was used to determine glutathione content by fluorimetry or flow cytometry. Pharmacological inhibitors of glutathione synthesis and recycling [buthionine-sulfoximine (BSO) and 6-Aminonicotinamide (6-AN), respectively] were used to investigate the impact of glutathione depletion on CSC-enriched cultures. Staining with propidium iodide (cell cycle), Annexin-V (apoptosis) and CD133 (CSC content) were determined by flow cytometry. Self-renewal was assessed by sphere formation assay and response to gemcitabine treatment was used as a readout for chemoresistance.

RESULTS

Analysis of our previously published RNAseq dataset E-MTAB-3808 revealed up-regulation of genes involved in the KEGG (Kyoto Encyclopedia of Genes and Genomes) Pathway Glutathione Metabolism in CSC-enriched cultures compared to their differentiated counterparts. Consistently, in pancreatic cancer patient samples the expression of most of these up-regulated genes positively correlated with a stemness signature defined by *NANOG*, *KLF4*, *SOX2* and *OCT4* expression ($P < 10^{-5}$). Moreover, 3 of the upregulated genes (*MGST1, GPX8, GCCT*) were associated with reduced disease-free survival in patients [Hazard ratio (HR) 2.2-2.5; $P = 0.03$-0.0054], suggesting a critical role for this pathway in pancreatic cancer progression. CSC-enriched sphere cultures also showed increased expression of different glutathione metabolism-related genes, as well as enhanced glutathione content in its reduced form (GSH). Glutathione depletion with BSO induced cell cycle arrest and apoptosis in spheres, and diminished the expression of stemness genes. Moreover, treatment with either BSO or the glutathione recycling inhibitor 6-AN inhibited self-renewal and the expression of the CSC marker CD133. GSH content in spheres positively correlated with intrinsic resistance to gemcitabine treatment in different PDXs $r = 0.96$, $P = 5.8 \times 10^{11}$). Additionally, $CD133^+$ cells accumulated GSH in response to gemcitabine, which was abrogated by BSO treatment ($P < 0.05$). Combined treatment with BSO and gemcitabine-induced apoptosis in $CD133^+$ cells to levels comparable to $CD133^-$ cells and significantly diminished self-renewal ($P < 0.05$), suggesting that chemoresistance of CSCs is partially dependent on GSH metabolism.

CONCLUSION

Our data suggest that pancreatic CSCs depend on glutathione metabolism. Pharmacological targeting of this pathway showed that high GSH content is essential to maintain CSC functionality in terms of self-renewal and chemoresistance.

Key Words: Pancreatic cancer; Cancer stem cells; Glutathione; Self-renewal; Chemoresistance; Redox

Core Tip: Several glutathione metabolism genes are upregulated in pancreatic cancer stem cells (CSCs), and their expression correlates with a stemness signature and predicts survival in clinical samples. Increased glutathione concentration in CSCs promotes viability, cell cycle progression and pluripotency gene expression. Inhibition of glutathione synthesis or recycling impairs CSC functionalities such as self-renewal and chemoresistance. Our data demonstrate a targetable metabolic vulnerability of this aggressive subpopulation of cancer cells.

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INTRODUCTION

Pancreatic cancer has the worst outcome of any cancer in the world, and is currently the 3rd most frequent cause of cancer-related deaths[1]. At the same time, the incidence of pancreatic cancer keeps increasing, with approximately 448000 new cases in 2019. This number is predicted to further increase in the coming years and, due to its extreme lethality and lack of effective treatments available[2], pancreatic cancer may even become the 2nd most frequent cause of cancer-related deaths by 2030[3].

One possible explanation for the poor outcome associated with pancreatic cancer is that, previously, only little attention had been paid to the efficient elimination of cancer stem cells (CSCs). Pancreatic cancer contains stem cell-like cells, which are the sole drivers of tumorigenesis, much like normal stem cells fuel proliferation and differentiation in normal tissue, and therefore have been termed CSCs[4-8]. While CSCs represent only a small fraction of all the cancer cells, they are extremely tumorigenic down to a single cell, and exclusively metastatic[4,6]. Current treatment strategies for pancreatic cancer spare CSCs due to their inherent chemoresistance[8-10] and therefore likely represent the key cellular source driving disease relapse. To develop more effective treatment strategies for pancreatic cancer, we need to obtain a thorough understanding of the regulatory machinery of CSCs, including their cellular metabolism.

Increasing evidence suggests that, similar to normal stem cells, cellular metabolism is highly regulated in CSCs and governs essential aspects of their functionality. Indeed, a reduced intracellular redox state with low reactive oxygen species (ROS) levels allows for self-renewal, while ROS accumulation induces differentiation[11]. Since relatively high amounts of ROS are formed as by-products of mitochondrial oxidative phosphorylation, glycolysis was traditionally considered the preferred metabolic pathway for both stem cells and CSCs[12]. However, CSCs across a wide range of cancers maintain low ROS levels[13-15], even if they favor mitochondrial metabolism as their dominant energy-producing pathway[16]. In particular, we have shown that pancreatic CSCs are dependent on mitochondrial oxidative phosphorylation for full stemness and tumorigenicity, in a process controlled by the balanced expression of c-MYC and the mitochondrial biogenesis factor PGC-1α[7]. We observed that pancreatic CSCs bear low levels of mitochondrial ROS and they are especially sensitive to the mitochondrial ROS inducer menadione, making the redox state of this organelle a relevant target for CSC elimination.

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH) is the most abundant non-protein antioxidant in eukaryotic cells. Although it is synthesized in the cytosol, glutathione levels are particularly high in mitochondria, where it maintains redox balance through ROS detoxification and protects phospholipids in the mitochondrial membrane[17]. Interestingly, glutathione levels are strongly increased in murine embryonic stem cells and mesenchymal stem cells, supporting defense against diverse insults and maintenance of stemness[18]. Similarly, glutathione content and specific GSH-related enzymes are up-regulated in CSCs from breast[13], liver[19] and gastric cancer[14]. Additionally, different components of the glutathione metabolism pathway have been shown to promote tumor initiation[20], metastasis[21,22] and chemoresistance[23]; features frequently associated with CSCs.

However, little is known about the role of this pathway in pancreatic CSCs. Here we now show that pancreatic CSCs display increased GSH content and expression of diverse genes involved in the glutathione metabolism pathway, which correlated with stemness and disease-free survival in pancreatic cancer patients. Depletion of GSH levels in sphere cultures with pharmacological inhibitors of glutathione synthesis or recycling induced cell cycle arrest and apoptosis. This translated into diminished self-renewal capacity and reduced expression of the CSC surface marker CD133. Importantly, GSH depletion sensitized CSCs to gemcitabine, suggesting an important role for glutathione in chemoresistance in pancreatic cancer.

MATERIALS AND METHODS

Primary human pancreatic cancer cells

Human pancreatic cancer Patient-Derived Xenografts [PDXs, either pancreatic ductal adenocarcinoma (PDAC) or pancreatic cancer of hepatobiliary origin] were obtained through the Biobank of the Spanish National Cancer Research Centre (CNIO), Madrid, Spain (reference 1204090835CHMH). For primary cultures, PDX tissue fragments previously expanded in nude mice (passages 1-13) were minced, enzymatically

digested with collagenase (Stem Cell Technologies, Vancouver, Canada) for 90 min at 37°C[24], and after centrifugation for 5 min at 1200 rpm the pellets were resuspended and cultured in RPMI, 10% fetal bovine serum (FBS), and 50 U/mL penicillin/streptomycin. For the experiments, cells were cultured in DMEM:F12 supplemented with B-27, L-Glutamine (all from Gibco, Life Technologies, Carlsbad, CA, USA), 50 U/mL penicillin–streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and β-FGF (PeproTech, Rocky Hill, NJ, USA).

CSC-enriching culture

Pancreatic cancer spheres were generated and expanded in supplemented DMEM-F12. A total of 10^4 cells/mL was seeded in ultra-low attachment plates (Corning, Corning, NY, USA) as described previously[25].

Sphere formation assay

Cells were seeded in triplicate in ultra-low attachment 24-well plates (Corning) at 10^4 cells/well in supplemented DMEM-F12 with or without the corresponding treatments, which were refreshed every other day. After 7 d, spheres were counted using a microscope at 20× magnification.

RNA preparation and real-time polymerase chain reaction

Total RNAs from human primary pancreatic cancer cells and spheres were extracted with the TRIzol kit (Life Technologies) according to the manufacturer's instructions. One microgram of total RNA was used for cDNA synthesis with SuperScript II reverse transcriptase (Life Technologies) and random hexamers. Quantitative real-time polymerase chain reaction (PCR) was performed using SYBR Green PCR master mix (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The list of utilized primers is detailed in Table 1.

GSH content

The glutathione-sensitive fluorescent probe monochlorobimane (mCLB, Sigma-Aldrich) was used to analyze the reduced intracellular glutathione content. Cells were trypsinized on the day of the experiment, washed, protected from light and incubated for 1 h at 37°C with 2 mmol/L mCLB diluted in phosphate buffered saline (PBS). Cells were then washed and resuspended in PBS and fluorescence (excitation: 380 nm, emission: 461 nm) was measured on a FLUOstar OPTIMA Microplate Reader. Values were corrected for cell content by measuring the protein concentration of the sample with the Bradford assay. Alternatively, cells were incubated with DAPI and analyzed by flow cytometry using a FACS Canto II (BD, Franklin Lakes, NJ, USA) and data were analyzed with FlowJo 9.2 software (Ashland, OR, USA) or Cytobank (Beckman Coulter, CA, USA).

CSC content by flow cytometry

To identify pancreatic CSCs, the anti-CD133/1 (APC or PE, Miltenyi Biotec, Bergisch Gladbach, Germany) or corresponding control Immunoglobulin G (IgG) 1 antibody were used. Briefly, cells were stained for 30 min on ice with gentle rocking. DAPI was used for exclusion of dead cells (eBiosciences, San Diego, CA, USA). All samples were analyzed by flow cytometry using a FACS Canto II (BD) and data were analyzed with FlowJo 9.2 software or Cytobank.

Apoptosis assay by flow cytometry

Five-day old spheres or adherent cultures were treated for 48 h in the presence of 100 mmol/L buthionine-sulfoximine (BSO) or BSO and gemcitabine (1 μmol/L). Attached and floating cells were collected, resuspended and stained with Annexin V (550474) diluted in Annexin V binding buffer (556454, all from eBiosciences) for 20 min at room temperature, following the manufacturer's instructions. Cells were then incubated with DAPI for an additional 5 min.

Cell-cycle analysis by flow cytometry

Spheres were trypsinized, washed in PBS, centrifuged, and pellets were fixed in 200 μL of 70% ethanol and stored at -20°C until use. The cells were centrifuged and pellets resuspended in 200 μL of PBS, 10 μg/mL of RNAse A was added and the cells were incubated for 1 h at 37°C. Subsequently, the cells were resuspended in propidium iodide solution (0.1% sodium citrate, 0.1% TritonX-100, and 50 μg/mL propidium iodide).

Table 1 List of utilized primers

Gene	Primer
HPRT	
Forward	TGACCTTGATTTATTTTGCATACC
Reverse	CGAGCAAGACGTTCAGTCCT
GCLC	
Forward	GCCGCTGAGCTGGGAGGAAA
Reverse	ATTCCACCTCATCGCCCCACT
GPX1	
Forward	TATCGAGAATGTGGCGTCCC
Reverse	GATGCCCAAACTGGTTGCAC
GPX2	
Forward	GGACATCAGGAGAACTGTCAGA
Reverse	TCAGGTAGGCGAAGACAGGA
GGT1	
Forward	CAACCTGCCCACAGTGAAGA
Reverse	TTCTTGGCCTCCATGACTGC
GGT2	
Forward	CCCTTCTTTCAGTGGGAGGG
Reverse	ATGCTGTCACCTTTGTGGCT
GSTM1	
Forward	AGGAAAAGAAGTACACGATGGG
Reverse	TTGCTCTGGGTGATCTTGTG
GSTA1	
Forward	CAACCTGCCCACAGTGAAGA
Reverse	TTCTTGGCCTCCATGACTGC
GSTA2	
Forward	CCCTTCTTTCAGTGGGAGGG
Reverse	ATGCTGTCACCTTTGTGGCT
GSTA	
Forward	TCCGTGAGATGGGTTTTAGC
Reverse	CTGTACCAACTTCATCCCGTC
IDH1	
Forward	CAAGTGACGGAACCCAAAAG
Reverse	ACCCTTAGACAGAGCCATTTG
IDH2	
Forward	ACAACACCGACGAGTCCATC
Reverse	GCCCATCGTAGGCTTTCAGT

RNAseq data of CSC-enriched conditions

Expression of the genes contained in the KEGG (Kyoto Encyclopedia of Genes and Genomes) Pathway Glutathione Metabolism was investigated in our published RNAseq dataset E-MTAB-3808 (Array Express), which compares primary PDX cells cultured either by adherence or as spheres[7]. All original bioinformatic analyses on this dataset E-MTAB-3808 were performed at the Bioinformatics Unit and Structural Biology and Biocomputing Programme, Spanish National Cancer Research Centre

(CNIO), Madrid 28029, Spain. Differential expression of genes across the different conditions was calculated with Cuffdiff[26].

Human data analysis

Expression data from pancreatic cancer and normal tissue from the TCGA and the GTEx projects were analyzed using the webserver GEPIA2[27]. Pearson correlation coefficient was calculated for correlation analysis of glutathione-related genes with a stemness signature defined by the combined expression of the pluripotency-related genes *NANOG*, *KLF4*, *SOX2* and *OCT4*. For disease-free survival analysis, the Hazard ratio (HR) was calculated using the Cox Proportional Hazards model for pancreatic cancer patients from upper and lower quartiles of expression of the indicated genes.

Statistical analyses

Results for continuous variables are presented as mean ± SE unless stated otherwise. Treatment groups were compared with the independent samples *t*-test. Pair-wise multiple comparisons were performed with the one-way ANOVA (two-sided) with Bonferroni adjustment. Correlation analysis was performed by calculating the Pearson correlation coefficient. P values < 0.05 were considered statistically significant. All analyses were performed using Prism GraphPad (version 5.04).

RESULTS

Connection of glutathione metabolism with stemness in human pancreatic cancer samples

In order to determine a possible connection between glutathione metabolism and stemness in pancreatic cancer, we first analyzed our previously published RNAseq dataset E-MTAB-3808 comparing 5 different PDAC PDX models cultured in differentiating (adherent) or CSC-enriching (spheres) conditions (Figure 1A)[7]. We focused on genes related to the KEGG Pathway Glutathione Metabolism, which we categorized into four different classes: Gamma-glutamyltransferases (GGTs), glutathione-s-transferases (GSTs), glutathione peroxidases (GPXs), and genes involved in glutathione synthesis and recycling. As summarized in Table 2, CSC-enriched cultures generally up-regulated genes from the four classes, although the specific genes varied among the different PDXs.

We have previously demonstrated that pancreatic CSCs show increased expression of several pluripotency-related genes such as *NANOG*, *KLF4*, *SOX2* and *OCT4*, which we have routinely used as a stemness signature[5-7]. In order to further support a connection between glutathione metabolism and stemness, we used the webserver GEPIA2 to analyze our target genes in human expression data from normal pancreas and PDAC tissues included in the TCGA and GTEx projects. Thus, we performed gene expression correlation studies between the different glutathione-related genes up-regulated in spheres and our defined stemness signature in normal *vs* PDAC samples. Interestingly, expression of 17 of the 25 genes up-regulated in CSCs positively correlated with the stemness signature in human samples, with P-values below 10^{-5} (Figure 1B). Since disease recurrence can be mainly attributed to CSCs due to their ability to regenerate tumors following treatment, we next investigated whether the expression of any of the 17 genes correlated with disease-free survival in patients (Figure 1C). We found that high expression of *MGST1*, *GPX8* and *GGCT* predicted between 2.2-2.5 times increased risk of recurrence in PDAC patients (P = 0.0054, 0.03 and 0.0054, respectively). Together, our results suggest a functional link between glutathione metabolism, stemness and the aggressiveness of pancreatic cancer.

Glutathione metabolism is enhanced in primary sphere cultures of pancreatic cancer PDXs

Next, we aimed to further validate the above RNAseq results. Therefore, we analyzed the expression of 2-4 genes from each subgroup by real-time PCR. We included two additional PDX models [one PDAC (PDX163) and one pancreatic tumor of hepatobiliary origin (PDX247)] resulting in a total of seven PDX models for this validation. As shown in Figure 2, we detected enhanced expression of glutathione metabolism genes in CSC-enriching conditions for all seven PDX models, ranging between 2.5 to 600-fold.

For further functional validation, we used the thiol-sensitive probe monochlorobimane to assess the content of intracellular glutathione content in its

Table 2 RNAseq reveals that glutathione metabolism genes are up-regulated in cancer stem cell-enriched cultures from pancreatic cancer patient-derived xenografts

PDX	GGTs	GSTs	GPXs	Synthesis and recycling
PDX185	*GGT1, GGT2, GGT3P, GGT6, GGT7*	*GSTA1, GSTA2, GSTT1, MGST2, MGST3*	*GPX2, GPX3*	*GCLC, GSR, PGD, IDH1*
PDX215	*GGT1, GGT2, GGT7, GGT8P*	*GSTA1, GSTA4, GSTM2, GSTM4, MGST2*		*OPLAH, IDH1*
PDX253	*GGT1, GGT2, GGT3P, GGT6*	*GSTA1, GSTA2, GSTA4, MGST1, MGST2, MGST3*	*GPX2, GPX8*	*GSR, IDH1*
PDX354		*GSTA1, GSTA2, MGST1, GSTM2*	*GPX3, GPX8*	*GCLC, GGCT, IDH1*
PDX265		*GSTA4, GSTM1, GSTM2, GSTM4*	*GPX2*	*GGCT, PGD, IDH1, IDH2*

PDX: Patient-derived xenograft; GGTs: Gamma-glutamyltransferases; GSTs: Glutathione-s-transferases; GPXs: Glutathione peroxidases.

reduced form (GSH). First, we measured the GSH content in primary cells cultured in adherent or sphere conditions using the same panel of seven PDX models. Interestingly, except for PDX247 (of hepatobiliary origin) GSH content was 2 to 8 times higher in CSC-enriching conditions (Figure 3A; P values < 0.05-0.01), reinforcing the results obtained by gene expression analysis. Next, we validated the increased GSH levels observed by analyzing $CD133^-$ and $CD133^+$ subpopulations by flow cytometry. As shown in Figure 3B and 3C, $CD133^+$ CSCs accumulated more intracellular GSH (1.94-fold, $P = 0.04$). In summary, our results indicate that expression of glutathione metabolism genes and GSH content are upregulated in CSC-enriched conditions.

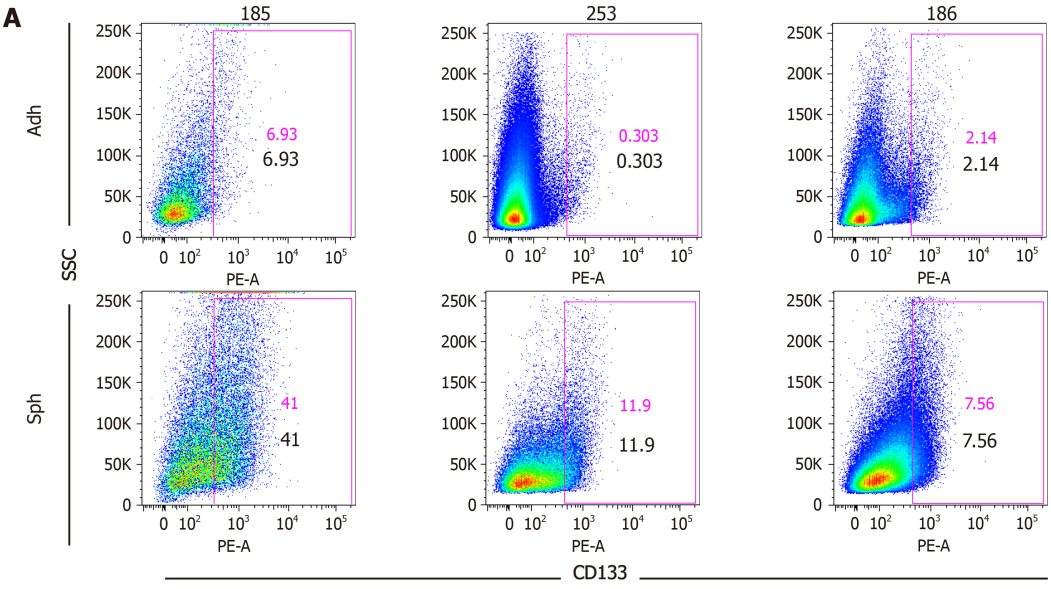
Depletion of GSH content impairs CSC functionality

In order to evaluate the significance of the increased GSH content for CSCs, we performed a series of GSH depletion assays using two distinct pharmacological approaches. We first tested the inhibitor of GSH synthesis BSO, which irreversibly blocks the enzyme gamma-glutamylcysteine synthetase (γ-GCS). Incubation of CSC-enriched spheres with increasing doses of BSO for 48 h resulted in a dose-dependent decline in GSH content, and with doses > 50 μmol/L depleted the GSH content below 50% (Figure 4A; P values ranging between 0.0023 and 0.00032). Treatment of CSC-enriched spheres with BSO at 100 μmol/L for 48 h resulted in the accumulation of cells in G1 phase, indicative of cell cycle arrest (Figure 4B). In addition, we observed an increase in the percentage of cells in both early and late apoptosis after BSO treatment, suggesting that full GSH content is necessary for proliferation and survival of CSCs (Figure 4C).

Considering these results, we next focused on alternative features directly linked to CSCs. First, BSO treatment decreased the expression of the aforementioned stemness signature defined by *NANOG*, *KLF4*, *SOX2* and *OCT4* (Figure 5A). Next, we measured the effect of BSO on CSC self-renewal. Since we had observed an up-regulation of genes involved in GSH recycling (Table 2), we also tested the GSH recycling inhibitor 6-aminonicotinamide (6-AN), which blocks the oxidative branch of the pentose phosphate pathway that is necessary for reduction of oxidized GSSG into GSH. Incubation with either BSO or 6-AN consistently reduced the number of spheres formed by day 7, indicative of diminished self-renewal capacity (Figure 5B). Consistently, the percentage of $CD133^+$ cells assessed by flow cytometry was also reduced following treatment with either inhibitor (Figure 5C). Together these results indicate that depletion of GSH content directly impacts CSC viability and self-renewal.

Depletion of GSH content in CSC-enriched cultures enhances response to gemcitabine

Apart from the ability to self-renew, another key feature of CSCs is chemoresistance. Notably, several glutathione-related enzymes such as GSTs are directly implicated in detoxification of xenobiotics, suggesting another potential link between glutathione metabolism and CSC features[28]. Indeed, we found that the absolute GSH concentration in spheres, but not adherent cultures, positively correlated with the percentage of surviving cells after gemcitabine treatment (Figure 6A; Pearson's $r = 0.96$, $P = 5.89 \times 10^{-11}$). Gemcitabine treatment induced GSH accumulation exclusively in $CD133^+$ cells (Figure 6B), which was abrogated by co-treatment with BSO. This translated in sensitization of $CD133^+$cells to treatment with gemcitabine, approximating levels of apoptosis observed in differentiated $CD133^-$ cells (Figure 6C),



B

Correlation with stemness genes

Gene	*R*	*P* value
GGT1	**0.41**	**2.4e-15**
GGT12	0.05	0.35
GGT3P	-0.048	0.37
GGT6	-0.16	0.025
GGT7	**0.37**	**5.8e-13**
GGT8P	**0.23**	**1.2e-5**
GSTA1	-0.1	0.056
GSTA2	-0.54	1.5e-27
GSTA4	-0.16	0.0025
GTSM1	**0.23**	**1.5e-5**
GSTM2	-0.35	9.3e-12
GSTM4	**0.47**	**0**
MGST1	**0.43**	**0**
MGST2	**0.73**	**0**
MGST3	**0.76**	**0**
GPX2	**0.64**	**0**
GPX3	-0.09	0.09
GPX8	**0.61**	**0**
GCLC	**0.34**	**1.0e-10**
GGCT	**0.69**	**0**
GSR	**0.63**	**0**
IDH1	**0.68**	**0**
IDH2	**0.19**	**0.00035**
PGD	**0.62**	**0**
OPLAH	**0.51**	**0**

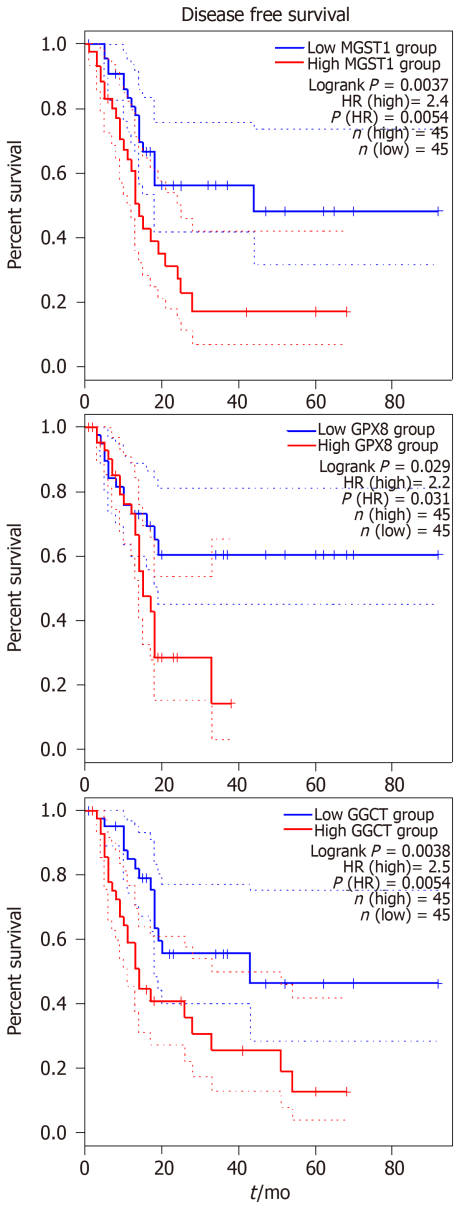


Figure 1 Expression of glutathione metabolism genes positively correlates with a stemness signature in human pancreatic cancer samples and predicts poor outcome. A: Enrichment in CD133$^+$ cells in sphere cultures *vs* adherent cultures, as determined by flow cytometry. Representative

flow cytometry histograms of the indicated patient-derived xenograft models; B: Correlation of genes that were up-regulated in cancer stem cell-enriched cultures with a stemness signature defined by combined expression of the pluripotency-related genes *NANOG*, *KLF4*, *SOX2* and *OCT4*. Pearson´s r and corresponding *P* values for individual correlations are shown; C: Disease-free survival in pancreatic cancer patients from upper and lower quartiles of expression of the indicated genes. Hazard ratio was calculated using the Cox Proportional Hazards model. Dotted lines represent the 95% confidence interval. Data in A and B were calculated using GEPIA2 (http://gepia2.cancer-pku.cn), using public data compiled in TCGA and GTEx. Sph: Sphere culture; Adh: adherent culture; HR: Hazard ratio.

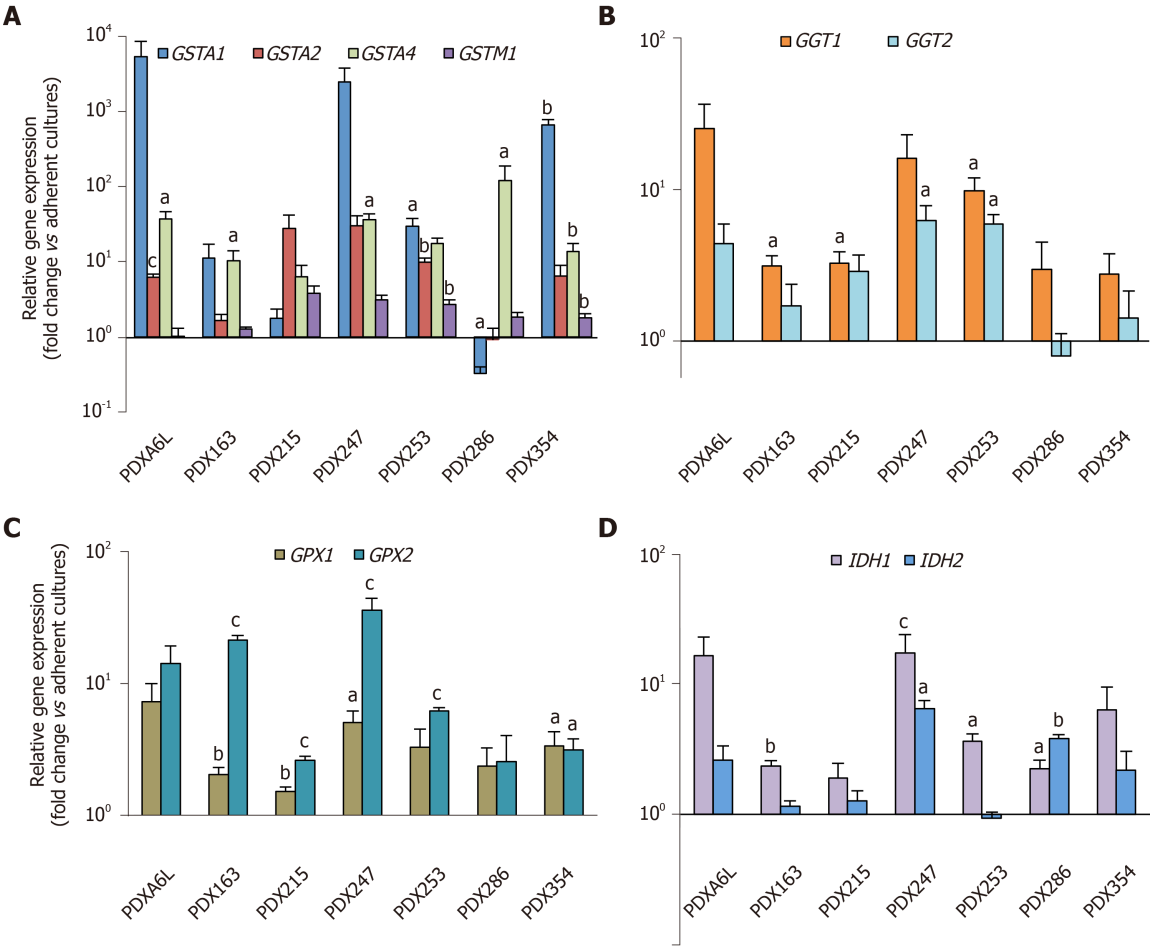


Figure 2 Glutathione metabolism-related genes are up-regulated in cancer stem cell-enriched conditions. Primary cells from different patient-derived xenograft models as indicated in the figure were cultured in adherent or low-attachment cancer stem cell-enriching conditions. On day 7 the expression of several glutathione (GSH)-related genes was evaluated by real-time polymerase chain reaction (PCR). A: Glutathione-S-Transferases A1, A2, A4, M1; B: Gamma-glutamyltransferases 1 and 2; C: Glutathione Peroxidases 1 and 2; D: Isocitrate Dehydrogenases 1 and 2. Data were normalized to HPRT and are shown as mean ± SE fold change expression levels of sphere *vs* adherent cultures in logarithmic scale. [a]*P* < 0.05; [b]*P* < 0.01; [c]*P* < 0.001. PDX: Patient-derived xenograft; GGT: Gamma-glutamyltransferase; GST: Glutathione-s-transferase; GPX: Glutathione peroxidase.

and diminished sphere formation as compared to single treatments (Figure 6D; $P < 0.05$). These results demonstrate that chemoresistance in CSCs is, at least in part, dependent on GSH synthesis.

In summary, our results suggest that glutathione metabolism plays an essential role in PDAC aggressiveness, supporting CSC survival, self-renewal and chemoresistance.

DISCUSSION

Any living cell produces ROS, either as a by-product of mitochondrial respiration or in a controlled manner by different oxidases to modulate cell signaling. Specifically, intracellular ROS levels regulate the complex balance between symmetric and asymmetric division in stem cells, thus controlling differentiation and self-renewal[29]. In these cells, avoiding ROS accumulation is particularly important, since it prevents the accumulation of hereditary mutations and premature senescence[30]. For these reasons, quiescent stem cells reside in a low oxygen niche favoring glycolysis over

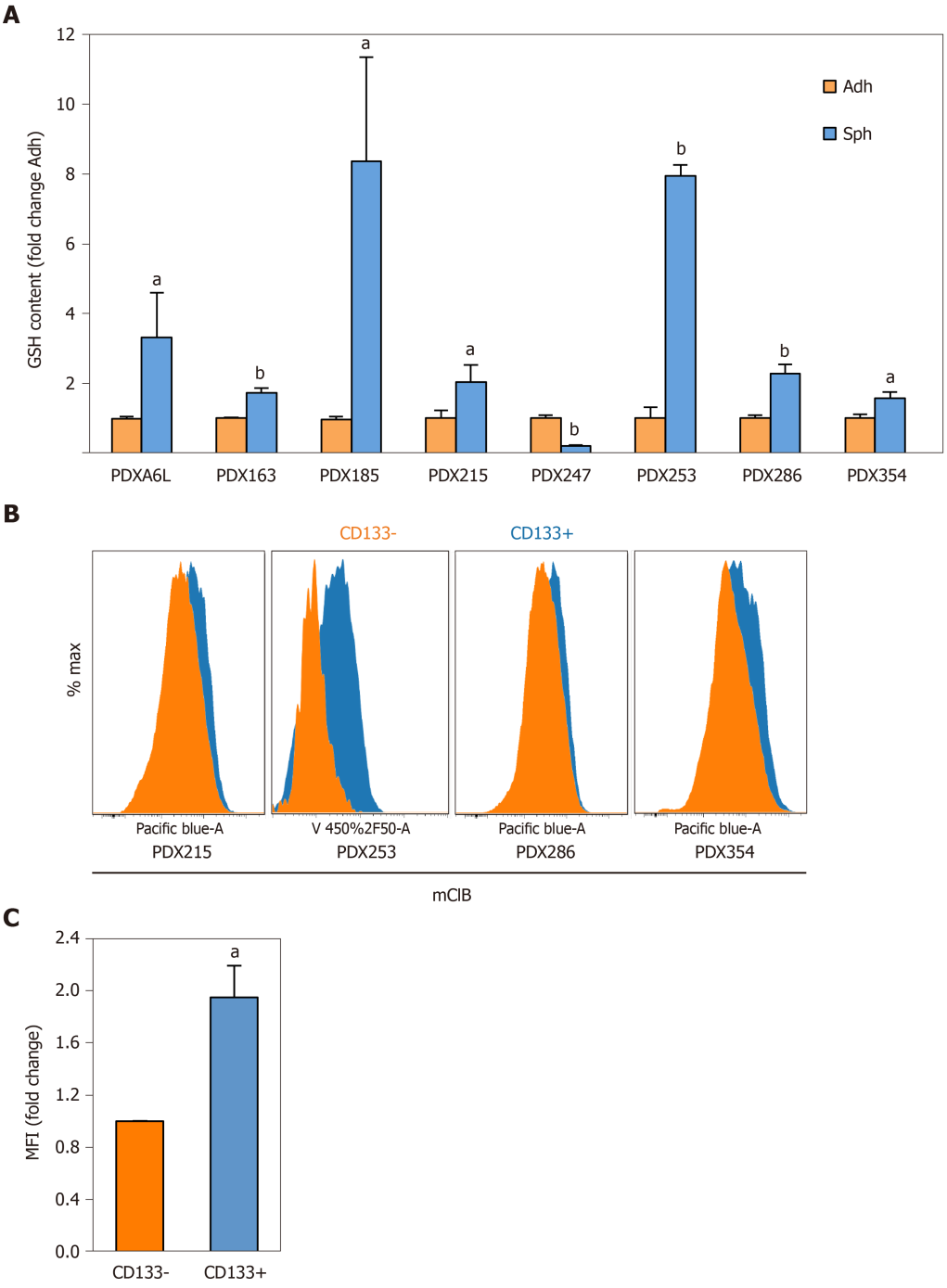


Figure 3 Reduced glutathione content is increased in cancer stem cell-enriching conditions. Reduced glutathione (GSH) content was measured using the fluorescent thiol-reactive probe monochlorobimane (mClB). A: GSH content in cellular lysates was assessed by fluorimetry. Primary cells from different patient-derived xenograft (PDX) models as indicated in the figure were cultured in adherent or low-attachment cancer stem cell-enriching conditions for 7 d. Data were normalized for protein content; B: GSH content in CD133 positive and negative subpopulations as determined by flow cytometry. Representative flow cytometry histograms of the indicated PDX models are shown, with the following mean fluorescence intensities (MFI) for $CD133^-$ and $CD133^+$ populations, respectively: PDX215 (2787 *vs* 4880), PDX286 (2748 *vs* 4364), PDX354 (4138 *vs* 6988); C: Pooled MFI data from PDX215, 286 and 354. Data in A and C are shown as mean ± SE fold change for sphere *vs* adherent cultures (A) or $CD133^+$ *vs* $CD133^-$. $^aP < 0.05$; $^bP < 0.01$. GSH: Glutathione content in its reduced form; PDX: Patient-derived xenograft; Sph: Sphere culture; Adh: adherent culture; MFI: Mean fluorescence intensities.

mitochondrial respiration and express high levels of antioxidant systems[18,31].

Similar to normal stem cells, publications on different cancer types indicate that CSCs have lower ROS levels than their differentiated counterparts to support self-renewal and tumorigenicity[13,14,19]. However, the mechanisms by which they keep a reduced redox state may differ, since many CSCs depend on mitochondrial oxidative metabolism[12,16]. In fact, while glycolysis maintains reduced ROS levels in gemcitabine-resistant pancreatic PaTU8988 cells[32], primary human pancreatic $CD133^+$ CSCs have low mitochondrial ROS concomitant with full oxidative metabolic (OXPHOS)

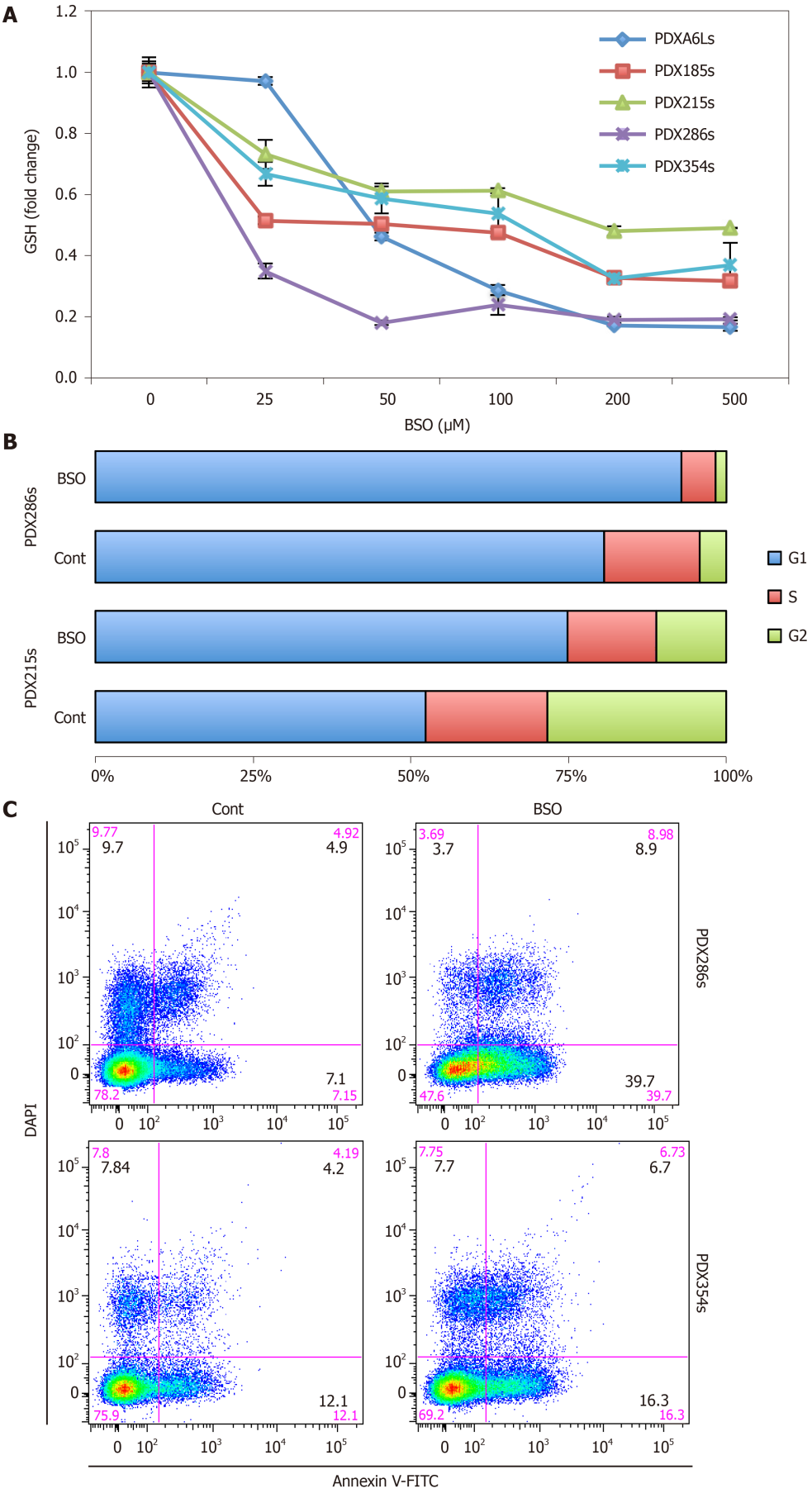


Figure 4 Inhibition of glutathione synthesis blocks cell cycle progression and induces apoptosis in cancer stem cell-enriched cultures. Cells from the indicated patient-derived xenograft models were grown in cancer stem cell-enriching conditions as spheres for 5 d and then treated for 48 h with 100

μmol/L buthionine-sulfoximine (BSO), unless indicated otherwise. A: Dose-dependent inhibition of glutathione (GSH) content by BSO measured by fluorimetry after staining with monochlorobimane (mClB); B: Percentage of cells in the different phases of the cell cycle as assessed by flow cytometry; C: Representative FACS plots of an Annexin-V/DAPI staining to detect apoptosis under the indicated conditions. GSH: Glutathione content in its reduced form; BSO: Buthionine-sulfoximine; PDX: Patient-derived xenograft.

activity[7]. These findings suggest that, rather than controlling ROS release from the primary production site, CSCs favor ROS detoxification as a common feature. Indeed, $CD44^+CD24^+$ Panc-1 cells survive radiation by blocking ROS accumulation, which also points to increased ROS scavenging in CSCs[33].

GSH is a key antioxidant defense in mammalian cells, maintaining intracellular redox homeostasis. Coupled to different enzymes such as GPXs or GSTs, GSH not only reacts with ROS and electrophiles, but also prevents oxidation of thiol groups in proteins, acts as a cysteine reserve pool, and detoxifies xenobiotics[28]. Interestingly, most of the representative works describing low ROS content in CSCs also reported high levels of GSH and/or GSH-related enzymes. For example, *GCLM* and *GSS* were upregulated in liver $CD13^+$N-cadherin$^-$ CSCs[19], and GSH content and GPXs expression were increased in gastric $CD44^+$ cells[14]. In addition, breast CSCs defined either as $CD24^-$/Low $CD44^+Lin^-$ or $ALDH^+$ showed elevated GSH concentration and glutathione synthesis enzymes[13,34], which, in the case of $ALDH^+$ cells, was further enhanced with upregulation of GPXs and GSTs[34,35].

To date, the few published reports connecting glutathione metabolism and stemness-related features in pancreatic cancer have focused on GPXs, with different isoforms playing contradictory roles. On the one hand, GPX1 was described to play a tumor suppressor role counteracting features attributed to CSCs in MiaPaca-2 cells. GPX1 overexpression inhibited clonogenicity and tumor growth *in vivo*[36], while GPX-1 silencing induced EMT (epithelial-to-mesenchymal transition) and chemoresistance[37]. On the other hand, Panc-1 spheroids up-regulate GPX1 and GPX4 in hypoxic conditions, but only GPX4 seems to be important for self-renewal and invasion under both normoxia and hypoxia[38].

Here, we show that CSC-enriched cultures established from 7 human PDX models not only up-regulate several GPX genes, but also numerous genes related to GSH synthesis and recycling, as well as GSTs (Table 2 and Figure 2). However, although there is a global upregulation of the pathway, our results suggest that expression patterns of the specific isoform(s) of GPXs and GSTs expressed vary considerably across patients (Table 2). This apparent heterogeneity argues against the universality of the previously mentioned studies on GPX isoforms, which were based on just one or two established cell lines[36-38]. Interestingly, our analysis of human normal or PDAC samples indicate a strong correlation of GSH-related genes with stemness and poor outcome (Figure 1). In addition, and in line with the gene expression data, intracellular GSH levels were also increased in spheres and $CD133^+$ cells, respectively, as compared to differentiated cells (Figure 2). Together, these results support the notion that pancreatic CSCs resemble CSCs from other cancer types in terms of glutathione metabolism, and corroborate the evidence for the crucial role of this pathway in cancer stemness.

Oxidative CSCs need to counteract ROS produced in cellular respiration. Notably, enhanced GSH metabolism is often linked to increased mitochondrial function, conferring protection from ROS produced during respiration and by detoxifying lipid hydroperoxides and electrophiles[28]. In fact, glutathione peroxidases such as GPX4 or transferases such as GSTP have been shown to maintain OXPHOS activity and preserve mitochondrial function[39,40]. Importantly, OXPHOS metabolism is linked to glutathione metabolism and antioxidative defense *via* a process controlled by NRF2, thereby promoting breast CSCs maintenance and self-renewal[34]. In this context, we have previously shown that PGC-1α promotes mitochondrial respiration and is required for full stemness, connecting both processes in pancreatic CSCs[7]. Interestingly, one of the main physiological functions of PGC-1α is to balance mitochondrial ROS production and scavenging by coordinating mitochondrial biogenesis and antioxidative response. Indeed, PGC-1α has been shown to control the expression of several antioxidants and glutathione-related enzymes such as GPXs and GSTs, likely *via* NRF2 activation[41,42]. Moreover, it modulates GSH levels by regulating its synthesis through direct gamma-glutamylcysteine ligase and glutathione synthase transcriptional control[41,43]. Therefore, glutathione metabolism could support stemness downstream of PGC-1α in pancreatic CSCs by detoxifying mitochondrial ROS.

Our results suggest that pancreatic CSCs are particularly sensitive to glutathione depletion. We have shown that inhibition of GSH synthesis induces cell cycle arrest

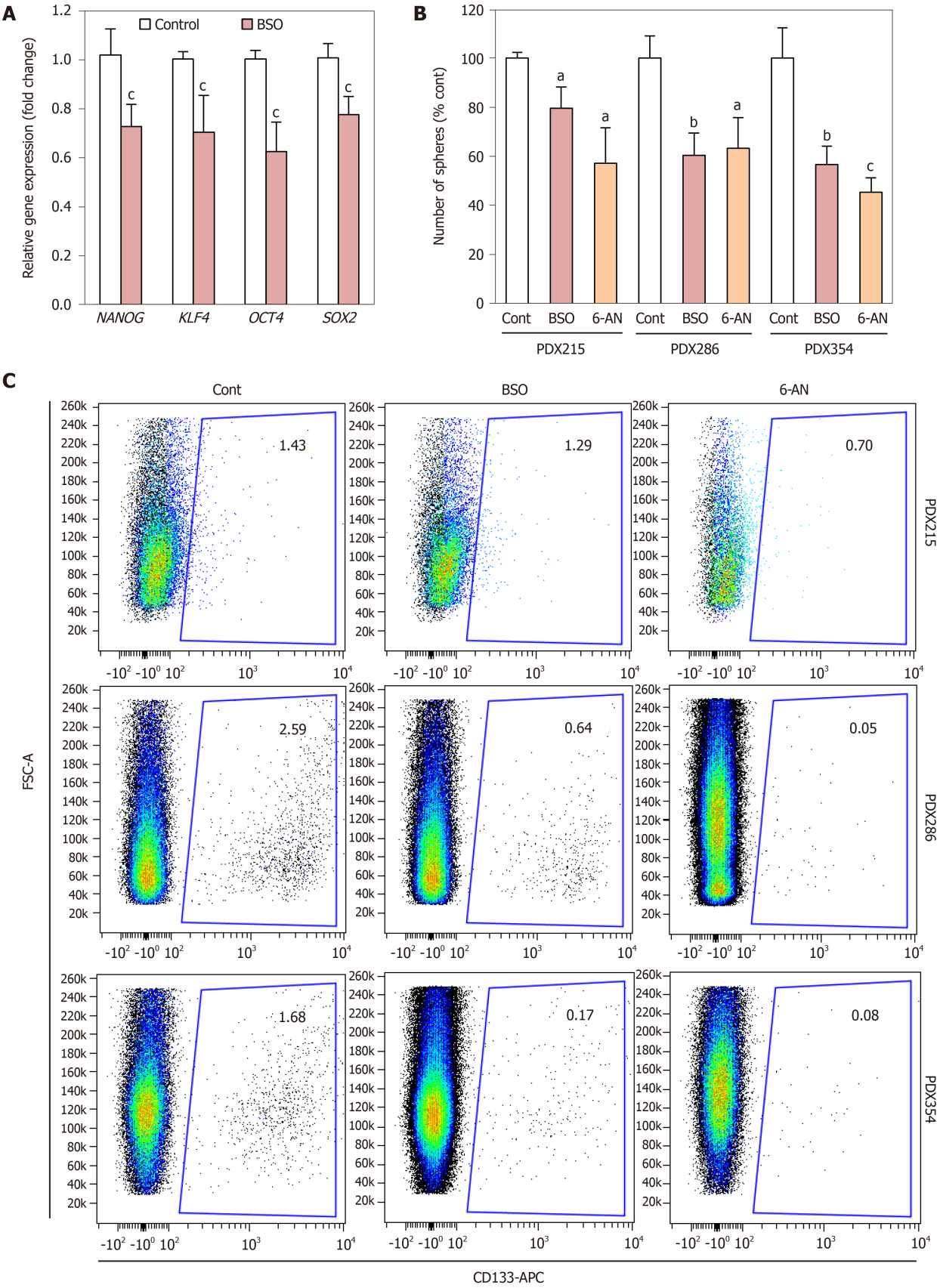


Figure 5 Inhibition of glutathione synthesis and recycling decreases self-renewal and CD133 expression. Cells from the indicated patient-derived xenografts (PDXs) were treated with 100 μmol/L buthionine-sulfoximine or 1 μmol/L 6-Aminonicotinamide as indicated. A: Expression of stemness gene expression following 72 h of treatment; B: Sphere formation ability after 7 d of treatment, replenished every other day. Data are shown as fold change *vs* untreated conditions (Cont) for each PDX model, mean ± SE; C: Representative flow cytometry plots for CD133 expression after 48 h of treatment. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. BSO: Buthionine-sulfoximine; PDX: Patient-derived xenograft; 6-AN: 6-Aminonicotinamide.

and apoptosis of pancreatic CSCs (Figure 4), resulting in reduced expression of stemness genes, self-renewal capacity and, consequently, $CD133^+$ content (Figure 5). In contrast, breast and colorectal CSCs seem to be resistant to BSO as a single treatment, and they up-regulate thioredoxins as a compensatory mechanism to counteract ROS and maintain self-renewal under BSO treatment[34,44]. Interestingly, inhibition of self-renewal was achieved not only with the glutathione synthesis inhibitor BSO, but also by pharmacological inhibition of GSH recycling using 6-AN. These results functionally validate our RNAseq data, in which we observed that pancreatic CSCs upregulated genes related to both synthesis (*GCLC*) and recycling processes (*GSR*, *PGD*), and suggest that GSH metabolism is highly dynamic in pancreatic CSCs. Of note, the specific balance of synthesis *vs* recycling processes determining the actual GSH content may vary between patients. For example, PDX215 cells were partially resistant to BSO, while they responded to 6AN similarly to the other PDXs tested (Figure 5).

Depletion of intracellular GSH with BSO has been shown to sensitize breast CSCs to radiotherapy, inducing oxidative DNA damage that leads to apoptosis[13,45]. However, glutathione synthesis inhibition has been mainly tested in combination with conventional chemotherapy: Treatment with BSO enhanced toxicity of melphalan[46], paclitaxel[47], cisplatin and gemcitabine[48] in different *in vitro* models, but showed no effect for 5-fluorouracil[46]. Our results further underscore the role of glutathione in chemoresistance, by regulating CSCs survival and functionality. First, we show that GSH content in CSC-enriched cultures, but not in adherent cultures, positively correlates with global survival under gemcitabine treatment (Figure 6A). Moreover, we found that $CD133^+$ cells, but not $CD133^-$, accumulate more intracellular GSH in response to gemcitabine (Figure 6B). Counteracting this defense response by BSO treatment induced apoptosis in both subpopulations to a similar degree (Figure 6C).

Other combination therapies involving glutathione synthesis inhibition have been tested in pancreatic cancer with reasonable success. For instance, inhibition of the PI3K/AKT pathway in combination with BSO blocked mRNA translation and impaired tumor growth *in vivo,* mimicking NRF2 loss in pancreatic cancer[49]. Although BSO is the most utilized treatment to achieve GSH depletion, other strategies such as inhibition of cysteine/glutamate transport showed a synergistic effect in combination with gemcitabine for inhibiting proliferation of pancreatic cancer cell lines[50].

CONCLUSION

In summary, we find that glutathione metabolism plays an essential role in pancreatic cancer aggressiveness, supporting CSC survival, self-renewal and chemoresistance. Our results point to a novel metabolic vulnerability of pancreatic CSCs that should be exploited for the design of new therapeutic strategies aimed at the elimination of this highly aggressive subpopulation of cancer cells.

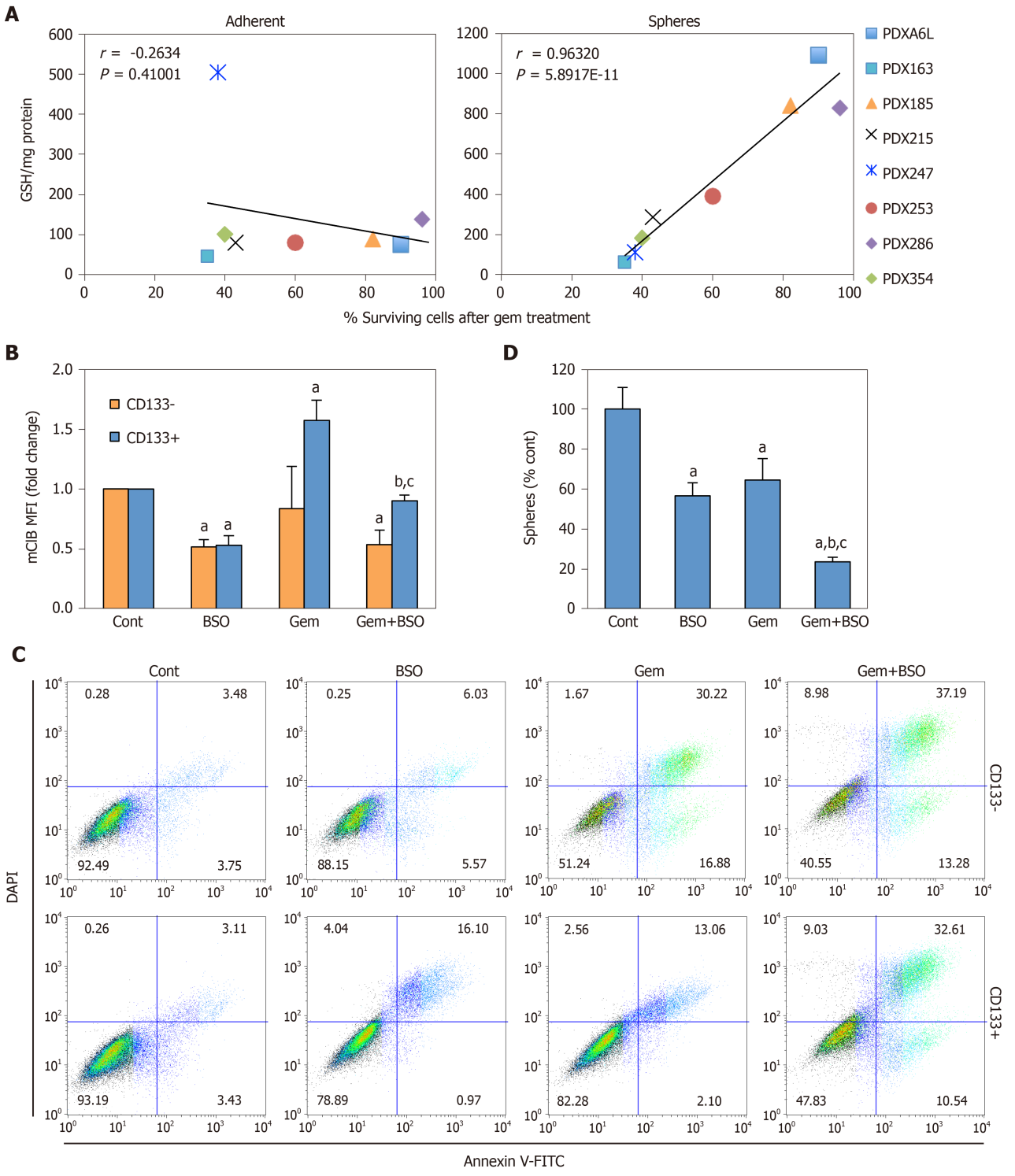


Figure 6 Depletion of glutathione (GSH) content in cancer stem cells enhances response to Gemcitabine. A: Correlation of the absolute glutathione (GSH) content per milligram of protein in lysates from adherent (left panel) or sphere (right panel) cultures *vs* the percentage of surviving cells following gemcitabine treatment (300 nM, 48 h). Values for Pearson´s r and corresponding *P* values are shown; B-D: Patient-derived xenograft 354 cells were treated with 100 μmol/L buthionine-sulfoximine (BSO) alone or in combination with 1 μmol/L Gemcitabine; B: mCIB staining for GSH content in $CD133^-$ *vs* $CD133^+$ cells after 48 h of treatment; C: Representative flow cytometry plots for Annexin V/DAPI staining for samples shown in B; D: Number of spheres following 7 d of treatment. Data in B and D are shown as mean fold change or mean percentage ± SE, with untreated conditions (Cont) set as 1.0 or 100%, respectively. $^aP < 0.001$ *vs* Cont; $^bP < 0.001$ *vs* BSO; $^cP < 0.001$ *vs* Gemcitabine. GSH: Glutathione content in its reduced form; PDX: Patient-derived xenograft; MFI: Mean fluorescence intensities; BSO: Buthionine-sulfoximine.

ARTICLE HIGHLIGHTS

Research background

Redox metabolism modulates stem cell and cancer stem cell (CSC) functionality in different model systems, regardless of their dominant metabolic phenotype. In fact, CSCs from several cancer types show increased glutathione content and associated enzymes.

Research motivation

Identification of metabolic vulnerabilities of highly aggressive CSCs can lead to the development of more effective treatment strategies for pancreatic cancer.

Research objectives

The present study aimed to determine the importance of glutathione metabolism for pancreatic CSCs as compared to their differentiated counterparts.

Research methods

Comparisons between CSCs and non-CSCs in primary pancreatic cancer cells of patient-derived xenografts were carried out by culturing in adherent or CSC-enriching sphere conditions and confirmed by CD133 staining by flow cytometry. Gene expression analyses were performed by RNAseq or real-time PCR. Public TCGA and GTEx RNAseq data from pancreatic cancer *vs* normal tissue samples were analyzed using the webserver GEPIA2. Staining for measurement of glutathione (monochlorobimane), cell cycle (propidium iodide) or apoptosis (Annexin-V) were determined by fluorimetry or flow cytometry. Pharmacological glutathione depletion was achieved with inhibitors of glutathione synthesis (buthionine-sulfoximine) and recycling (6-Aminonicotinamide). Self-renewal was assessed by sphere formation assay and response to gemcitabine treatment was used as a readout for chemoresistance.

Research results

Several glutathione metabolism genes were upregulated in pancreatic CSCs, and their expression correlates with a stemness signature and predicts survival in clinical samples. Increased glutathione concentration in CSCs promotes viability, cell cycle progression and pluripotency gene expression. Inhibition of glutathione synthesis or recycling impairs CSC functionalities such as self-renewal and chemoresistance.

Research conclusions

Our data suggest that pancreatic CSCs depend on glutathione metabolism. Pharmacological targeting of this pathway showed that high GSH (glutathione in its reduced form) content is essential to maintain CSC functionality in terms of self-renewal and chemoresistance.

Research perspectives

Our data demonstrate a targetable metabolic vulnerability of this aggressive subpopulation of cancer cells, which could be exploited for therapeutic purposes.

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