AWARD NUMBER: W81XWH-19-1-0596

TITLE: Glutamine-Mediated Tumor-Stromal Interaction: A Novel Target for Pancreatic Cancer Treatment

PRINCIPAL INVESTIGATOR: Xiaoyu Yang

CONTRACTING ORGANIZATION: University of Texas Health Science Center at San Antonio

REPORT DATE: JUNE 2022

TYPE OF REPORT: Final Technical Report

PREPARED FOR: U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
JUNE 2022	FINAL	15Aug2019 – 14Feb2022

4. TITLE AND SUBTITLE

Glutamine-Mediated Tumor-Stromal Interaction: A Novel Target for Pancreatic Cancer Treatment

5a. CONTRACT NUMBER
W81XWH-19-1-0596

5b. GRANT NUMBER
CA181275

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Xiaoyu Yang

E-Mail: yangx3@livemail.uthscsa.edu

5d. PROJECT NUMBER
0011349283

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Texas Health Science Center at San Antonio
7703 Floyd Curl Dr.
San Antonio TX 78229-3901

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Pancreatic ductal adenocarcinoma is a devastating disease with appallingly poor outcome. Conventional therapeutic approaches including gemcitabine – based combination chemotherapy offer modest survival benefit at the cost of increased toxicity. We have previously demonstrated that pancreatic stellate cells (PSCs) secrete glutamine (Q) to promote the growth of pancreatic cancer cells (PCCs), which can be attenuated by a natural compound palmatine (PMT). However, the precise mechanisms associated with Q- and PMT- mediated biological outcome have not been fully understood. Here, we demonstrated that PMT inhibits Q-stimulated STAT3 phosphorylation at both tyrosine 705 and serine 727, its downstream target survivin, and Q-stimulated increased proliferation, clonogenicity, anchorage independent growth, migration and invasion. Furthermore, RNA-seq analysis revealed that gene expression profile of PMT treatment under Q stimulation condition mimics STAT3 knockdown. These data suggest that PMT abrogates glutamine-induced biological outcome in part through STAT3. Given that both STAT3 and survivin are involved in therapeutic resistance of GEM and Abraxane (Abr), we tested the combination of PMT with GEM and Abr and found that PMT potentiates anti-proliferative effect of GEM and Abr in PSCs and PCCs and identified potential feedback activation mechanism that resist to PMT and GEM treatment. Taken together, this study demonstrated the potential clinical utility of PMT in the management of pancreatic cancer through inhibition of STAT3.

15. SUBJECT TERMS
Pancreatic cancer; Therapeutic resistance; Palmatine; Glutamine; Pancreatic stellate cells; Gemcitabine; Abraxane

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	36	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

1. **INTRODUCTION:** Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease with dismal survival rate and limited treatment options. Due to occupational exposure, military personnel are at elevated risk of developing and dying from PDAC. Conventional therapeutic approaches including gemcitabine (GEM) – based combination chemotherapy [mainly GEM and Abraxane (Abr)] offer modest survival benefit at the cost of increased toxicity. Moreover, development of therapeutic resistance remains a major challenge limiting the effectiveness of treatment. Desmoplasia, a prominent feature of pancreatic ductal adenocarcinoma, has been known to contribute to therapeutic resistance caused by excessive deposition of extracellular matrix components by pancreatic stellate cells (PSCs). Additionally, the reciprocal crosstalk between PSCs and pancreatic cancer cells (PCCs), resulting in disease progression is an attractive target for pancreatic cancer treatment. Previous studies from our laboratory identified that PSCs secrete glutamine (Q) that enhances proliferation of PCCs. I have discovered that protein levels of pSTAT3 and its target gene survivin were increased under conditions of Q-stimulation which were attenuated by a novel small molecule called palmatine (PMT). Since both STAT3 and survivin are associated with resistance to GEM and Abr, the **purpose** of this research is to decipher the role of STAT3 and survivin in Q-mediated PSC-PCC communication, and to determine the effectiveness of PMT to inhibit this interplay as an approach to potentiate response to standard of care. In this study, we tested the hypothesis that Q triggers PSC-PCC communication through STAT3/survivin upregulation to promote hallmarks of cancer and that PMT inhibits this process to improve response to conventional therapeutic agents.

2. **KEYWORDS:** Pancreatic cancer; Therapeutic resistance; Palmatine; Glutamine; Pancreatic stellate cells; Gemcitabine; Abraxane

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

Major goals	**Completion**
1. Determine if PSC-secreted Q is responsible for the changes of cancer hallmarks in PCCs	95%
2. Determine the effects of PMT on Q release from PSCs and on cancer hallmarks in PCCs	100%
3. Establish the causal relationship between Q treatment, STAT3 and Survivin's promoter activity and verify the role of PMT in this process	95%
4. Determine the effectiveness of PMT to potentiate conventional therapy in PSCs and PCCs	95%
5. Determine the therapeutic activity of PMT with GEM plus Abr in preclinical model	5%

What was accomplished under these goals?

Major goal 1: Determine if PSC-secreted glutamine (Q) is responsible for the changes of cancer hallmarks in PCCs

1) Glutamine stimulation increased multiple cancer hallmarks in pancreatic cancer cells

We first determined the effect of glutamine on multiple cancer hallmarks including proliferation, migration and invasion in pancreatic cancer cells (MIA PaCa-2, BxPC-3, PANC-1, Capan-2). To rule out cell line specific effects, we tested on multiple cell lines with different KRAS mutation, differentiation and metabolic status (Table 1). We observed that glutamine significantly increased proliferation ($p<0.05$) in all the cell lines examined at both 24h and 48h (Figure 1 A-D).

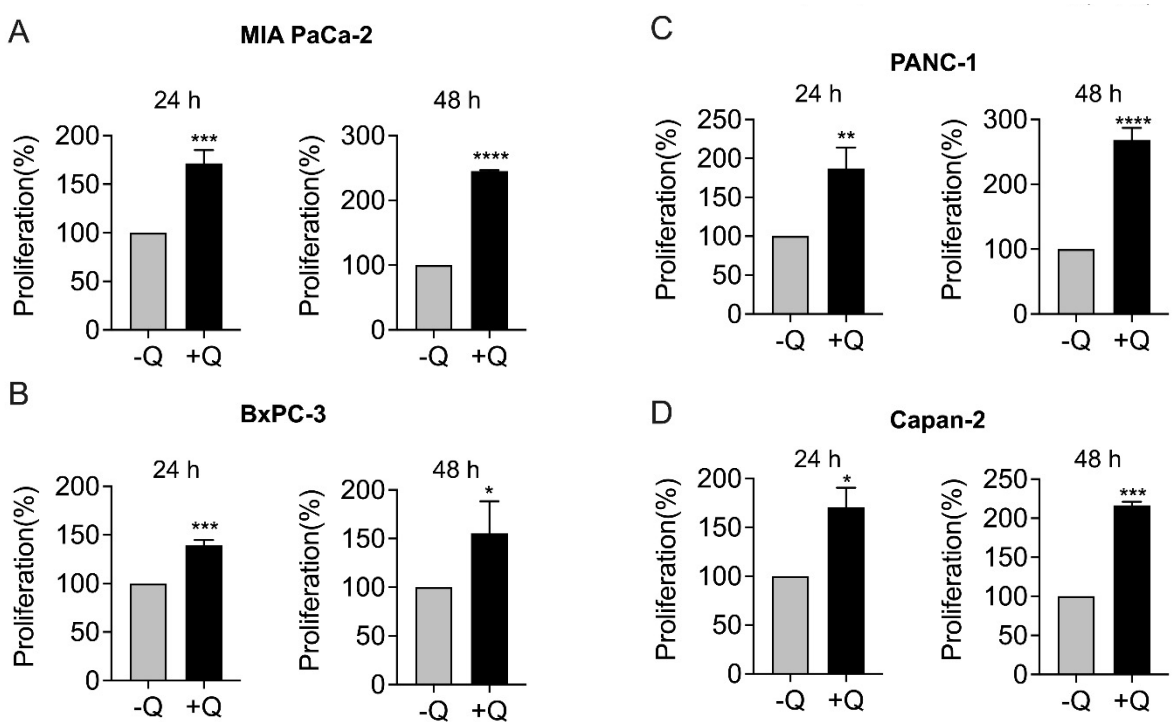


Figure 1 Glutamine Stimulation Increased Proliferation in Pancreatic Cancer Cells Independent of STAT3 [A-D] MIA PaCa-2 (n=3) **(A)**, BxPC-3 (n=3) **(B)**, PANC-1 (n=3) **(C)** and Capan-2 (n=2) **(D)** cells were starved in glutamine-deficient media overnight, and stimulated with 2 mM glutamine for 24h and 48h. Cell proliferation was measured by MTT assay (which measures intracellular metabolic activity). Data presented is an average ± SD of two to three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, *** $P < 0.0001$ (Student's t-test).

2) Glutamine stimulation increased the ability of clonogenicity, anchorage-independent growth, migration and invasion in pancreatic cancer cells

As an independent approach, we used clonogenic assay (also called colony formation assay) that measures the ability of single cell to form colonies and can be used to assess the sensitivity of cancer cells to radiation, chemotherapeutic agent or other treatment. We observed significantly increased clonogenicity with glutamine stimulation in MIA PaCa-2 ($p<0.0001$) and PANC-1 ($p=0.0216$) cells (Figure 2 A-B). Anchorage-independent growth is the ability of cells to grow without a solid surface. It is usually tested by soft agar assay and has been a hallmark of cancer but not normal cells. Despite not reaching statistical significance ($p=0.0666$), we found a ~1.5 fold increase in anchorage-independent growth with glutamine stimulation in MIA PaCa-2 cells (Figure 2

C). We also observed significantly increased migration (p=0.0001) (Figure 2 D) and invasion (p=0.0119) (Figure 2 E) capacities of MIA PaCa-2 cells with glutamine stimulation. Taken together, these data suggest that glutamine stimulation supports multiple cancer hallmarks.

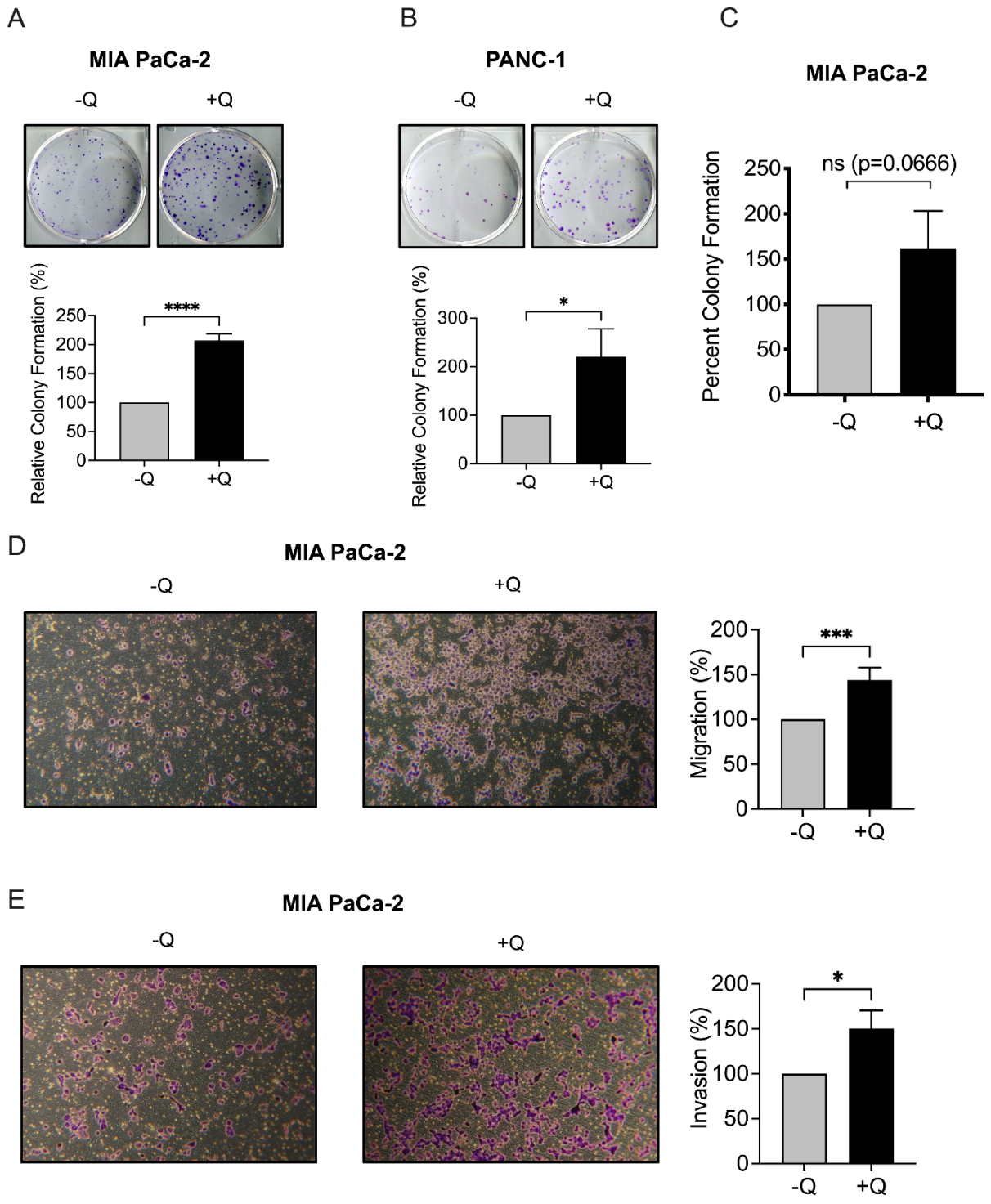


Figure 1 Glutamine Stimulation Increased the Ability of Clonogenicity, Anchorage-independent Growth, Migration and Invasion in Pancreatic Cancer Cells. [A-B] Logarithmically growing MIA PaCa-2 (n=3) and PANC-1 (n=3) cells were starved in glutamine-deficient media overnight, and stimulated with 2 mM glutamine for 24 h (MIA PaCa-2) and 48 h (PANC-1). Cells were then trypsinized and viable cell concentration was determined by trypan blue assay. 1000 (MIA PaCa-2) or 500 (PANC-1) live cells were seeded in complete media in 6-well plates. Cells were maintained 7-14 days until colonies are formed. Colonies were stained with 0.1% crystal violet in methanol. Quantification was done by solubilizing crystal violet staining with 10% acetic acid and measuring absorbance at 570 nm. Data presented is an average ± SD of three independent experiments.

* P < 0.05, *** P < 0.001 (Student's t-test) **[C]** Logarithmically growing MIA PaCa-2 cells were starved in glutamine-deficient media overnight, and stimulated with 2 mM glutamine for 24h. Cells were then trypsinized and viable cell concentration was determined by trypan blue assay. 10,000 viable cells were seeded in soft agar and maintained for 7-10 days. Anchorage-independent growth was quantified following the manufacturer's instructions using CytoSelect 96-well Cell Transformation Assay (Cell Biolabs, San Diego, CA). Data presented is an average ± SD of three independent experiments. ns: not significant (Student's t-test) **[D-E]** MIA PaCa-2 cells starved from glutamine were stimulated with glutamine for 24h. Cells were then trypsinized and viable cell concentration was determined by trypan blue assay. 300,000 cells (for migration assay) **(D)** or 500,000 cells (for invasion assay) **(E)** suspended in serum-free media were seeded in the cell culture insert with a porous membrane for cell migration. A thin layer of MATRIGEL Basement Membrane Matrix served as reconstituted basement membrane was included in the invasion chamber **(E)**. Complete media (containing serum) was added to the bottom chamber as chemoattractant. Cells were incubated for 24h **(D)** or 28h **(E)**. Cells on the lower surface of the membrane were stained with 0.2% crystal violet in methanol. Images were taken with an inverted Zeiss PrimoVert light microscope. Quantification was done by solubilizing crystal violet staining with 30% acetic acid and measuring absorbance at 570 nm. Data presented is an average ± SD of five **(D)** or three **(E)** independent experiments. * P < 0.05, *** P < 0.001 (Student's t-test). Abbreviations: Glutamine (Q)

3) Glutamine stimulation enhanced pSTAT3 levels in pancreatic cancer cells

We then tested the effect of glutamine on time-dependent changes in protein levels of total and phosphorylated STAT3 at tyrosine 705 (Y705) using MIA PaCa-2, BxPC-3 and PANC-1 cells. The dose of glutamine (2 mM) was determined from published literature showing sustained growth of various pancreatic cancer cells including MIA PaCa-2 and PANC-1 cells. Cells were cultured in glutamine-deficient media overnight (16 h), followed by stimulation with 2 mM glutamine for up to 48h. Following stimulation, whole cell lysates were prepared at 0h, 2h, 6h and 24h for MIA PaCa-2 cells; 0h, 6h, 24h and 48h for BxPC-3 and PANC-1 cells and examined by western blot analysis.

We observed significantly increased pSTAT3 levels at 24h in MIA PaCa-2 cells (p=0.0003) and at 48h in BxPC-3 cells (p=0.038). A significant increase in pSTAT3 was also observed at 6h (p=0.0019) and 24h (p=0.0004) in PANC-1 cells, but pSTAT3 levels was drastically reduced at 48h, which is possibly caused by exhaustion of glutamine at this time point due to high demand of glutamine in PANC-1 cells due to its KRAS-mutation and metabolic features (Table 1) (Figure 3 A-C). There was a trend towards increased total STAT3, in MIA PaCa-2 and BxPC-3 cells at 24h and 48h respectively. However, we noticed a statistically significant increase in total STAT3 level in PANC-1 cells at 48h (p=0.0183), indicating that glutamine stimulation also increased total STAT3 levels. To examine if glutamine stimulation affects STAT3 activation levels, we calculated the ratio of pSTAT3/STAT3, and observed significantly increased STAT3 activation (pSTAT3/STAT3) at 6 h (p=0.0474) and 24 h (p=0.0001) glutamine stimulation in MIA PaCa-2 cells, 48 h (p=0.0003) glutamine stimulation in BxPC-3 cells and 24h (p=0.0284) glutamine stimulation in PANC-1 cells (Figure 3). These data suggest that glutamine stimulation activates STAT3 signaling in pancreatic cancer cells.

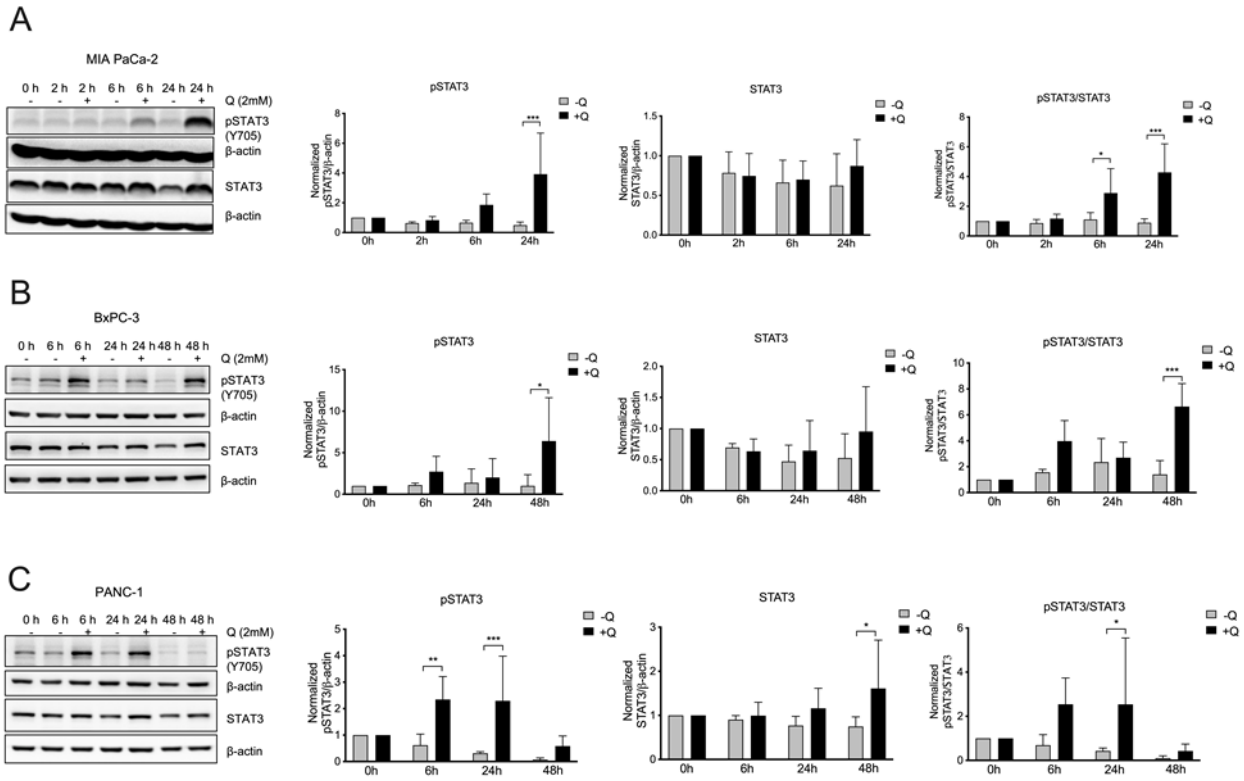


Figure 3 Glutamine Stimulation Enhanced pSTAT3 Levels in Pancreatic Cancer Cells. [A-C] MIA PaCa-2 (n=4) **(A)**, BxPC-3 (n=3) **(B)** and PANC-1 (n=5) **(C)** cells were starved in glutamine-deficient media overnight and stimulated with 2 mM glutamine for 0 – 24h (for MIA PaCa-2) and 0 – 48h (PANC-1 and BxPC-3). Whole cell lysates were collected to detect total STAT3 and pSTAT3 Y705 levels using immunoblotting. The ratio of normalized pSTAT3 to total STAT3 levels were calculated and plotted. Data presented represent an average ± SD of three to five independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, relative to 0 mM glutamine. (Two-way ANOVA followed by post-hoc Sidak test). Abbreviations: glutamine (Q)

Table 1 Differences between pancreatic cancer cells

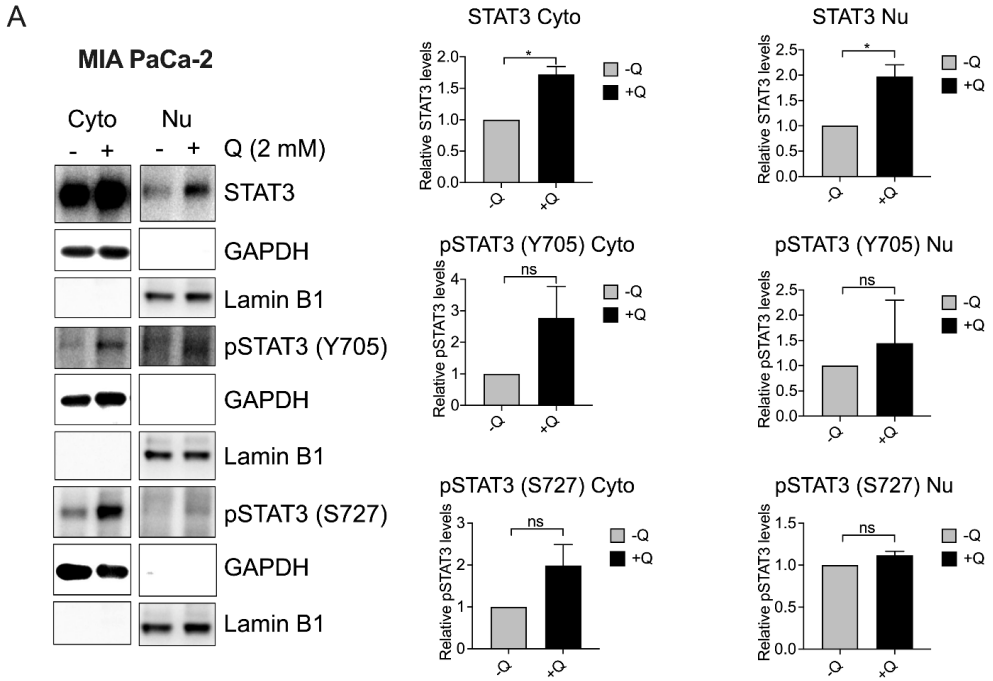
Cell line	Differentiation	KRAS mutation	Derivation	Doubling Time	Metabolic Phenotype	STAT3 levels	Others
MIA PaCa-2	Poor	G12C	primary tumor	40h	Glycolytic	Medium	
PANC-1	Poor	G12D	primary tumor	52h	Lipogenic	High	high pERK
BxPC-3	Moderate to Poor	WT	primary tumor	48–60h	Lipogenic	Medium	high COX-2
Capan-2	Well	G12V	primary tumor	96h	Slow Proliferating	Medium	

4) Glutamine stimulation increased nuclear levels of pSTAT3 (Y705) in pancreatic cancer cells

Given that in canonical STAT3 signaling, pSTAT3 translocates to the nucleus and binds to the consensus sequence in the promoter region of target genes to activate target gene expression, we examined the effect of glutamine stimulation on STAT3 and pSTAT3 (Y705) using nuclear and cytoplasmic fractions in MIA PaCa-2 cells. Logarithmically growing MIA PaCa-2 cells were seeded at a density of 2 million cells per dish in 10 cm dishes. Following attachment (~24h), cells were starved overnight in glutamine-deficient DMEM supplemented with 10% FBS plus 5% horse serum, followed by stimulation with or without 2 mM glutamine for 24 h. Nuclear

and cytoplasmic protein fractions were isolated using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA) essentially as described by the manufacturer. Lamin B1 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as loading controls for nuclear and cytoplasmic fractions respectively. We observed that glutamine stimulation increased levels of total STAT3 in both cytoplasmic (p=0.0142) and nuclear (p=0.0269) fractions. On the other hand, we observed trend albeit not statistically significant towards increased pSTAT3 (Y705) in both cytoplasmic and nuclear fractions (Figure 4A). Given the role of pSTAT3 (S727) in mitochondria activities, we explored the effects of glutamine stimulation on pSTAT3 (S727). We observed that the majority of pSTAT3 (S727) in the cytoplasmic fraction, which is in line with its functions in the mitochondria to enhance electron transport chain activity and ATP production. We also observed a minor portion of pSTAT3 (S727) in the nuclear fraction, which did not respond to glutamine stimulation (Figure 4A).

As an independent approach, we performed immunofluorescence (IF) analysis in MIA PaCa-2 cells and observed increased nuclear pSTAT3 with glutamine stimulation (Figure 4B). These observations suggest that glutamine-stimulation had no significant impact on nuclear translocation of STAT3. Nevertheless, results from both subcellular fractionation and IF demonstrated a trend towards increased nuclear levels of pSTAT3 (Y705), implying activated STAT3 signaling with glutamine stimulation.



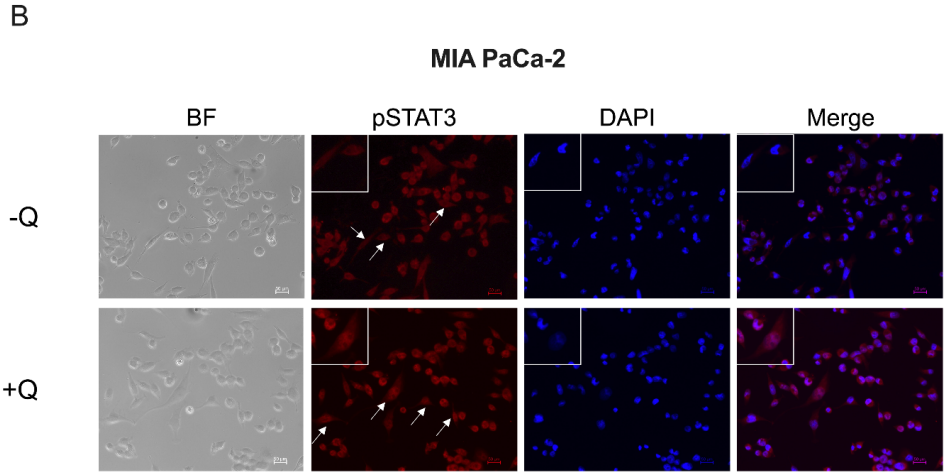


Figure 4 Glutamine Stimulation Increased Nuclear Levels of pSTAT3 (Y705) in Pancreatic Cancer Cells. **[A]** Cytoplasmic and nuclear levels of STAT3, pSTAT3 (Y705) and pSTAT3 (S727) in MIA PaCa-2 cells treated with the presence and absence of 2 mM glutamine for 24 h were examined with immunoblotting. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and Lamin B1 were used as cytoplasmic and nuclear loading controls, respectively. Quantification was presented as average ± SD of two independent experiments. * $P < 0.05$ (Student's t-test). **[B]** MIA PaCa-2 cells treated with the presence and absence of 2 mM glutamine for 24 h were examined with IF staining of pSTAT3 (red) (Magnification = 20x). DAPI (blue) was used for nuclear staining. Arrows indicate pSTAT3 staining in and out of the nucleus. Image present is a representation of two independent experiments. Abbreviations: Glutamine (Q); Cytoplasmic fraction (Cyto); Nuclear fraction (Nu); Bright field (BF).

5) Glutamine stimulation increased survivin levels and promoter activity in pancreatic cancer cells

The above data suggest that glutamine stimulation activates STAT3 signaling in pancreatic cancer cells. As a master transcription factor, STAT3 mediates various downstream target genes in a broad range of cellular processes such as proliferation and survival, migration, invasion and metastasis, angiogenesis and immune evasion. Survivin is one of the downstream target genes of STAT3 and is associated with poor survival in human pancreatic tumors. Studies also showed the involvement of survivin in PDAC development, metastatic spread and therapeutic resistance. We therefore tested the effect of glutamine on protein levels and transcriptional activity of survivin in pancreatic cancer cells. We observed that glutamine stimulated a trend increase of survivin levels in MIA PaCa-2 cells in a dose-dependent manner (Figure 5 A). Furthermore, depending on the cell line, the increased levels of survivin were observed at either 24h or 48h of stimulation (Figure 5 B-D). Specifically, we observed trend but non-significant increase of survivin levels at 24h in MIA PaCa-2 and PANC-1 cells. Interestingly, although survivin levels in BxPC-3 cells have dropped at 24h and 48h compared to baseline 0h, survivin protein levels were still higher (non-significant) under glutamine stimulation compared to glutamine starvation. We measured survivin reporter activity by transfecting MIA PaCa-2 and PANC-1 cells with human survivin promoter-luciferase construct (pLuc-survivin) (containing STAT3 binding sites) followed by glutamine stimulation. We observed significantly increased survivin reporter activity with glutamine stimulation in MIA PaCa-2 cells at 6h ($p=0.023$) and at 24h in PANC-1 cells ($p=0.0004$; Figure 5 E-F). Taken together, these data suggest that glutamine stimulation activates transcription of survivin.

To understand whether glutamine activates survivin's promoter activity via STAT3, we used MIA PaCa-2 cells with stably silenced STAT3. STAT3 knockdown (KD) efficiency was evaluated by immunoblot analysis. As shown in Figure 5 G, we observed significant reduction (>80%) of STAT3 levels in STAT3-KD compared to non-targeted control (NTC) cells (Figure 5 G). Transient expression assays using survivin-promoter showed that glutamine stimulation significantly increased survivin's promoter activity in both NTC ($p=0.0262$) and STAT3 KD ($p=0.0232$) cells (Figure 5 H). These data imply that the increase in survivin's promoter activity upon glutamine stimulation is not solely mediated by STAT3.

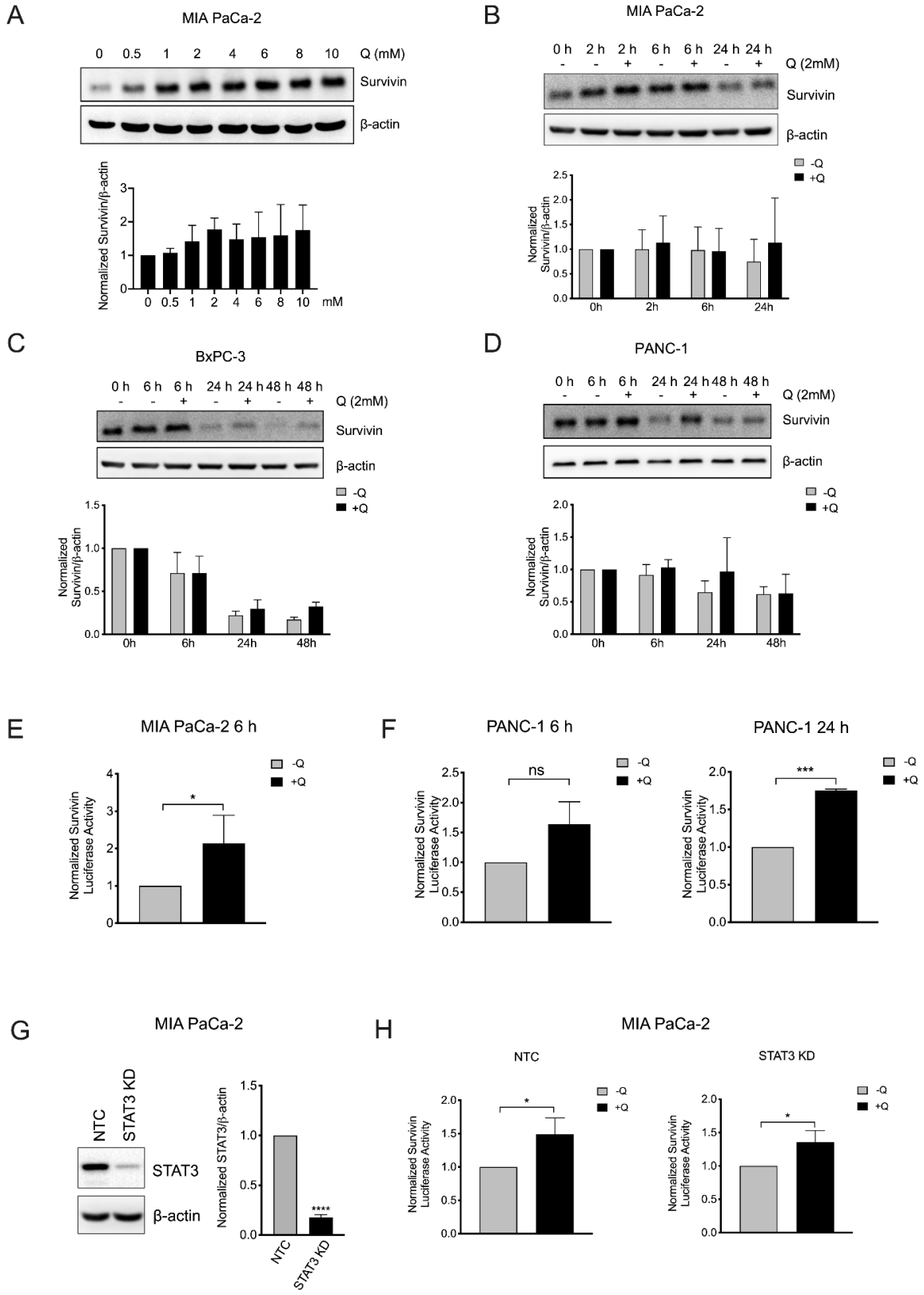


Figure 5 Glutamine Stimulation Increased Survivin Levels and Promoter Activity in Pancreatic Cancer Cells. [A] MIA PaCa-2 cells were starved from glucose and glutamine overnight, and stimulated with 0 – 10 mM glutamine for 24h. Whole cell lysates were harvested to detect survivin levels using immunoblotting. Data presented is an average ± SD of three independent experiments. **[B-D]** MIA PaCa-2 (n=4) **(B)**, BxPC-3 (n=3) **(C)** and PANC-1 (n=5) **(D)** cells were starved from glucose and glutamine overnight, and stimulated with 2 mM glutamine for 0 – 24h (MIA PaCa-2) and 0 – 48h (PANC-1 and BxPC-3). Whole cell lysates were collected to detect survivin levels using immunoblotting. **[E-F]** pLuc-survivin containing STAT3 binding site were transiently transfected into MIA PaCa-2 (n=4) **(E)** and PANC-1 (n=2) **(F)** cells with renilla luciferase construct.

24 h after transfection, cells were stimulated with glutamine for 6 h for MIA PaCa-2 cells, 6h and 24h for PANC-1 cells. Cells were then collected, lysed, and luciferase/renilla activity was measured and calculated in each group. Data presented is an average ± SD of two to four independent experiments. ns: not significant, * P < 0.05, *** P < 0.001, relative to 0 mM glutamine (-Q) group (Student's t-test). **[G]** Protein levels of STAT3 by immunoblotting in NTC and STAT3 KD MIA PaCa-2 cells. Quantification is an average ± SD of three independent experiments. **** P < 0.0001 (Student's t-test). **[H]** pLuc-survivin containing STAT3 binding site were transiently transfected into MIA PaCa-2 NTC and STAT3 KD cells with renilla luciferase construct. 24 h after transfection, cells were stimulated with glutamine for 6 h. Cells were then collected, lysed, and luciferase/renilla activity was measured and calculated in each group. Data presented is an average ± SD of three independent experiments. * P < 0.05, relative to 0 mM glutamine (-Q) group (Student's t-test). Abbreviations: Glutamine (Q); non-targeted control (NTC); knockdown (KD)

6) The effect of glutamine synthetase (GS) knockdown (KD) on glutamine levels in PSC-conditioned media (CM)

To determine the role of PSC-secreted glutamine on cancer hallmarks, I generated stable GS-KD PSCs. Validation of GS-KD is shown in Figure 6A. To test the effect of GS-KD on glutamine levels in the PSC-CM, I collected CM from PSCs at 24h, 48h and 72h after plating cells at 300,000/dish in 60mm dishes. Glutamine concentration in the CM was measured using glutamine/glutamate-Glo Assay Kit (Promega, Madison, WI). Our result showed similar levels of glutamine in the CM of NTC and GS-KD PSCs (Figure 6B). It is possible that the remaining GS in GS-KD cells is sufficient to synthesize glutamine to a comparable level in the NTC cells. Therefore, GS knockout PSCs may be needed to evaluate the role of PSC-secreted glutamine on pancreatic cancer cells.

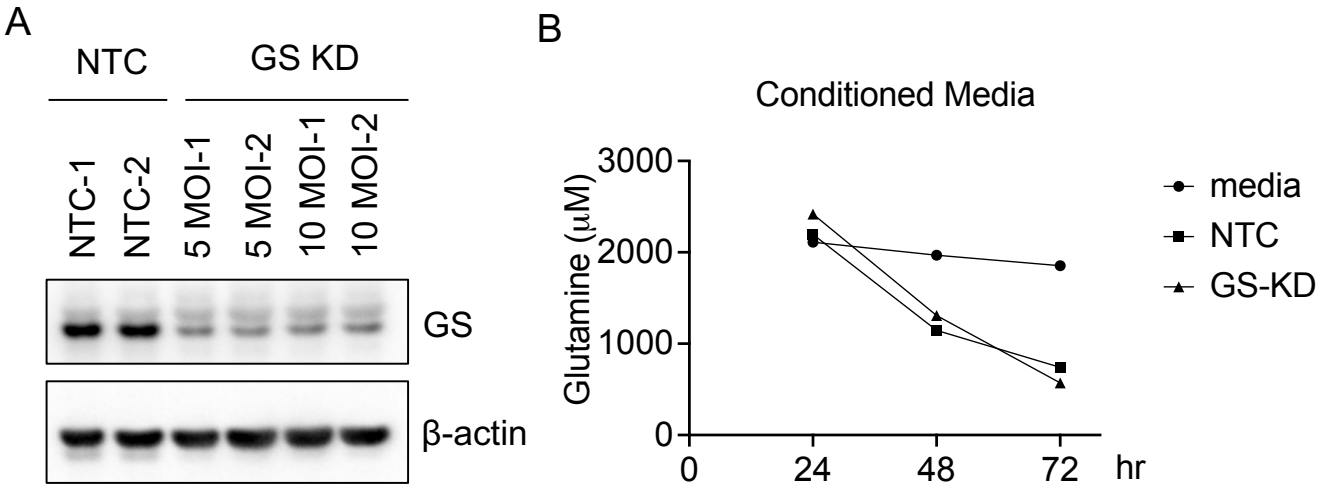


Figure 6. The effect of glutamine synthetase (GS) knockdown (KD) on glutamine levels in PSC-conditioned media (CM). [A] Validation of glutamine synthetase (GS) knockdown (KD) in pancreatic stellate cells (PSCs). PSCs were transducted with lentiviral particles carrying shRNA against non-targeted control (NTC) and Glutamine synthase (KD). Whole cell protein extracts prepared from NTC and KD cells were examined by Western Blot. **[B]** PSCs were plated at 300,000/plate in 60mm dishes. CM was collected at 24h, 48h and 72h after plating and glutamine concentration in the CM was measured using glutamine/glutamate-Glo Assay Kit (Promega, Madison, WI).

Major goal 2: Determine the effects of PMT on glutamine release from PSCs and on cancer hallmarks in PCCs

1) PMT reduces glutamine levels in the conditioned media of PSC

To test the effect of PMT on glutamine-release from PSCs, I treated PSCs with increasing doses of PMT followed by measurement of glutamine concentration in the conditioned media and cell lysate with glutamine/glutamate-Glo Assay Kit (Promega, Madison, WI). Our results reveal that PMT treatment reduces secreted levels of Q in the CM but not intracellular levels (Figure 7).

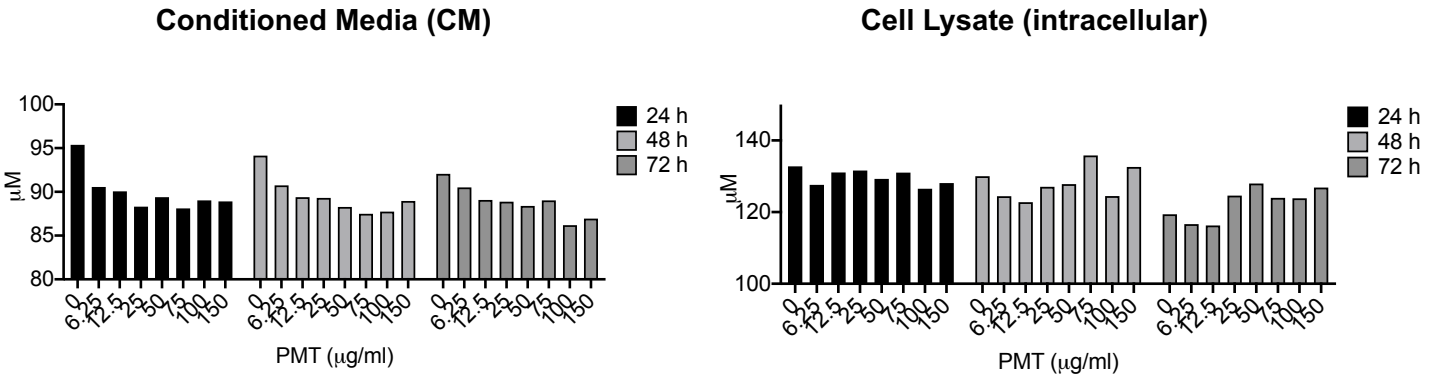


Figure 7. PMT reduces Q levels in PSC CM. PSCs were treated with increasing doses of PMT for 24h – 72h. CM and cell lysate was collected and measured following the manufacturer's instructions of Glutamine/glutamate-Glo Assay Kit (Promega, Madison, WI).

2) PMT suppresses glutamine-induced proliferation in pancreatic cancer cells

To examine the functional significance of PMT on glutamine-stimulation induced biological outcome, we performed proliferation assays in human pancreatic cancer cell lines with different KRAS mutation, differentiation and metabolic status (Table 1). MIA PaCa-2, BxPC-3, PANC-1 and Capan-2 cells were starved in glutamine-deficient media overnight and stimulated with or without 2 mM glutamine in the presence and absence of 0-50 μg/ml PMT (MIA PaCa-2, BxPC-3 and Capan-2 cells) or 0-100 μg/ml PMT (PANC-1 cells) for 48 h. We observed that PMT reduced glutamine-induced proliferation in all cell lines tested in a statistically significant manner with MIA PaCa-2 being the most sensitive (Figure 8 A-D). PMT treatment reduced glutamine-induced proliferation starting at doses as low as 3.125 μg/ml in MIA PaCa-2 cells (Figure 8 A). BxPC-3 and Capan-2 cells were less sensitive compared to MIA PaCa-2 to PMT treatment displaying proliferation inhibition starting at 12.5 μg/ml PMT (Figure 8 B, D). In contrast, we found PANC-1 cells are the least sensitive relative to other cell lines tested given the requirement of 50 μg/ml PMT to exert anti-proliferative effects (Figure 8 C).

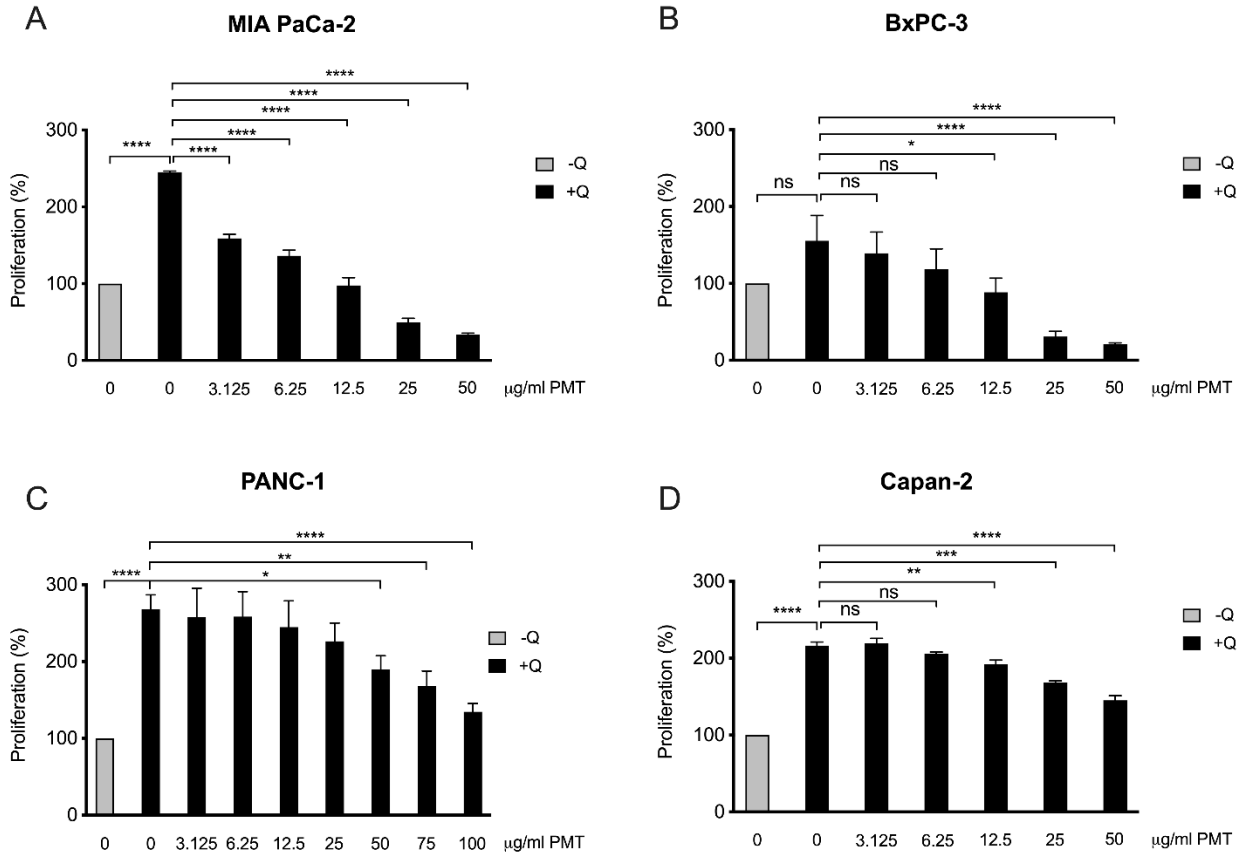


Figure 8 Impact of PMT on Glutamine–induced Proliferation in Pancreatic Cancer Cells. [A-D] MIA PaCa-2 (n=3) **(A)**, BxPC-3 (n=3) **(B)**, PANC-1 (n=3) **(C)** and Capan-2 (n=2) **(D)** cells were starved in glutamine-deficient media overnight, and stimulated with or without 2 mM glutamine and 0-50 µg/ml PMT (MIA PaCa-2, BxPC-3 and Capan-2 cells) or 0-100 µg/ml PMT (PANC-1 cells) for 48 h. Cell proliferation was measured by MTT assay (which measures intracellular metabolic activity). Data presented is an average ± SD of two to three independent experiments. ns: not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, *** $P < 0.0001$ (One-way ANOVA followed by post-hoc Tukey test). Cell proliferation was measured by MTT assay. Data presented is an average ± SD of three to four independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, *** $P < 0.0001$ (Two-way ANOVA followed by post-hoc Tukey test). Abbreviations: Glutamine (Q); Palmatine (PMT); Non-targeted control (NTC); Knockdown (KD).

3) PMT suppresses glutamine-induced multiple cancer hallmarks in pancreatic cancer cells

Based on the above observations, we examined the effect of PMT on additional cancer hallmarks, including clonogenicity, migration and invasion using MIA PaCa-2 cells. Clonogenic assays showed that PMT significantly ($p=0.0029$) reduced glutamine-induced colony formation in MIA PaCa-2 at 25 µg/ml (Figure 9 A). However, PMT had no effect on glutamine-induced colony formation ability in PANC-1 cells (Figure 9 B). In addition, PMT reduced glutamine-induced anchorage-independent growth starting at 6.25 µg/ml (Figure 9 C), migration at 25 µg/ml ($p<0.0001$; Figure 9 D) and invasion (Figure 9 E) at both 6.25 ($p=0.0257$) and 25 ($p=0.0102$) µg/ml. Taken together, these data suggest that PMT suppresses glutamine-induced clonogenicity, anchorage-independent growth, migration and invasion in MIA PaCa-2 cells.

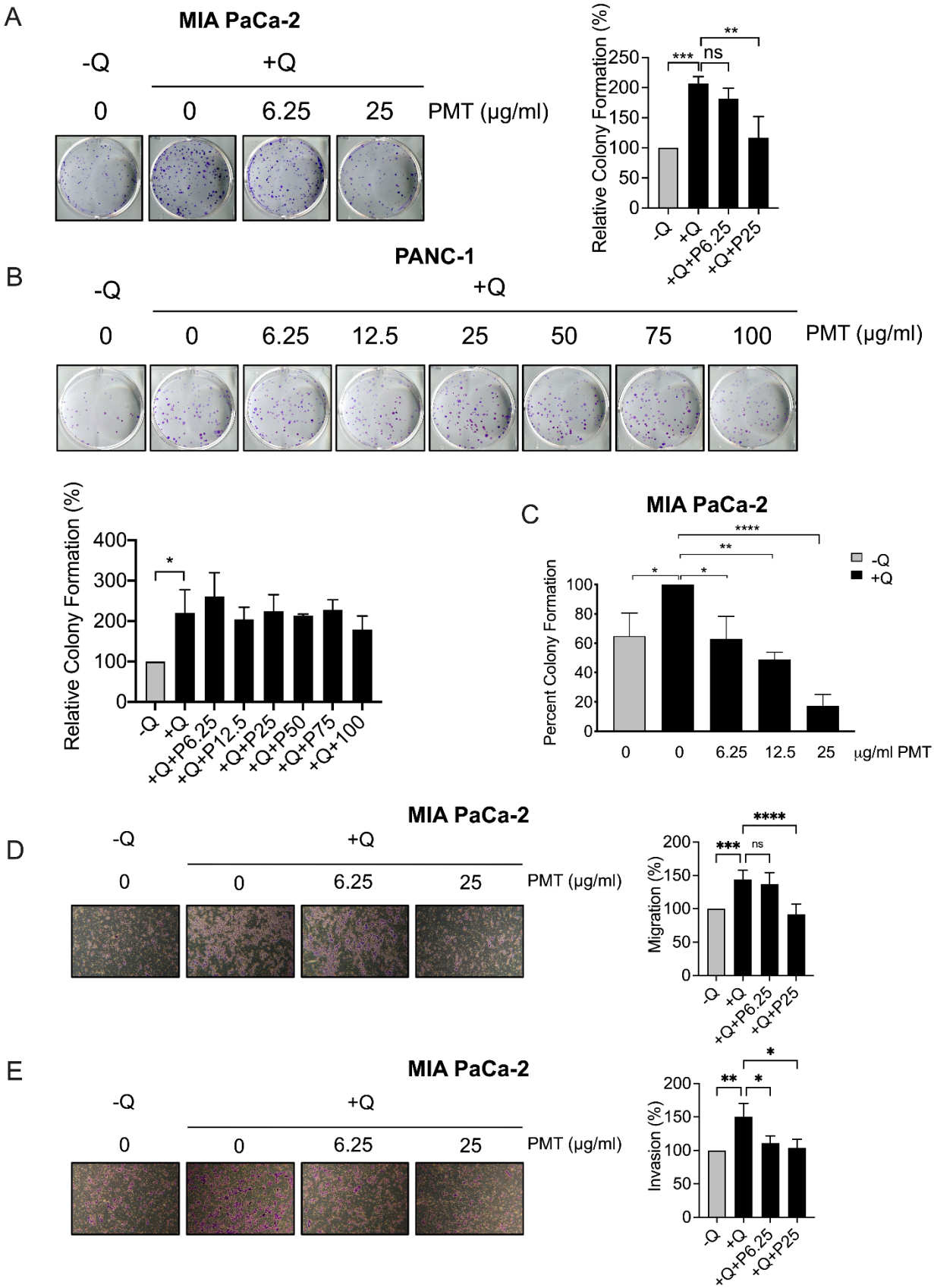

A
MIA PaCa-2
-Q
+Q
0
0
6.25
25
PMT (µg/ml)
Relative Colony Formation (%)
***
ns
**
-Q
+Q
+Q+P6.25
+Q+P25
B
PANC-1
-Q
+Q
0
0
6.25
12.5
25
50
75
100
PMT (µg/ml)
Relative Colony Formation (%)
*
-Q
+Q
+Q+P6.25
+Q+P12.5
+Q+P25
+Q+P50
+Q+P75
+Q+100
C
MIA PaCa-2
Percent Colony Formation
-Q
+Q
0
0
6.25
12.5
25
µg/ml PMT
D
MIA PaCa-2
-Q
+Q
0
0
6.25
25
PMT (µg/ml)
Migration (%)
***
ns
****
-Q
+Q
+Q+P6.25
+Q+P25
E
MIA PaCa-2
-Q
+Q
0
0
6.25
25
PMT (µg/ml)
Invasion (%)
**
*
*
-Q
+Q
+Q+P6.25
+Q+P25

Figure 9 PMT Suppresses Glutamine–induced Clonogenicity, Anchorage-independent Growth, Migration and Invasion in MIA PaCa-2 Cells. [A-B] Logarithmically growing MIA PaCa-2 (n=3) **(A)** and PANC-1 (n=3) **(B)** cells were starved in glutamine-deficient media overnight, and stimulated with or without 2 mM glutamine with increasing concentrations of PMT for 24h (MIA PaCa-2) and 48h (PANC-1). Cells were then trypsinized and viable cell concentration was determined by trypan blue assay. 1000 (MIA PaCa-2) or 500 (PANC-1) live cells were seeded in complete media in 6-well plates. Cells were maintained 7-14 days until colonies are formed. Colonies were stained with 0.1% crystal violet in methanol. Quantification was done by solubilizing crystal violet staining with 10% acetic acid and measuring absorbance at 570 nm. Data presented is an average ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (One-way ANOVA followed by post-hoc Tukey test) **[C]** Logarithmically growing MIA PaCa-2 cells were starved in glutamine-deficient media overnight, and treated with or without 2 mM glutamine with increasing concentrations of PMT (0-25 μg/ml) for 24h. Cells were then trypsinized and viable cell concentration was determined by trypan blue assay. 10,000 viable cells were seeded in soft agar and maintained for 7-10 days. Anchorage-independent growth was quantified following the manufacturer's instructions using CytoSelect 96-well Cell Transformation Assay (Cell Biolabs, San Diego, CA). Data presented is an average ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (One-way ANOVA followed by post-hoc Sidak test) **[D-E]** MIA PaCa-2 cells starved in glutamine-deficient media were treated with the presence or absence of glutamine (2mM) and PMT (6.25 and 25 μg/ml) for 24h. Cells were then trypsinized and viable cell concentration was determined by trypan blue assay. 300,000 cells (for migration assay) **(D)** or 500,000 cells (for invasion assay) **(E)** suspended in serum-free media were seeded in the cell culture insert with a porous membrane for cell migration **(D)**. A thin layer of MATRIGEL Basement Membrane Matrix served as reconstituted basement membrane was included in the invasion chamber **(E).** Complete media (with serum) was added to the bottom chamber as chemoattractant. Cells were incubated for 24h for migration assay **(D)** or 28h for invasion assay **(E)**. Cells on the lower surface of the membrane were stained with 0.2% crystal violet in methanol. Images were taken with an inverted Zeiss PrimoVert light microscope. Quantification was done by solubilizing crystal violet staining with 30% acetic acid and measuring absorbance at 570 nm. Data presented is an average ± SD of five **(D)** or three **(E)** independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (One-way ANOVA followed by post-hoc Sidak test) Abbreviations: Glutamine (Q); Palmatine (PMT of P).

Major goal 3: Establish the causal relationship between glutamine (Q) treatment, STAT3 and Survivin's promoter activity and verify the role of PMT in this process

1) PMT treatment reduces glutamine–induced pSTAT3 levels in pancreatic cancer cells in a cell line specific manner

In Major Goal 1, we established that glutamine-stimulation increased pSTAT3 levels in pancreatic cancer cells MIA PaCa-2. Here, we evaluated the role of PMT on glutamine-induced pSTAT3 levels in multiple cancer cells. Briefly, MIA PaCa-2, PANC-1 and BxPC-3 cells were starved in glutamine-deficient media overnight and stimulated with 2 mM glutamine in the presence and absence of 6.25 μg/ml PMT for 0 – 48h. Whole cell lysates were collected to detect total STAT3 and pSTAT3 Y705 levels using western blotting. Glutamine stimulation significantly enhanced pSTAT3 levels both at 6h ($p=0.0254$) and 24h ($p<0.0001$) in MIA PaCa-2 cells, and that treatment with PMT resulted in significant reduction in glutamine-induced stimulation of pSTAT3 at 24h ($p=0.0009$) (Figure 10A). Similarly, in PANC-1 cells, glutamine stimulation significantly enhanced pSTAT3 ($p<0.0001$), that was reduced with PMT treatment ($p=0.016$) at 24h (Figure 10B). Although glutamine stimulation resulted in a significantly increased pSTAT3 in BxPC-3 cells at 48h ($p<0.0001$), PMT treatment had no significant effect on glutamine-induced pSTAT3 levels in BxPC-3 cells (Figure 10C). The different sensitivity between cell lines can be attributed to their different KRAS mutation, metabolic subtype, doubling time and cell-specific signaling pathways depicted in Table 1. Furthermore, glutamine stimulation also resulted in increased levels of total STAT3 in all three cell lines tested. However, the observed increase reached statistical significance only in BxPC-3 cells ($p=0.0053$). Importantly, PMT treatment showed no significant effect on glutamine-induced total STAT3 levels in all three cell lines tested (Figure 10 A-C).

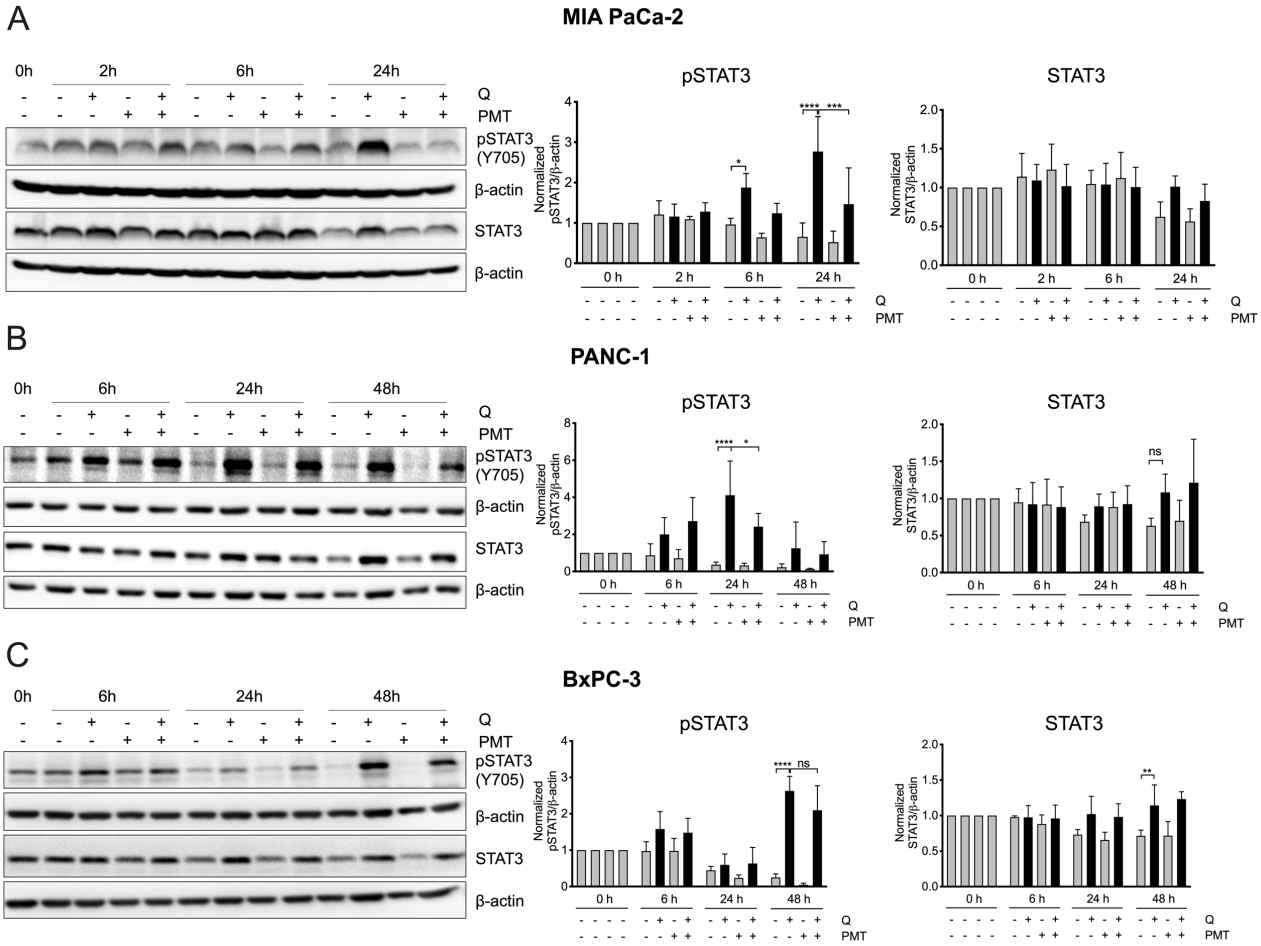
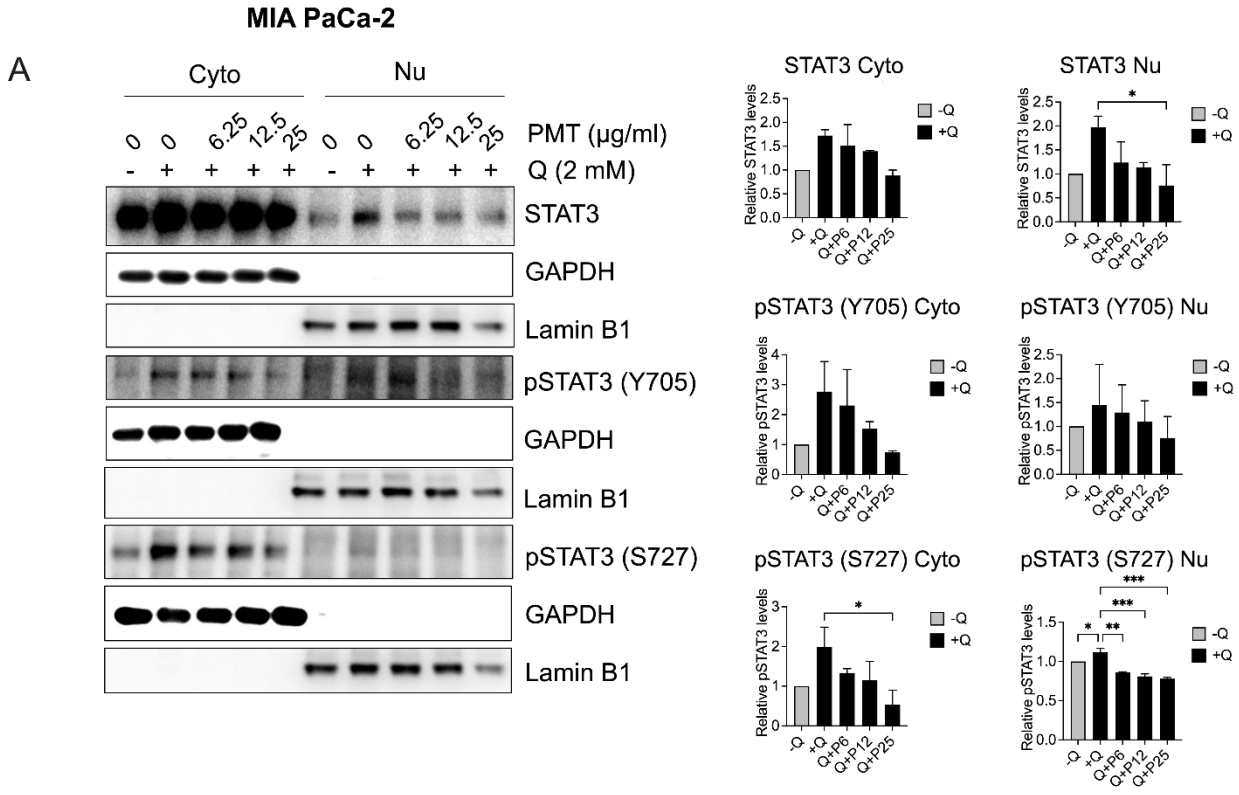


Figure 10 PMT Treatment Reduces Glutamine–induced pSTAT3 Levels in Pancreatic Cancer Cells in a Cell Line Specific Manner. [A-C] MIA PaCa-2 (n=3) **(A)**, PANC-1 (n=4) **(B)** and BxPC-3 (n=3) **(C)** cells were starved in glutamine-deficient media overnight, and stimulated with 2 mM glutamine in the presence and absence of 6.25 µg/ml PMT for 0 – 48h. Whole cell lysates were collected to detect total STAT3 and pSTAT3 Y705 levels using western blotting. Data presented is an average ± SD of three to four independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. (Two-way ANOVA followed by post-hoc Tukey test). Abbreviations: Glutamine (Q); Palmatine (PMT).

2) PMT treatment reduces glutamine-induced nuclear levels of pSTAT3 (Y705)

Given that PMT treatment significantly reduced levels of pSTAT3 with no impact on total levels, we examined the effect of PMT on the subcellular localization of pSTAT3 (Y705 and S727) in MIA PaCa-2 cells. Subcellular fractionation experiments revealed that PMT treatment resulted in significantly (p=0.0456) reduced nuclear STAT3 levels at 25 µg/ml in these cells. On the other hand, we observed trend towards reduction in the levels of pSTAT3 (Y705) in both cytoplasmic and nuclear fractions (Figure 11 A). Interestingly, we found that PMT treatment (25 µg/ml) significantly (p=0.0447) reduced cytoplasmic levels of pSTAT3 (S727). There was also slight but significant reduction of nuclear pSTAT3 (S727) with PMT treatment at 6.25 µg/ml (p=0.0013), 12.5 µg/ml (p=0.0006) and 25 µg/ml (p=0.0004) (Figure 11 A). Using immunofluorescence (IF) as an independent approach, we observed that PMT treatment reduced glutamine-induced pSTAT3 (Y705) levels in the nucleus, as indicated by the overlap between pSTAT3 staining (red) and nuclear staining by DAPI (blue) (Figure 11 B). These data suggest that PMT treatment reduced glutamine-induced STAT3 signaling in MIA PaCa-2 cells.


MIA PaCa-2
A
Cyto
Nu
0 0 6.25 12.5 25 0 0 6.25 12.5 25
PMT (μg/ml)
- + + + + - + + + +
Q (2 mM)
STAT3
GAPDH
Lamin B1
pSTAT3 (Y705)
GAPDH
Lamin B1
pSTAT3 (S727)
GAPDH
Lamin B1
STAT3 Cyto
STAT3 Nu
pSTAT3 (Y705) Cyto
pSTAT3 (Y705) Nu
pSTAT3 (S727) Cyto
pSTAT3 (S727) Nu
Relative STAT3 levels
Relative pSTAT3 levels
-Q
+Q
-Q +Q Q+P6 Q+P12 Q+P25

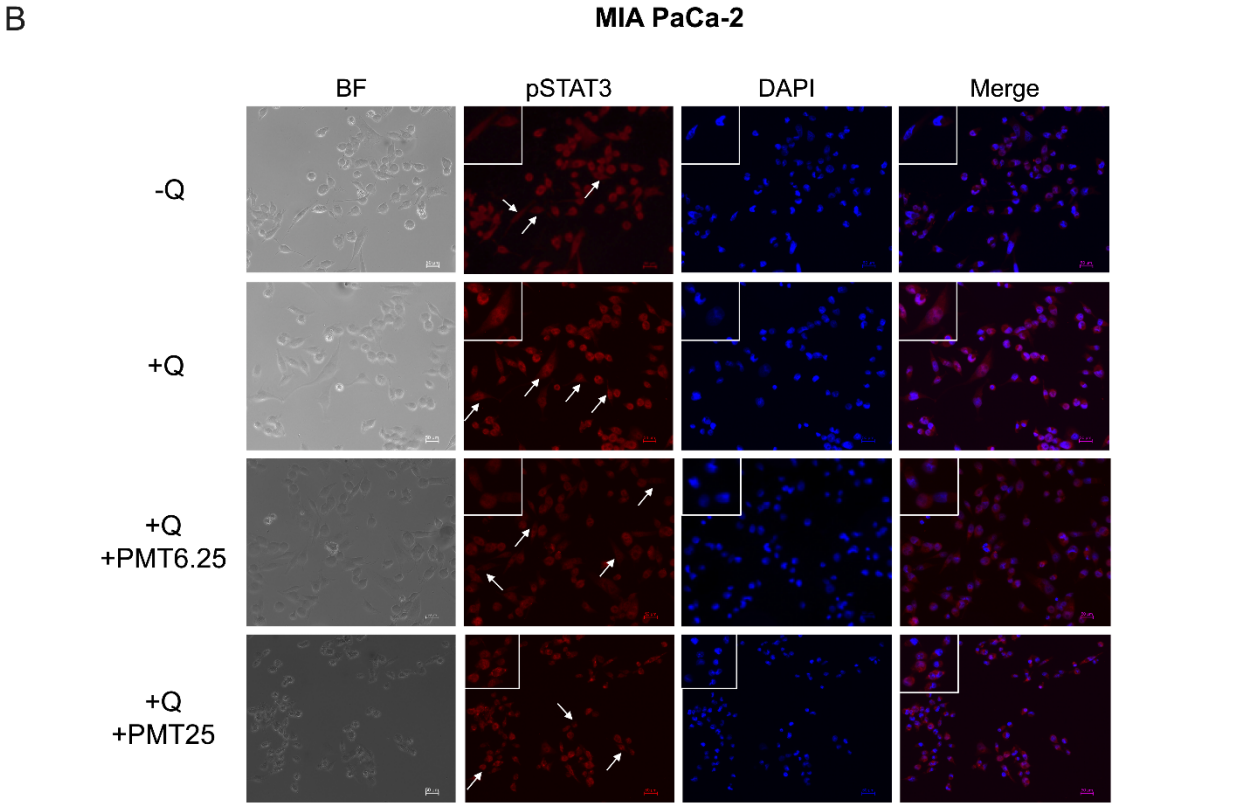

B
MIA PaCa-2
BF
pSTAT3
DAPI
Merge
-Q
+Q
+Q
+PMT6.25
+Q
+PMT25

Figure 11 Effect of PMT on Glutamine–induced Nuclear Levels of pSTAT3 (Y705) in Pancreatic Cancer Cells. [A] MIA PaCa-2 cells were starved in glutamine-deficient media overnight, and treated with the presence or absence of 2 mM glutamine and 0-25 μg/ml PMT for 24h. Cytoplasmic and nuclear levels of STAT3, pSTAT3 (Y705) and pSTAT3 (S727) were examined with western blotting. GAPDH and Lamin B1 were used as cytoplasmic and nuclear loading control, respectively. Quantification was presented as average ± SD of two independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (One-way ANOVA followed by post-hoc Tukey test). **[B]** MIA PaCa-2 cells were starved in glutamine-deficient media overnight and treated with the presence and absence of glutamine (2mM) and PMT (6.25 and 25 μg/ml) for 24 h. Cells were examined with immunofluorescent staining of pSTAT3 (Y705) (red) (Magnification = 20x). DAPI (blue) was used for nuclear staining. Image present is a representation of two independent experiments. Abbreviations: Glutamine (Q); Palmatine (PMT); Cytoplasmic fraction (Cyto); Nuclear fraction (Nu); Bright field (BF).

3) PMT suppresses glutamine–induced survivin's promoter activity in pancreatic cancer cells in a cell line-specific manner

We showed that glutamine stimulation results in increased promoter activity of survivin, one of the well-known downstream targets of STAT3. Given the association between survivin with PDAC progression, treatment resistance and low patient survival, we examined the effect of PMT on survivin levels in pancreatic cancer cells.

We first treated MIA PaCa-2, PANC-1 and BxPC-3 cells with increasing concentrations (0-150 μg/ml) of PMT for 24h and 48h in complete media. We found that PMT treatment significantly reduced survivin levels in MIA PaCa-2 at 24 h (using 50 μg/ml) but not at 48 h (Figure 12 A); in PANC-1cells at 24 h using 50 μg/ml and 48 h with 25 μg/ml) and in BxPC-3 cells at 24 h using 100 μg/ml and 48 h with 25 μg/ml) (Figure 12 A-C). In order to test the effect of PMT on glutamine induced survivin levels, we starved MIA PaCa-2, PANC-1 and BxPC-3 cells in glutamine-deficient media overnight and stimulated with 2 mM glutamine in the presence of 6.25 μg/ml PMT. Under these experimental conditions, we observed a trend towards reduction of survivin levels in MIA PaCa-2 cells and PANC-1 cells albeit not statistically significant (Figure 12 D-E). However, PMT treatment showed no effect on glutamine-induced survivin levels in BxPC-3 cells (Figure 12 F).

We also examined survivin's promoter activity by transfecting MIA PaCa-2 and PANC-1 cells with human survivin promoter-luciferase plasmid containing STAT3 binding sites (pLuc-survivin) under conditions of glutamine stimulation. Interestingly, our results show that PMT significantly inhibited glutamine-induced increase in survivin promoter activity in MIA PaCa-2 using 6.25 ($p=0.0144$) and 25 μg/ml ($p=0.0023$) (Figure 12 G). However, PMT showed no effect on inhibiting glutamine-induced survivin's promoter activity in PANC-1 cells (Figure 12 H).

Furthermore, we also tested whether the PMT-induced reduction in survivin's promoter activity in MIA PaCa-2 cells is mediated by STAT3 using STAT3 KD cells generated (Figure 12 G). We found that silencing STAT3 partially rescued the effect of PMT in reducing glutamine-stimulated promoter activity of survivin under these experimental conditions ($p=0.0327$). These data suggest that PMT suppresses glutamine-induced survivin's promoter activity at least in part through STAT3 (Figure 12 I).

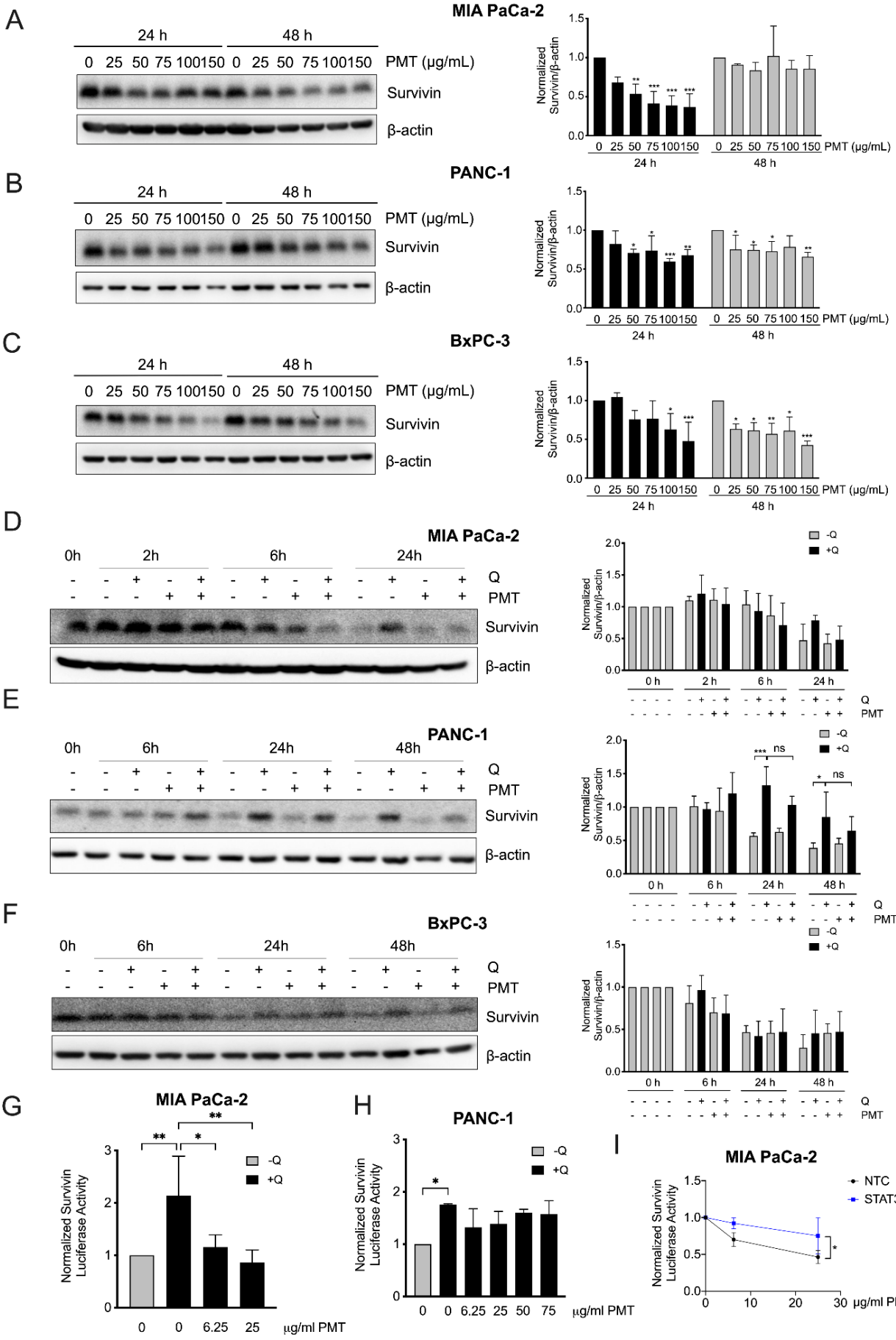

A
MIA PaCa-2
24 h
48 h
0 25 50 75 100150 0 25 50 75 100150 PMT (μg/mL)
Survivin
β-actin
Normalized Survivin/β-actin
0 25 50 75 100150 0 25 50 75 100 150 PMT (μg/mL)
24 h
48 h
B
PANC-1
C
BxPC-3
D
MIA PaCa-2
0h 2h 6h 24h
Q
PMT
-Q
+Q
E
PANC-1
0h 6h 24h 48h
ns
F
BxPC-3
G
MIA PaCa-2
Normalized Survivin Luciferase Activity
0 0 6.25 25 μg/ml PMT
H
PANC-1
0 0 6.25 25 50 75 μg/ml PMT
I
MIA PaCa-2
NTC
STAT3 KD
0 10 20 30 μg/ml PMT

Figure 12 PMT Suppresses Glutamine–induced Survivin's Promoter Activity in Pancreatic Cancer Cells. [A-C] MIA PaCa-2 (n=3) **(A)**, PANC-1 (n=3) **(B)** and BxPC-3 (n=3) **(C)** cells were treated with increasing concentrations of PMT (0-150 μg/ml) for 24 h and 48 h. Whole cell lysates were collected to analyze survivin levels with western blotting. Data presented is an average ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (Two-way ANOVA followed by post-hoc Dunnett test). **[D-F]** MIA PaCa-2 (n=3) **(D)**, PANC-1 (n=3) **(E)** and BxPC-3 (n=3) **(F)** cells were starved in glutamine-deficient media overnight, and stimulated with glutamine (2 mM) and PMT (6.25 μg/ml) for 0 – 24h (MIA PaCa-2) and 0-48h (PANC-1 and BxPC-3). Whole cell lysates were collected to detect survivin levels using immunoblotting. Data presented is an average ± SD of three independent experiments. **[G-H]** pLuc-survivin containing STAT3 binding site were transiently transfected into MIA PaCa-2 (n=4) **(G)** and PANC-1 (n=2) **(H)** cells along with Renilla luciferase construct. 24 h after transfection, cells were stimulated with the presence or absence of 2 mM glutamine with or without 0-25 μg/ml PMT for 6 h (MIA PaCa-2) and 0-75 μg/ml PMT for 24 h (PANC-1). Lysates were prepared to measure luciferase activity. Survivin-luciferase and Renilla-Luciferase activity was quantified using Dual Luciferase Reporter Assay system (Promega, Madison, WI). Data presented is an average ± SD of two to four independent experiments. * $P < 0.05$, ** $P < 0.01$ (One-way ANOVA followed by post-hoc Dunnett test). **[I]** NTC and STAT3 KD MIA PaCa-2 cells were transiently transfected with pLuc-survivin and Renilla luciferase construct. 24 h after transfection, cells were stimulated with the 2 mM glutamine and 0-25 μg/ml PMT for 6 h. Lysates were prepared to measure luciferase activity. Survivin-luciferase and Renilla-Luciferase activity was quantified using Dual Luciferase Reporter Assay system (Promega, Madison, WI). Data presented is an average ± SD of three independent experiments. * $P < 0.05$. (Two-way ANOVA followed by post-hoc Sidak test). Abbreviations: Glutamine (Q); Palmatine (PMT); Non-targeted control (NTC); Knockdown (KD).

4) Gene expression profile of PMT treatment mimics STAT3 KD

To gain deeper insight into the mechanism associated with PMT-mediated down regulation of STAT3 signaling under glutamine stimulation, we conducted RNA-seq analysis using MIA PaCa-2 cells under the conditions of glutamine starvation (control), and glutamine stimulation (2mM; GS), glutamine stimulation in the presence of low and high dose PMT (6.25 and 12.5 μg/ml) (LD-PMT and HD-PMT). We further used MIA PaCa-2 STAT3 KD cells and their respective NTC cells to identify STAT3 altered pathways under the experimental conditions described above. We have identified 2118, 131 and 500 differentially expressed genes (DEGs) by adjusted p-value < 0.05, at least a 2-fold change under conditions of glutamine stimulation (GS) versus Control (CTRL) (no glutamine condition); low dose PMT (LD-PMT) versus GS; and high dose PMT (HD-PMT) versus GS, respectively (Figure 13 A). Among the DEGs, 4 genes were changed in the same direction in GS_vs_CTRL and HD-PMT_vs_GS. 114 were changed in the same direction in HD-PMT_vs_GS and LD-PMT_vs_GS comparison. In addition, 343 DEGs were identified in STAT3 KD versus NTC cells (Figure 13 C). To determine the gene expression profile of STAT3 regulated genes in relation to glutamine stimulation, we ranked all DEGs by fold change in STAT3 KD vs NTC condition and performed Gene Set Enrichment Analysis (GSEA) analysis in the gene sets upregulated or downregulated by glutamine (GS vs CTRL_UP; GS vs CTRL_DOWN). We found that genes upregulated by glutamine stimulation (GS vs CTRL_UP) were enriched in NTC cells, and that genes downregulated by glutamine stimulation (GS vs CTRL_DOWN) were enriched in STAT3 KD cells. These data suggest that gene expression profile of STAT3 KD is negatively associated with glutamine stimulation (Figure 13 D). To understand gene expression profile of STAT3 KD in relation to PMT treatment under glutamine stimulation condition, we ranked all DEGs by fold change in STAT3 KD vs NTC condition and performed GSEA in the gene sets upregulated and downregulated in HD-PMT vs GS condition (HD-PMT vs GS_UP; HD-PMT vs GS_DOWN). We observed that genes upregulated by HD-PMT treatment vs glutamine stimulation condition are enriched in STAT3 KD, while genes downregulated by HD-PMT treatment vs glutamine stimulation are enriched in NTC. These data suggest that gene expression profile of PMT treatment under glutamine stimulation condition mimics STAT3 KD (Figure 13 D).

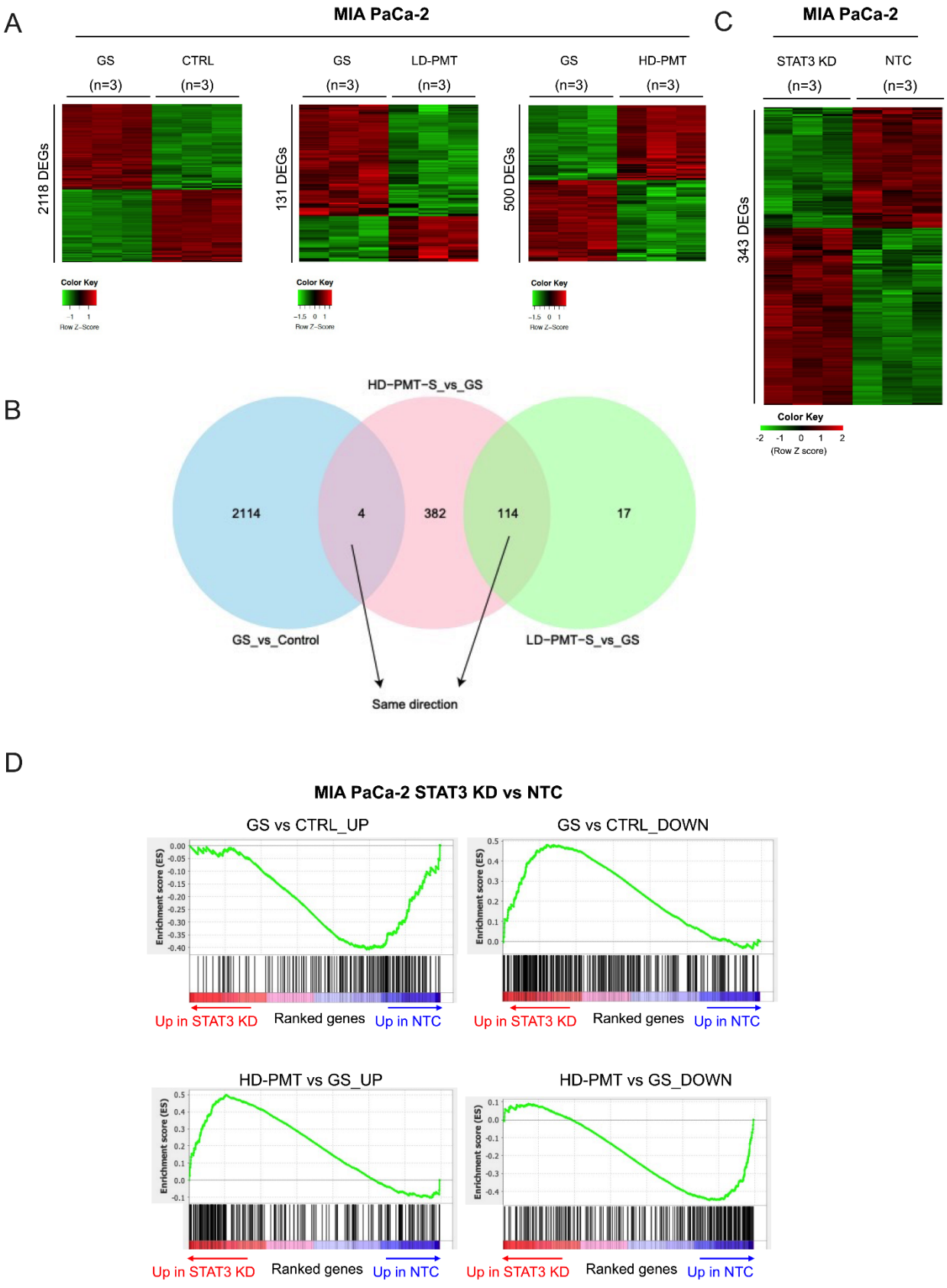


Figure 13 Gene Expression Profile of PMT Treatment Mimics STAT3 KD. [A] RNA-seq analysis on MIA PaCa-2 cells under the conditions of control (CTRL; glutamine starvation), glutamine stimulation (GS; 2mM), glutamine stimulation plus low dose PMT (LD-PMT; 6.25 μg/ml) and glutamine stimulation plus high dose

PMT (HD-PMT; 12.5 µg/ml). DEGs were selected based on adjusted p-value < 0.05 and fold change > 2. Heatmaps showing selected DEGs under GS vs CTRL, GS vs LD-PMT and GS vs HD-PMT. **[B]** Venn diagram showing DEGs with same direction in GS vs CTRL, LD-PMT vs GS and HD-PMT vs GS conditions. **[C]** Heatmap represents selected DEGs under STAT3 KD and NTC condition. **[D]** Enrichment of STAT3-regulated transcriptome under STAT3 KD and NTC condition in gene sets of GS vs CTRL_UP, GS vs CTRL_DOWN, HD-PMT vs GS_UP, HD-PMT vs GS_DOWN. Abbreviations: Glutamine stimulation (GS); Control (CTRL); low dose palmatine (LD-PMT); high dose palmatine (HD-PMT); knockdown (KD); Non-targeted control (NTC); Differentially expressed genes (DEGs).

Major goal 4: Determine the effectiveness of PMT to potentiate conventional therapy in PSCs and PCCs

1) Dose-response studies with PMT, GEM and Abr in pancreatic stellate and cancer cells

In order to determine the appropriate doses for combination treatment, human PSCs and pancreatic cancer cells (MIA PaCa-2 and PANC-1) were treated with increasing concentrations of PMT, GEM, and Abr for 48 h. Cell proliferation was determined by MTT assay (Figure 14). PMT treatment alone reduced proliferation by 9.1%, 33% and 28% in PSCs, MIA PaCa-2 and PANC-1 cells respectively at 25, 50 and 86 µg/ml. Abr also reduced proliferation by 28%, 22% and 30% in PSCs, MIA PaCa-2 and PANC-1 cells at 25 nM, 5 nM and 25 nM respectively. Although GEM reduced proliferation by 9% and 29% in PSCs and MIA PaCa-2 cells using 5 nM and 25 nM respectively, it had no significant effect in PANC-1 cells under our experimental conditions. Analysis of these data indicated that the half maximal inhibitory concentrations (IC50) for PMT range from 64-200 µg/ml [PSCs (>100 µg/ml), MIA PaCa-2 cells (64.60 µg/ml), PANC-1 cells (~200 µg/ml)]; for GEM in PSC's (30.72 nM), MIA PaCa-2 cells (>500 nM), PANC-1 cells (>1 µM) and for Abr in PSC's (36.78 nM), MIA PaCa-2 cells (~25 nM) and PANC-1 cells (~1 µM) (Table 2).

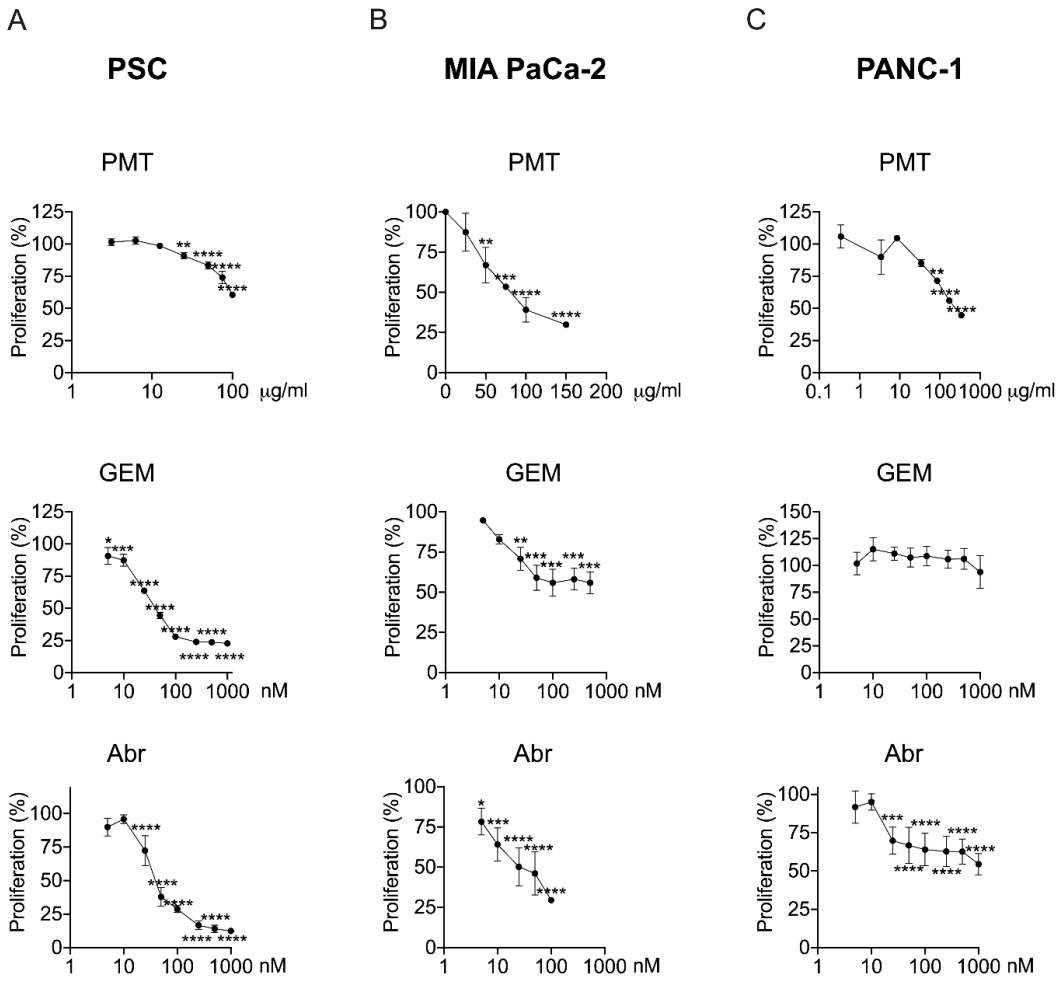


Figure 14 Dose-response Studies with PMT, GEM and Abr in Pancreatic Stellate and Cancer Cells. [A-C] PSCs **(A)**, MIA PaCa-2 **(B)** and PANC-1 **(C)** cells were treated with increasing concentrations of PMT [PSC (n=3), MIA PaCa-2 (n=3), PANC-1 (n=4)], GEM [PSC (n=3), MIA PaCa-2 (n=2), PANC-1 (n=4)] and Abr [PSC (n=3), MIA PaCa-2 (n=4), PANC-1 (n=4)] for 48h. Cell proliferation was measured by MTT assay (which measures intracellular metabolic activity). Data presented is an average ± SD of two to four independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, *** $P < 0.0001$ (One-way ANOVA followed by post-hoc Dunnett test). Abbreviations: Palmatine (PMT); Gemcitabine (GEM); Abraxane (Abr).

Table 2 IC_{50} of PSCs and Pancreatic Cancer Cells (MIA PaCa-2 and PANC-1 cells) Treated with Palmatine, Gemcitabine and Abraxane

	IC_{50}		
	PSC	MIA PaCa-2	PANC-1
Palmatine	>100 μg/ml (292 μM)	64.60 μg/ml (189 μM)	~200 μg/ml (584 μM)
Gemcitabine	30.72 nM	>500 nM	>1 μM
Abraxane	36.78 nM	25 nM	~1 μM

2) PMT potentiates growth inhibitory effect of gemcitabine (GEM) and Abraxane (Abr) in pancreatic stellate and cancer cells.

In order to determine the combinatorial benefits of PMT plus GEM and Abr in vitro, we selected doses of each drug inhibiting less than 60% of cell proliferation in each cell line and tested the combination effect using MTT assay. Dose selections for combination treatment are PSCs (100 μg/ml PMT, 25 nM GEM, 25 nM Abr); MIA PaCa-2 cells (50 μg/ml PMT, 50 nM GEM, 50 nM Abr) and PANC-1 cells (75 μg/ml PMT, 500 nM GEM, 50 nM Abr).

PMT treatment alone reduced proliferation by 34% ($p<0.0001$) in PSCs. GEM alone treatment caused 51% inhibition ($p<0.0001$), which was increased to 65% (a 14% increase) in combination with PMT (PMT+GEM vs GEM, $p=0.0327$). Abr alone showed 18% proliferation inhibitory effect ($p=0.005$), however in combination with PMT, the observed inhibitory effect was enhanced to 49% (a 31% increase; PMT+Abr vs Abr, $p<0.0001$). Combination of GEM + Abr showed 57% proliferation inhibition, a modest 6% increase compared to GEM alone but significant 39% increase relative to Abr alone (GEM+Abr vs Abr, $p<0.0001$). Triple combination of PMT plus GEM+Abr showed 71% inhibition (PMT+GEM+Abr vs GEM+Abr, $p=0.0357$). Taken together, these observations suggest that PMT+GEM+Abr triple combination is better than GEM plus Abr combination in terms of proliferation inhibition in PSCs under our experimental conditions (Figure 15 A and Table 3).

In MIA PaCa-2 cells, PMT treatment alone inhibited proliferation by 37% ($p<0.0001$). Consistent with our observations in PSCs, addition of PMT to GEM enhanced proliferation inhibition to 53% from 32% with GEM alone (PMT+GEM vs GEM, $p=0.006$). Although similar results were obtained with Abr plus PMT (54% with Abr alone to 64% with Abr plus PMT), the observed increase was not statistically significant. Furthermore, under these experimental conditions, surprisingly we found that Abr alone (54% inhibition) was much better than GEM plus Abr (43% inhibition) in terms of inhibiting proliferation of MIA PaCa-2 cells. In addition, unlike PSCs, triple combination (PMT plus GEM plus Abr) showed marginal non-significant change in proliferation inhibition (58% inhibition) relative to standard GEM plus Abr (43% inhibition). Overall, combination of PMT plus Abr appears to be the best relative to triple or GEM plus Abr combination (21% greater; PMT+Abr vs GEM+Abr, $p=0.0052$) in MIA PaCa-2 cells (Figure 15 B, Table 3).

In PANC-1 cells, PMT and Abr alone caused 32% ($p=0.0004$) and 25% proliferation inhibition respectively, while GEM had minimal impact with 2% inhibition. Furthermore, although combining PMT with GEM had no significant impact compared to PMT alone, in combination with Abr, PMT enhanced proliferation inhibitory effects to 51% from 25% observed with Abr alone (PMT+Abr vs Abr, $p=0.0037$). Additionally, combination of

GEM plus Abr or PMT with GEM plus Abr though exhibited enhanced proliferation inhibition, combination of PMT plus Abr appears to be the best with statistically significant 42% increased inhibition compared to GEM+Abr combination (PMT+Abr vs GEM+Abr, $p<0.0001$) in these cells (Figure 15 C, Table 3).

We also assessed the impact of combining PMT (50 µg/ml) with GEM (50 nM) and Abr (50 nM) on anchorage-independent growth using MIA PaCa-2 cells. Cells were pre-treated with PMT, GEM, Abr alone or combination of PMT+GEM, PMT+Abr, GEM+Abr and PMT+GEM+Abr for 48 h. Following treatment, cells were trypsinized and 10,000 live cells from each treatment group were seeded on soft agar plates and maintained for 7-10 days until colony formation. Anchorage-independent growth was quantified using CytoSelect 96-well Cell Transformation Assay (Cell Biolabs, San Diego, CA). We observed that PMT alone demonstrated 77% inhibition of anchorage-independent growth ($p=0.0002$). Although GEM alone treatment appears to enhance the anchorage-independent growth by 36%, the observed change was not statistically significant (GEM vs CTRL, ns). However, this increased anchorage-independent growth was attenuated in combination with PMT, which showed 76% inhibition (112% reduction from GEM alone) (PMT+GEM vs GEM, $p<0.0001$). Abr alone decreased anchorage-independent growth by 61% inhibition ($p=0.003$) that was increased to 79% in combination with PMT which was not significant compared to Abr alone. The combination of GEM+Abr displayed 44% inhibition, which attenuated anchorage-independent growth of GEM alone treatment by 80% (GEM+Abr vs GEM, $p<0.0001$). The triple combination of PMT+GEM+Abr demonstrated 77% inhibition which was not significant when compared to GEM plus Abr combination. Although the combination of PMT+Abr appears to be the best (79%) regimen to inhibit anchorage-independent growth, as a single agent PMT alone caused 77% decreased growth in these cells. Taken together, these data suggest that PMT alone potently inhibited anchorage- independent growth in MIA PaCa-2 cells, which is comparable to the inhibitory effect of double or triple combination (PMT+Abr or PMT+GEM+Abr) (Figure 15 D, Table 3).

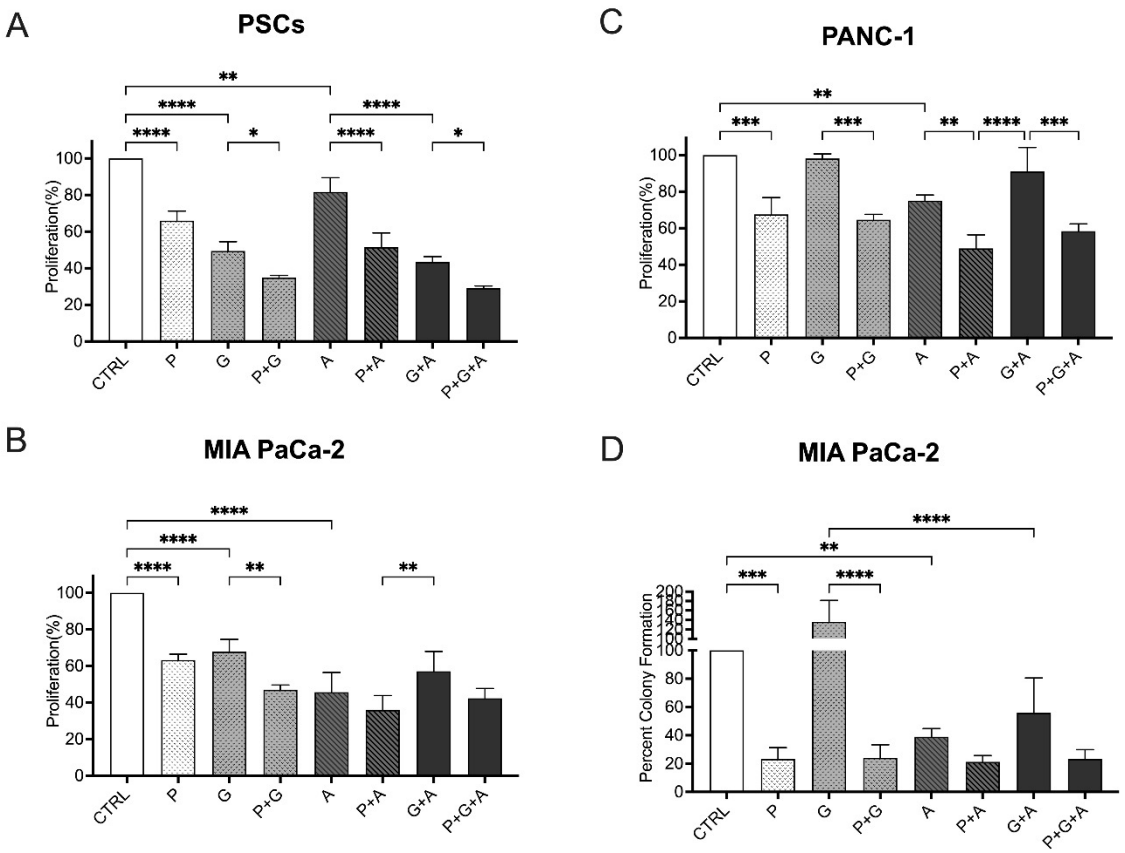


Figure 15 PMT Potentiates Growth Inhibitory Effect of GEM and Abr in Pancreatic Stellate and Cancer Cells. [A-C] PSC (n=3) **(A)**, MIA PaCa-2 (n=4) **(B)** and PANC-1 (n=3) **(C)** cells were treated with PMT, GEM and Abr or combinations or PMT+GEM, PMT+Abr, GEM+Abr, PMT+GEM+Abr for 48h. Doses selections are PSCs (100 µg/ml PMT, 25 nM GEM, 25 nM Abr), MIA PaCa-2 cells (50 µg/ml PMT, 50 nM GEM, 50 nM Abr), PANC-1 cells (75 µg/ml PMT, 500 nM GEM, 50 nM Abr). Cell proliferation was measured by MTT assay (which measures intracellular metabolic activity). Data presented is an average ± SD of three to four independent experiments. ns: not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, *** $P < 0.0001$ (One-way ANOVA followed by post-hoc Tukey test). **[D]** MIA PaCa-2 cells were treated with 50 µg/ml PMT, 50 nM GEM, 50 nM Abr or combination and 48h. Cells were then trypsinized and viable cell concentration was determined by trypan blue assay. 10,000 viable cells were seeded in soft agar and maintained for 7-10 days. Anchorage-independent growth was quantified following the manufacturer's instructions using CytoSelect 96-well Cell Transformation Assay (Cell Biolabs, San Diego, CA). Data presented is an average ± SD of four independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, *** $P < 0.0001$ (Two-way ANOVA followed by post-hoc Tukey test). Abbreviations: Control (CTRL); Palmatine (P); Gemcitabine (G); Abraxane (A).

Table 3 Impact of Combination Regimen on Proliferation, Anchorage-independent Growth and Protein Levels of Survivin and pSTAT3(Y705) in Pancreatic Cancer Cells

	Inhibition (%)				Survivin		pSTAT3	
	PSC	MIA PaCa-2	MIA PaCa-2 (AI)	PANC-1	Inhibition (%) (MIA PaCa-2)		Inhibition (%) (MIA PaCa-2)	
P	34	37	77 *d	32	44	↓↓	98	↓↓↓ *i
G	51	32	-36	2	-104	↑↑↑	23	↓
A	18	54	61	25	3	no effect	38	↓↓
P + G	65*a	53	76 *e	35	29	↓ *h	94	↓↓↓ *j
P + A	49	64 *c	79 *f	51 *g	51	↓↓	95	↓↓↓ *k
G + A	57	43	44	9	-58	↑↑	35	↓↓
P + G + A	71*b	58	77	42	0	no effect	90	↓↓↓ *l

Abbreviations: Palmatine (P); Gemcitabine (G); Abraxane (A); Anchorage-independent growth (AI)
*a – significance relative to GEM alone (p=0.0327)
*b – significance relative to GEM + Abr (p=0.0357)
*c – significance relative to GEM + Abr (p=0.0052)
*d – significance relative to Control (p=0.0002)
*e – significance relative to GEM (p<0.0001)
*f – significance relative to GEM + Abr (p=0.0052)
*g – significance relative to GEM + Abr (p<0.0001)
*h – significance relative to GEM (p<0.0166)
*i – significance relative to Control (p<0.0001)
*j – significance relative to GEM (p=0.0001)
*j – significance relative to GEM +Abr (p=0.0008)
*k – significance relative to GEM + Abr (p=0.0007)
*l – significance relative to GEM + Abr (p=0.0018)

3) Impact of combination regimen on STAT3/survivin signaling

Intrigued by the observed biological significance of the combination effects, we explored whether underlying mechanism involves alterations in the levels of STAT3 and survivin. Whole cell extracts prepared from MIA PaCa-2 cells treated with PMT or GEM or Abr singly or in combination (PMT 50 µg/ml, GEM 50 nM, Abr 50

nM or combinations of PMT+Abr, PMT+GEM, Abr+GEM and triple combination of PMT+GEM+Abr) for 48h were used in these experiments. Our result showed reduction in the protein levels of pSTAT3 (Y705) with PMT ($p<0.0001$) or Abr ($p=0.0407$) alone but not with GEM treated cells. Combining PMT with GEM ($p=0.0001$) or Abr ($p=0.0012$) resulted in further reduction in pSTAT3 protein levels of relative to GEM or Abr alone. Furthermore, triple combination of PMT, GEM and Abr decreased pSTAT3 levels ($p=0.0018$) relative to GEM plus Abr combination. Remarkably, combination of PMT plus Abr also reduced pSTAT3 compared to GEM plus Abr ($p=0.0007$). However, no changes in the protein levels of total STAT3 were observed under any of these experimental conditions (Figure 16 and Table 3). We calculated pSTAT3/STAT3 ratio to evaluate single or combination treatment on STAT3 activation level, and observed PMT alone but not Abr alone or GEM alone significantly ($p=0.0002$) reduced > 90% STAT3 activation (as indicated by pSTAT3/STAT3 ratio). While combining PMT with GEM ($p=0.0006$), Abr ($p=0.0018$) or the combination of GEM+Abr ($p=0.0324$) further significantly reduced STAT3 activation compared to GEM alone, Abr alone, or GEM+Abr combination, PMT alone treatment achieved the best inhibitory effect on STAT3 activation compared to all other treatment conditions. Based on these findings suggesting that PMT alone or combination regimen selectively inhibits STAT3 activation, we examined the protein levels of survivin under these conditions. Although PMT or Abr alone had no effect, GEM treatment resulted in ~2-fold increased survivin levels nevertheless it did not reach statistical significance. However, combination with PMT decreased the observed GEM-increased levels of survivin ($p=0.0166$). Furthermore, combination of PMT plus Abr treatment reduced survivin levels better than GEM plus Abr combination (Figure 16 and Table 3). Since both pSTAT3 and survivin are involved in therapeutic resistance associated with GEM and Abr, these data indicate that PMT has the potential to reduce the acquired resistance to GEM and Abr at least in part through downregulation of pSTAT3 and survivin levels.

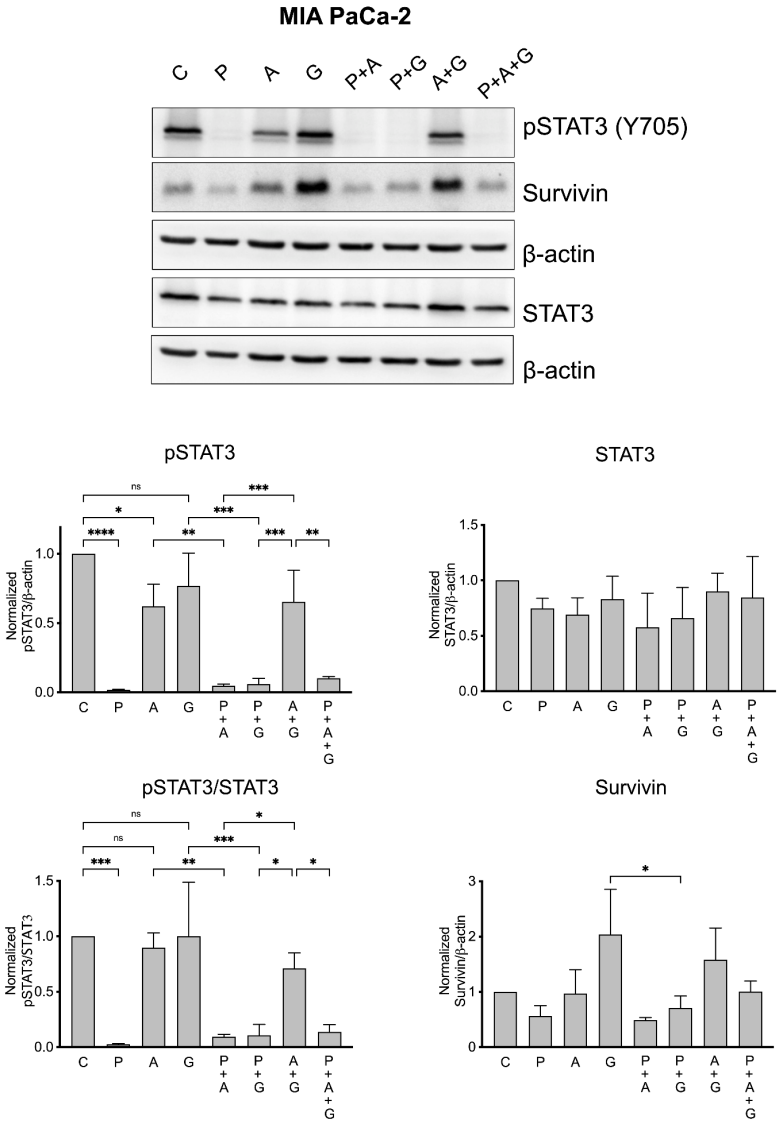


Figure 16 Impact of Combination Regimen on STAT3/Survivin Signaling. MIA PaCa-2 cells were treated with 50 μg/ml PMT, 50 nM GEM, 50 nM Abr or combinations of PMT+Abr, PMT+GEM, Abr+GEM and triple combination of PMT+GEM+Abr for 48h. Whole cell lysates were harvested to detect total STAT3, pSTAT3 (Y705) and Survivin levels using immunoblotting. The ratio of normalized pSTAT3 to total STAT3 levels were calculated and plotted. Data presented is an average ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, *** $P < 0.0001$ (One-way ANOVA followed by post-hoc Tukey test). Abbreviations: Control (C); Palmatine (P); Gemcitabine (G); Abraxane (A).

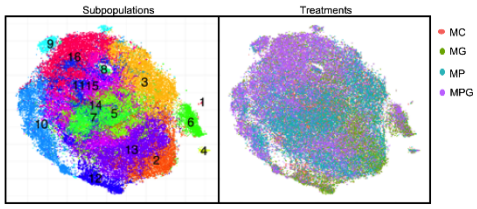
4) CyTOF analysis revealed heterogeneous pSTAT3 expression in pancreatic cancer cells and association with response to treatment

Above data suggest differential response of tumor cells and stellate cells to treatment with single or combination agents. To understand the underlying mechanism associated with such differential response, we used CyTOF to analyze key proteins in cell signaling [AXL, JAK1, pErk1, epidermal growth factor receptor (EGFR), VEGF, c-MET, SOX9, programmed cell death 1 receptor (PD-1), programmed cell death ligand 2 (PD-L2), Ki67], epithelial-mesenchymal transition (EMT) [ZO-1, EpCAM, Twist-related protein 1 (TWIST1), SNAI1, β-catenin, Vimentin, Fibronectin, ZEB1, ZEB2, Slug, N-Cadherin], and extracellular matrix (ECM) [collagen type V alpha 2 chain (COL5A2), collagen type IV alpha 1 Chain (COL4A1), Arginase1, ITGA5, CD44] based on their involvement in pancreatic cancer progression, metastasis and therapeutic resistance in human pancreatic cancer cells (MIA PaCa-2 and PANC-1 cells treated with GEM, PMT or the combination of GEM plus PMT for 48h). MIA PaCa-2 cells were treated with 50 μg/ml PMT or 50 nM GEM or combination of both, and PANC-1 cells with 12.5 μg/ml PMT or 5 μM GEM or combination of both agents. Following treatment, single cell profiling was performed using CyTOF. Analysis of these data identified 16 and 15 subpopulations in MIA PaCa-2 and PANC-1 cells respectively based on t-SNE scatter plots (Figure 17 A and E). A heatmap was generated showing the changes of all the proteins analyzed among different clusters (Figure 17 B and F). To explore the intratumor cell heterogeneity, we categorized the identified subpopulations into four classes based on the expression levels of pSTAT3 (pSTAT3-low, pSTAT3-intermediate and pSTAT3-high in MIA PaCa-2 cells and pSTAT3 (pSTAT3-low, pSTAT3-intermediate, pSTAT3-intermediate high and pSTAT3-high in PANC-1 cells). Identified subpopulations were aligned with increasing levels of pSTAT3 and we found an association between levels of pSTAT3 with cell signaling, EMT, and ECM proteins in both cell lines (Figure 17 C and G). The percentage of each sub-population of cells was calculated and plotted (Figure 17 D and H). As shown in figure, majority of subgroups are assigned to pSTAT3-intermediate (category II, 67.1%) followed by pSTAT3-high (category III, 19.3%) and pSTAT3-low (category I, 13.5%) in untreated MIA PaCa-2 cells. Treatment with GEM resulted in increased percentage of category I (pSTAT3 low, 41%) and decreased category II (40.4%) cells. PMT treatment resulted in decreased category III (14.5%) and increased category I (22.6%) while combination increased category III subgroups (37.2%). In untreated PANC-1 cells, majority of subgroups were assigned to category II (pSTAT3-intermediate, 54.5%); and treatment with GEM alone and combination of PMT plus GEM increased category IV (pSTAT3 high) from 4.5% to 13.8% and 17.2%, respectively. On the other hand, PMT treatment resulted in decreased percentage of category IV (from 4.5% to 2.7%) and increased category I (from 13.6% to 18.8%) subgroups (Figure 17D and H).

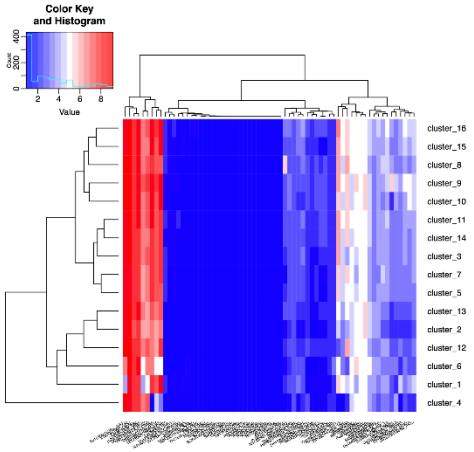
PMT treatment reduced population of cells with pSTAT3-high subgroups in both MIA PaCa-2 and PANC-1 cells. However, pSTAT3-high subgroup was slightly reduced with GEM treatment in MIA PaCa-2 cells but increased in PANC-1 cells, suggesting that PMT alone might be a better option for PANC-1 cells. Furthermore, PMT plus GEM treatment increased pSTAT3-high subgroup in both cell lines, indicating the activation of additional resistance signaling pathways. Interestingly, we found a positive correlation between pSTAT3 levels with proteins involved in cell signaling (such as AXL, EGFR, JAK1), EMT and ECM in both cell lines. It is likely that in the presence of PMT plus GEM, AXL or EGFR or JAK1 activation might serve as a bypass mechanism contributing to therapeutic resistance directly or in part through STAT3 reactivation.

MIA PaCa-2

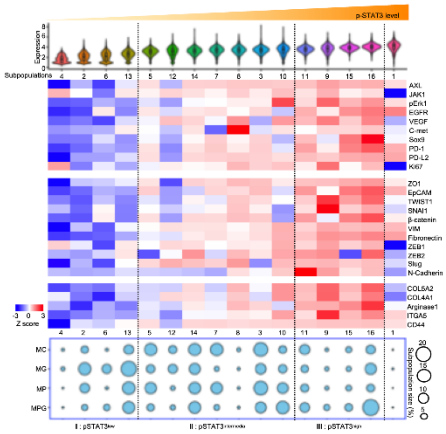
A



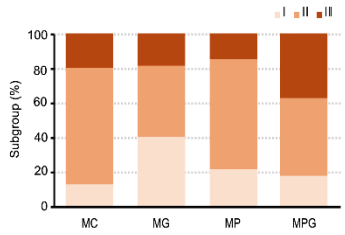
B



C

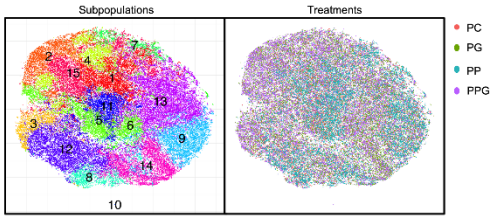


D

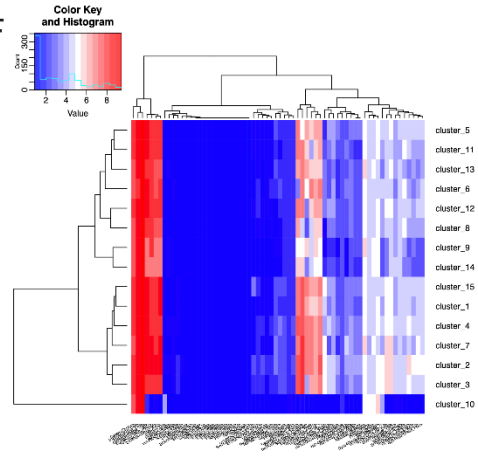


PANC-1

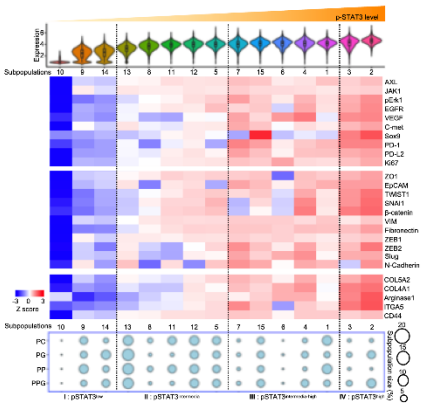
E



F



G



H

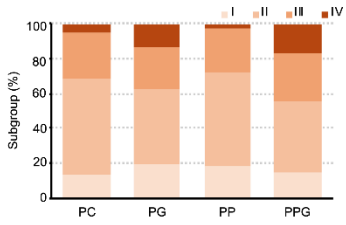


Figure 17 CyTOF Analysis Revealed Heterogeneous pSTAT3 Expression in Pancreatic Cancer Cells and Association with Response to Treatment. [A-D] MIA PaCa-2 and **[E-H]** PANC-1 cells were treated with PMT, GEM, or combination for 48h. Dose selections are MIA PaCa-2 (50 μg/ml PMT, 50 nM GEM) and PANC-1 (12.5 μg/ml PMT, 5 μM GEM). Cells were stained with pSTAT3 other antibodies before running on Helios CyTOF mass cytometer. **[A, E]** Subpopulations of cells based on their protein expression profile (left) and their corresponding treatment (right). **[B, F]** Heatmaps showing the changes of all the proteins analyzed among different clusters. **[C, G]** Heatmap ranking the subpopulations based on pSTAT3 expression and their association with proteins in cell signaling, EMT and ECM. Percentage of each subpopulation in each treatment group was calculated and shown in circles. Based on pSTAT3 expression levels, subpopulations were divided into pSTAT3-low, pSTAT3-intermediate and pSTAT3-high in MIA PaCa-2 cells and pSTAT3-low, pSTAT3-intermediate, pSTAT3-intermediate high and pSTAT3-high in PANC-1 cells. **[D, H]** The percentage of each pSTAT3 subgroups in each treatment conditions. Abbreviations: MC, MG, MP, MPG represent MIA PaCa-2 cells in control, gemcitabine, palmatine and combination (palmatine plus gemcitabine) groups respectively; PC, PG, PP, PPG represent PANC-1 cells in control, gemcitabine, palmatine and combination (palmatine plus gemcitabine) groups respectively.

Major goal 5: Determine the therapeutic activity of PMT with GEM plus Abraxane in preclinical model

Obtained ACURO and IACUC approval

What opportunities for training and professional development has the project provided?

- Through participating in Cancer Biology Journal Club and seminars at UT Health San Antonio, I have kept up with breakthroughs in cancer and biomedical research
- **Technical skills:** The trainings throughout the years have provided me great opportunities to gain and sharpened my technical skills (such as CyTOF, immunofluorescence and subcellular fractionation). My collaborative work has resulted in 4 co-authored publications since the year 2020. I also have another first-authored publication under preparation.
- **Presentation skills:** I've improved my presentation skills through presenting journal articles at Cancer Biology Journal Club, dissertation committee meeting, lab meetings, student seminars and at the UTHSA Mays Cancer Center (MCC) Retreat as well as in my dissertation defense.
- **Scientific writing skills:** I have improved my scientific writing skills through dissertation writing and abstract preparation.
- **Mentoring skills:** I have developed my mentoring skills by mentoring undergraduate student in Dr. Kumar's lab.
- **Critical thinking skills:** I've improved my critical thinking skills through weekly one-on-one meetings to discuss my findings with my mentor Dr. Kumar, our monthly lab meetings, my discussions with my mentor Dr. Kumar during the process of dissertation writing and preparation for my dissertation defense.

How were the results disseminated to communities of interest?

I have disseminated my research findings at the UTHSA Mays Cancer Center (MCC) Retreat, UTHSA-Molecular Medicine Retreat/Research Day and UT Health San Antonio student seminars, to clinicians and researchers who may or may not be aware of the role of pancreatic cancer microenvironment and its role in the resistance to chemotherapy.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Impact on the base of knowledge of pancreatic cancer: the pancreatic cancer microenvironment plays a critical role in the initiation, progression and therapeutic resistance of pancreatic cancer. The reciprocal crosstalk between pancreatic stellate cells (PSCs) and pancreatic cancer cells (PCCs) resulting in disease progression is an attractive target for pancreatic cancer treatment. However, the biological outcome and molecular mechanisms of PSC-PCC communication have not been fully elucidated. Our findings added to the knowledge that PSC-secreted glutamine stimulates growth and supports metastatic spread of pancreatic cancer. Additionally, our findings strengthened the current understanding between glutamine and STAT3, a master regulator in pancreatic cancer pathogenesis.

Impact on pancreatic cancer drug development: pancreatic cancer is a devastating disease with limited treatment options. Current therapeutic regimens displayed modest survival benefit at the cost of considerable toxicity. Despite numerous studies identifying the tumor-promoting role of STAT3 in pancreatic cancer, no direct STAT3 inhibitor has been approved for pancreatic cancer or any cancer. Our findings identified a non-toxic STAT3 inhibitor, namely palmatine, that provides therapeutic sensitization by inhibiting glutamine-induced signaling pathways and demonstrated anti-proliferative effect of palmatine in gemcitabine-resistant pancreatic cancer cells.

What was the impact on other disciplines?

Constitutive activation of STAT3 has been found in various types of human cancers, including leukemia and tumors of the head and neck, breast, lung, prostate, ovary, colon and pancreas. Furthermore, persistent STAT3 activation is also found in autoinflammatory conditions such as rheumatoid arthritis and psoriasis. Our findings identified that palmatine as a non-toxic STAT3 inhibitor can be expended to other types of tumor types beyond pancreas, and the treatment of autoinflammatory diseases.

What was the impact on technology transfer?

We have submitted a patent on palmatine before the start of this project, that has the potential to lead to the initiation of a start-up company, or to be used in the government or industry.

What was the impact on society beyond science and technology?

Pancreatic cancer is a high economic burden to both the patients and the healthcare system. Part of this economic burden is caused by therapeutic resistance and treatment-associated adverse events. We have identified a natural compound, which is non-toxic (demonstrated by our previous study and findings from other groups) and reduces therapeutic resistance of conventional therapeutics gemcitabine and Abraxane. This study can lead to the development of a clinical trial testing the combination of palmatine and current therapeutics in pancreatic cancer patients. This will have major impact in reducing the economic burden caused by pancreatic cancer in the society.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

Due to COVID-19 related hurdles we were not able to obtain necessary reagents for our preclinical study.

Changes that had a significant impact on expenditures

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Due to COVID-19 related delays, we were unable to obtain reagents and compounds necessary for our preclinical study and were therefore unable to complete the planned animal study.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

  Journal publications.

  Nothing to Report. A manuscript describing the findings is under preparation.

  Books or other non-periodical, one-time publications.

  Nothing to Report.

  Other publications, conference papers and presentations.

Presentations:

8-23-2019	**UT Health San Antonio (UTHSA) Graduate School of Biomedical Sciences (GSBS), Cancer Biology Journal Club**
	Topic: EGFR-Pak Signaling Selectively Regulates Glutamine Deprivation-Induced Macropinocytosis
8-29-2019	**Lab Meeting Presentation - Journal presentation**
	Topic: Nutritional cues regulate pancreatic tumor's "cell drinking"
10-10-2019	**Lab Meeting Presentation - Journal presentation**
	Topic: Proteomic analysis of ECM during pancreatic ductal adenocarcinoma progression reveal different contributions by tumor and stromal cells
11-06-2019	**UTHSA Mays Cancer Center Retreat**
	Topic: Targeting glutamine addiction in pancreatic cancer
11-22-2019	**UTHSA GSBS - Graduate student Committee Meeting**
	Topic: Novel role of glutamine in the tumor-stromal interaction of pancreatic ductal adenocarcinoma
5-7-2020	**Lab Meeting Presentation**
	Topic: Research Progress Update
5-13-2020	**UTHSA GSBS - Graduate student Committee Meeting**
	Topic: Novel role of glutamine in the tumor-stromal interaction of pancreatic ductal adenocarcinoma
5-29-2020	**UTHSA GSBS Cancer Biology Discipline - Student seminar presentation**
	Topic: Novel role of glutamine in the tumor-stromal interaction of pancreatic ductal adenocarcinoma
10-2-2020	**UT Health San Antonio (UTHSA) Graduate School of Biomedical Sciences (GSBS), Cancer Biology Journal Club**
	Topic: CAR-T cells and oncolytic viruses: joining forces to overcome the solid tumor challenge
12-3-2020	**UTHSA GSBS - Graduate student Committee Meeting**
	Topic: Novel role of glutamine in the tumor-stromal interaction of pancreatic ductal adenocarcinoma
1-13-2021	**UTHSA Mays Cancer Center Retreat**
	Topic: Targeting glutamine/STAT3 axis to overcome therapeutic resistance in pancreatic cancer
9-8-2021	**UTHSA GSBS – Dissertation Defense**
	Topic: Targeting glutamine/STAT3 axis in pancreatic ductal adenocarcinoma
11-12-2021	**UTHSA-Molecular Medicine Retreat/Research Day**
	Topic: Targeting glutamine/STAT3 axis in pancreatic ductal adenocarcinoma

- **Website(s) or other Internet site(s)**

Nothing to Report.

- **Technologies or techniques**

  Nothing to Report.

- **Inventions, patent applications, and/or licenses**

  We have submitted a patent on palmatine before the start of this project

- **Other Products**

  Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

PI: Xiaoyu Yang (no change)

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report.

9. APPENDICES:

1) Abstract of UTHSA Mays Cancer Center Retreat

Targeting glutamine/STAT3 axis to overcome therapeutic resistance in pancreatic cancer

Xiaoyu Yang[1], Kelsey Sidell[1], Michelle Villarreal[1], Haiyong Han[9], Robert Reddick[4], Chia-Nung Hung[1], Tim Huang[1], Zhao Lai[7], Li-Ju Wang[7], Yidong Chen[7], Danielle Fritze[5,6], Glenn Halff[5,6], Rita Ghosh[1,2,3] and Addanki P Kumar[1,2,3,6,8]

Departments of Molecular Medicine[1], Urology[2], Pharmacology[3], Pathology[4], Surgery[5], Mays Cancer Center[6], Greehey Children's Cancer Research Institute[7], University of Texas Health San Antonio, South Texas Veterans Health Care System[8], Translational Genomics Research Institute, AZ[9]

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease with poor outcome and risk increases with advancing age. Conventional therapeutic approaches including gemcitabine (GEM)-based combination chemotherapy offer modest survival benefit. Development of therapeutic resistance remains another major challenge limiting the effectiveness of treatment. Targeting interactions between pancreatic stellate and cancer cells (PSC-PCCs) that contributes to desmoplasia is an attractive strategy for treatment of PDAC. We have

previously shown that palmatine (PMT), a natural compound inhibits growth of PCCs by preventing glutamine-induced PSC-PCC communication and enhances the anti-proliferative activity of GEM. Subsequently, we found upregulation of STAT3 and survivin in response to glutamine (Q). Given that both STAT3 and survivin are involved in therapeutic resistance, we hypothesized that STAT3 inhibition using PMT could be a strategy for effective management of PDAC. We determined the efficacy of PMT alone and the combination with GEM or Abx *in vitro* and *in vivo*. We further used RNA-seq to explore global transcriptional changes under conditions of (i) Q-stimulation in the presence and absence of PMT, (ii) PMT & PMT plus GEM treatment and (iii) stably silenced for STAT3. We used C57BL/6 mice implanted with pancreatic tumor cells originating from Pdx1-Cre; LSL-KRas$^{G12D/+}$; LSL-Tp53$^{R172H/+}$ (KPC) mouse model to test PMT's *in vivo* efficacy. After the establishment of tumors, mice were treated singly with PMT or GEM or combination of PMT plus GEM. Tumor growth was monitored over the course of the experiment. Our results show (i) that PMT (a) attenuates Q-mediated enhanced proliferation, clonogenicity and anchorage independent growth in part through STAT3; (b) reduces Q-induced survivin promoter activity; (c) potentiates growth inhibitory effect of GEM and Abr; and (ii) gene expression profile of PMT or PMT plus GEM treatment mimics STAT3 KD. *In vivo*, we observed significant reduction of tumor growth as evidenced by tumor-associated bioluminescence in both combination and GEM alone group. Notably, majority of animals treated with GEM alone but not in combination with PMT exhibited body weight loss >10-15% and metastasis to lungs. To understand if intra or inter tumor heterogeneity plays a role in therapeutic response, we determined single cell changes in proteins using CyTOF in (i) cells treated with mono and combination therapy and (ii) human pancreatic tumors. Analysis of these data revealed an association between pSTAT3 expression with response to treatment and tumor differentiation. Patient-derived cells cultured *ex vivo* or organoids obtained from tumors showed sensitivity to combination treatment. Taken together, these data show potential clinical utility for the combination of PMT plus GEM in the treatment of pancreatic cancer. Supported in part through DOD Horizon Award (XY) VA Merit Award BX3876 and Owens Foundation (APK).

2) Abstract of UTHSA Molecular Medicine Retreat/Research Day

Targeting glutamine/STAT3 axis in pancreatic ductal adenocarcinoma

BACKGROUND: Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease with appallingly poor outcome. Conventional therapeutic approaches including gemcitabine (GEM) – based combination offer modest survival benefit at the cost of increased toxicity. Desmoplasia, a prominent feature of PDAC, has been known to contribute to therapeutic resistance caused by excessive deposition of extracellular matrix components by pancreatic stellate cells (PSCs). Additionally, the reciprocal crosstalk between PSCs and pancreatic cancer cells (PCCs) resulting in disease progression is an attractive target for pancreatic cancer treatment. We have previously demonstrated that PSCs secretes glutamine to promote the growth of PCCs and identified a natural compound namely palmatine (PMT) that inhibits glutamine-stimulated proliferation and potentiates GEM activity in PSCs and PCCs. Despite these promising results indicating the potential of PMT in targeting glutamine-stimulated signaling in pancreatic cancer cells, the precise mechanisms associated with glutamine- and PMT- mediated biological outcome have not been fully understood. Previous we demonstrated that Nexrutine (the natural product PMT is derived from) reduced signal transducer and activator of transcription 3 (STAT3) signaling in PCCs and preclinical models of pancreatic cancer. These findings prompted us to study the roles of glutamine and PMT in STAT3 signaling and related biological effects. We observed that PMT inhibits glutamine-stimulated STAT3 phosphorylation, its downstream target survivin, glutamine-stimulated increased proliferation, clonogenicity, anchorage independent growth, migration and invasion. Given that both STAT3 and survivin have been shown to be involved in therapeutic resistance of GEM and Abraxane (Abr), we set out to determine the therapeutic benefit of PMT in combination with GEM and Abr in PSCs and PCCs. Our results show that PMT potentiates anti-proliferation effect of GEM and Abr in PSCs and PCCs.

KEY CURRENT RESULTS: To gain deeper insight into the mechanism associated with PMT-mediated downregulation of STAT3 signaling under glutamine stimulation, we conducted RNA-seq analysis using MIA PaCa-2 cells under the conditions of (i) glutamine starvation (control), glutamine stimulation, and glutamine

stimulation in the presence of PMT and (ii) MIA PaCa-2 STAT3 knockdown (KD) cells and their respective non-targeted control (NTC). Our results suggest that gene expression profile of PMT treatment under glutamine stimulation condition mimics STAT3 KD. Our preclinical study using KPC model showed significantly reduced tumor growth accompanied by extensive fibrosis and increased pSTAT3 in GEM alone and combination (PMT and GEM) groups but not in PMT group. Although various possibilities exist, interestingly, we found that the lack of effect in PMT alone group is possibly resulting from feedback activation of STAT3 signaling through systemically elevated levels of leukemia inhibitory factor (LIF). CyTOF analysis in pancreatic cancer cell lines and patient- derived primary cells also identified an association between pSTAT3 and proteins in cell signaling, epithelial-mesenchymal transition and extracellular matrix formation, suggesting the activation of additional resistance signaling under combination treatment of PMT and GEM. Intrigued by the combinatorial benefit of PMT with conventional therapeutics in vitro, we tested PMT alone and in combination with GEM and/or Abr in in vivo and ex vivo models. We observed association between increased population of high-pSTAT3 cells (as evidenced by CyTOF analysis), reduced sensitivity to PMT and combination treatment and patient prior-treatment history. Taken together, this study demonstrated the potential clinical utility of PMT in the management of pancreatic cancer through inhibition of glutamine-STAT3 axis. Supported in part through DOD Horizon Award (XY) VA Merit Award BX3876 and Owens Foundation (APK).