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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

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1. **INTRODUCTION:** Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease with dismal survival rate and limited treatment options. Military personnel are at elevated risk of developing and dying from PDAC due to occupational exposure. 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 using cell culture and preclinical models.

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	80%
2. Determine the effects of PMT on Q release from PSCs and on cancer hallmarks in PCCs	90%
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 nuclear levels of pSTAT3 (Y705) in pancreatic cancer cells

In last year's annual report (YI annual report), we have established that glutamine-stimulation enhanced pSTAT3 levels in different pancreatic cancer cells (MIA PaCa-2, BxPC-3, PANC-1 and Capan-2). These pancreatic cancer cells have different differentiation status, KRAS-mutation status, doubling time, metabolic phenotype, STAT3 levels and other cell line-specific signaling pathways which is depicted in Table 1. 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 1A). 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 1A).

As an independent approach, we performed immunofluorescence (IF) analysis in MIA PaCa-2 cells and observed increased nuclear pSTAT3 with glutamine stimulation (Figure 1B). 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.

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	

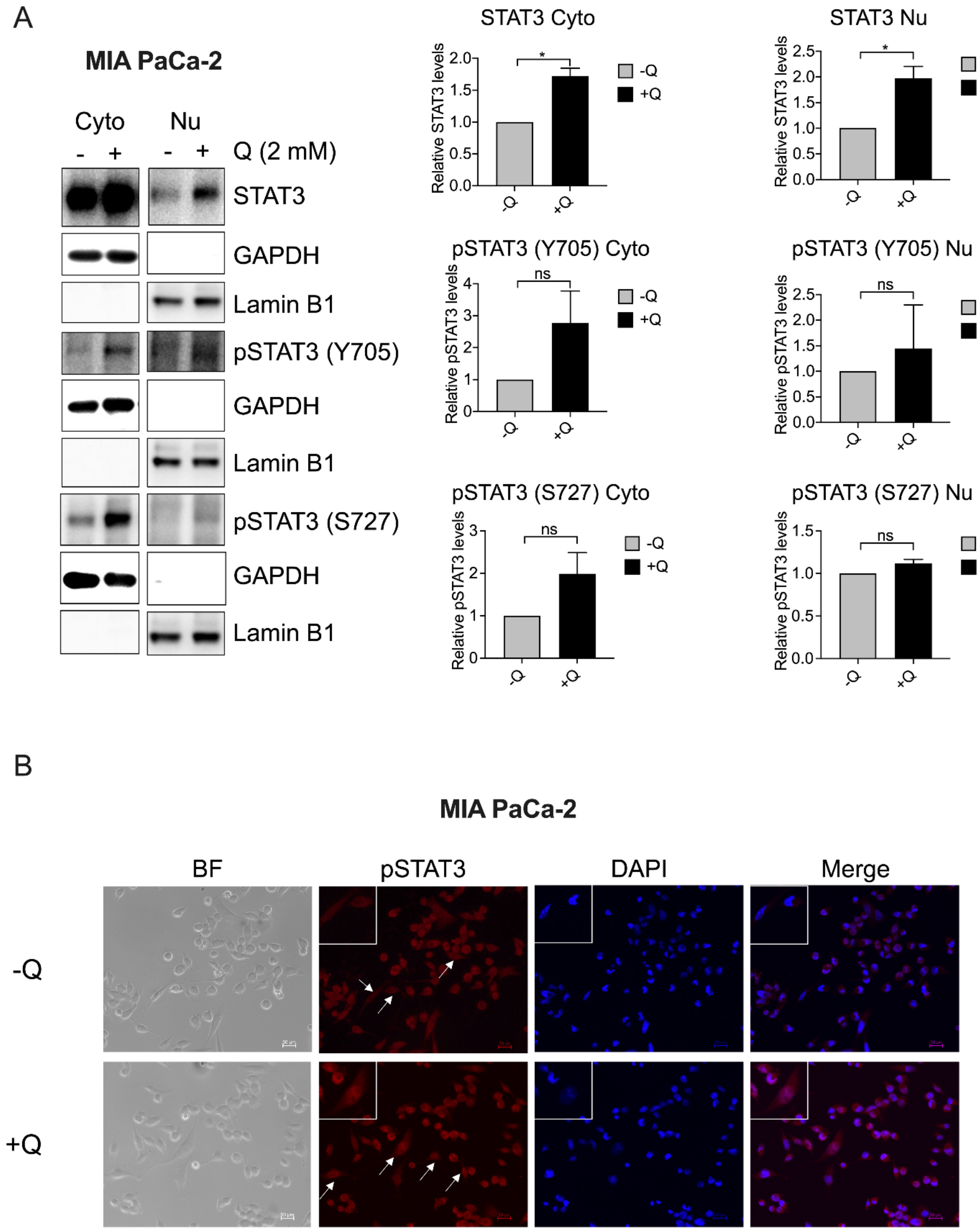


Figure 1 Glutamine Stimulation Increased Nuclear Levels of pSTAT3 (Y705) in Pancreatic Cancer Cells. [A] Cytoplasmic and nuclear levels of STAT3, pSTAT3 (Y705) and pSTAT3 (S727) in MIA PaCa-2 cells treated with the presence and absence of 2 mM glutamine for 24 h were examined with immunoblotting. Glyceraldehyde-3-Phosphate 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).

2) Glutamine-stimulation increased multiple cancer hallmarks in pancreatic cancer cells.

In YI annual report, we showed that glutamine-stimulation enhanced proliferation in multiple pancreatic cancer cells (MIA PaCa-2, BxPC-3, PANC-1, Capan-2), anchorage-independent growth in MIA PaCa-2 cells (Figure 2C) and colony formation in PANC-1 cells (Figure 2B). This year we explored the role of glutamine on colony formation, migration and invasion capacities in MIA PaCa-2 cells. Logarithmically growing MIA PaCa-2 cells were seeded at a density of 300,000 per well in 6-well plates. After attachment (~24h), cells were starved overnight in glutamine-deficient media, followed by stimulation with or without 2 mM glutamine for 24 h. After treatment, cells were trypsinized and cell viability was determined by trypan blue assay and viable cells were used for colony formation, migration and invasion assay. 1) For colony formation assay, 1000 (MIA PaCa-2) viable cells were seeded in complete media in 6-well plates. Cells were maintained for additional 7-14 days for colonies to form. 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. 2) For migration assay 300,000 viable cells suspended in serum-free media were seeded on to the top compartment of a Boyden Chamber which is sealed with a porous membrane at the bottom for cell migration. Complete media (containing serum) was added to the bottom chamber as chemoattractant. Cells were incubated in the tissue culture incubator at 37°C for additional 24 h. 3) For invasion assay 500,000 viable cells suspended in serum-free media were seeded to the top compartment of a Boyden Chamber which is sealed with a porous membrane at the bottom and containing a thin layer of MATRIGEL Basement Membrane Matrix for cell invasion (Corning, Corning, NY). Complete media (containing serum) was added to the bottom chamber as chemoattractant. Cells were incubated in the tissue culture incubator at 37°C for 28 h. For both migration and invasion assay, after incubation, cells on the upper side of the membrane were gently wiped by a wet cotton applicator and cells on the lower surface of the membrane were stained with 0.2% crystal violet in methanol.

We observed significantly increased clonogenicity with glutamine stimulation in MIA PaCa-2 ($p<0.0001$) (Figure 2 A). 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.

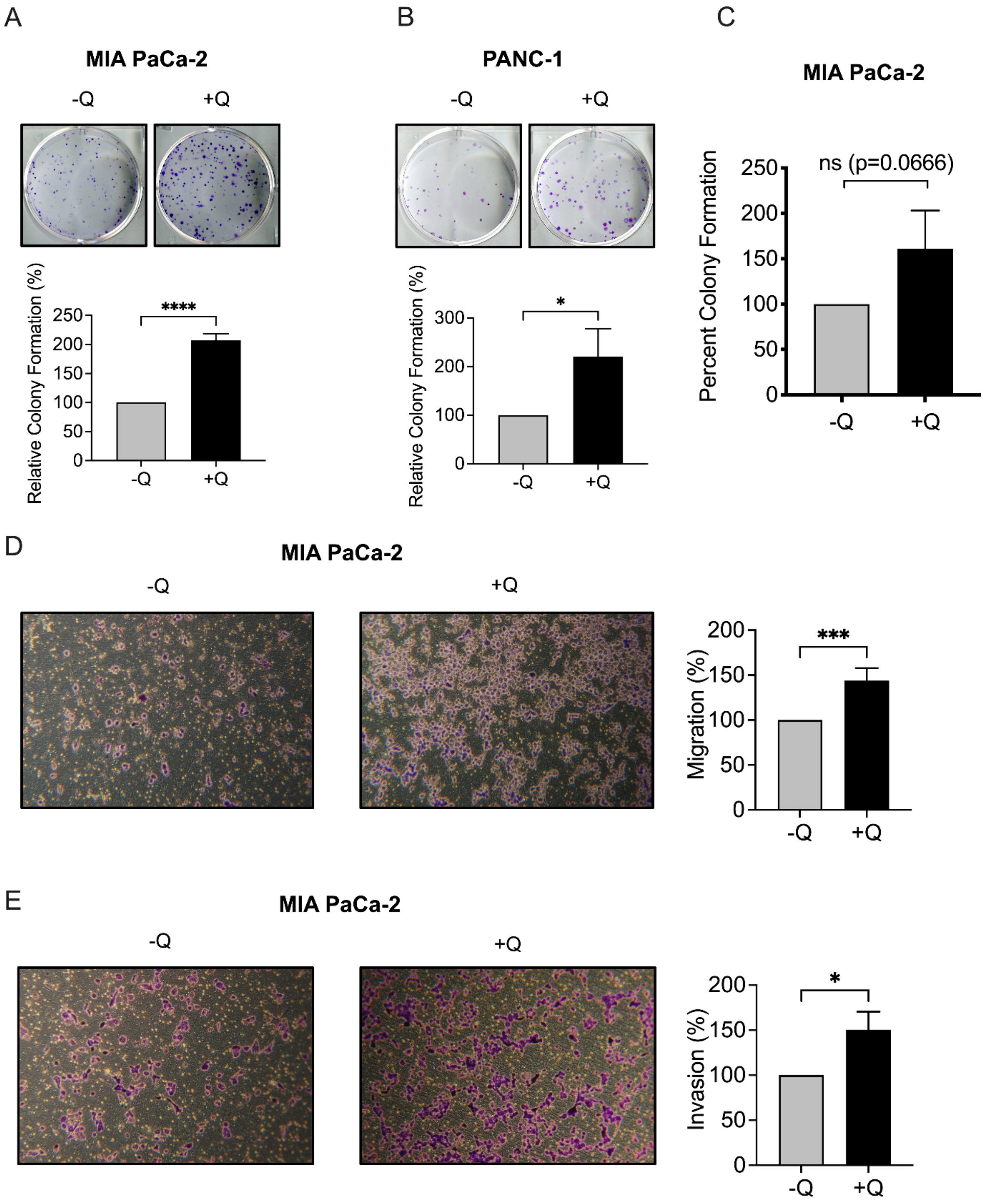


Figure 1 Glutamine Stimulation Increased the Ability of Clonogenicity, Anchorage-independent Growth, Migration and Invasion in Pancreatic Cancer Cells. [A-B] Logarithmically growing MIA PaCa-2 (n=3) and PANC-1 (n=3) cells were starved in glutamine-deficient media overnight, and stimulated with 2 mM glutamine 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)

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

1) PMT had no impact on glutamine levels in conditioned media from PSCs

In YI report, we showed from a preliminary experiment showing that PMT reduces secreted levels of glutamine in the conditioned media (CM) from PSCs. To examine if this trend reduction is significant, we performed three independent repeats. Briefly, PSCs seeded in a 96-well plate at 10k/well. 24h after seeding, cells were treated with increasing concentrations of PMT (0-150 μg/ml) for 24 h, followed by measurement of glutamine concentration in the conditioned media (CM) with glutamine/glutamate-Glo Assay Kit (Promega, Madison, WI). Our results from the three independent repeats combined suggest that PMT has no impact on glutamine levels in PSC-CM under these experimental settings (Figure 3).

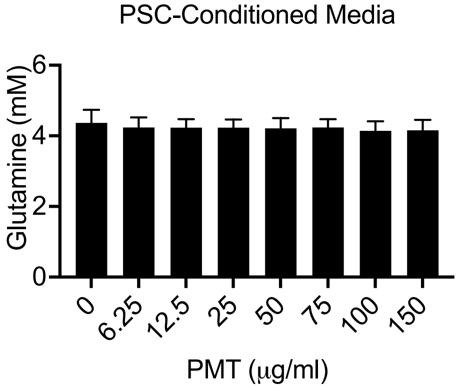


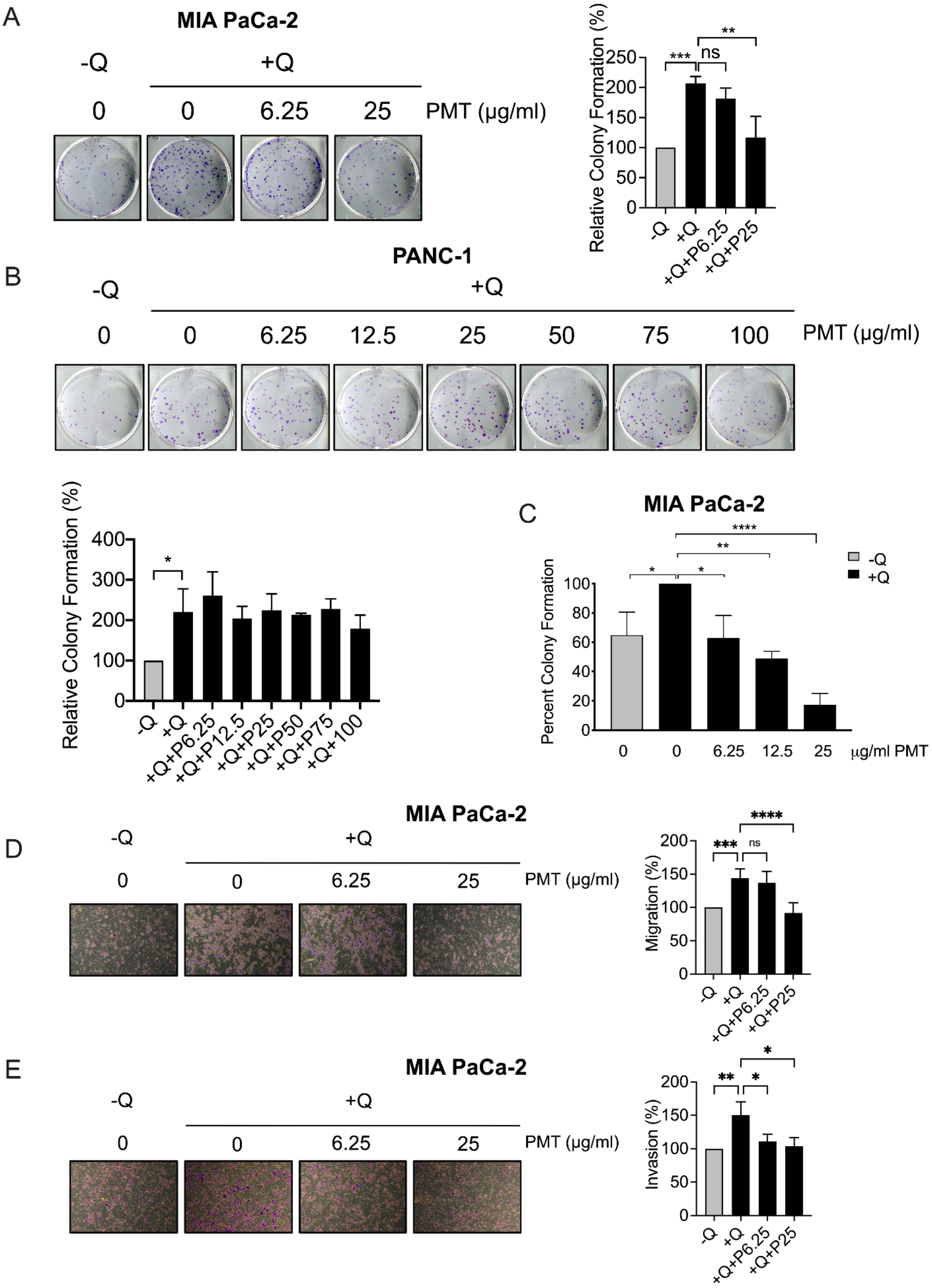
Figure 3 PMT had no impact on glutamine levels in PSC-CM.

PSCs were seeded in a 96-well plate at 10k/well. 24h after seeding, cells were treated with 0, 6.25, 12.5, 25, 50, 75, 100, 150 μg/ml PMT for 24h. CM were collected and measured following the manufacturer's instructions of Glutamine/glutamate-Glo Assay Kit (Promega, Madison, WI). Data presented is an average ± SD of three independent experiments. (One-way ANOVA followed by post-hoc Tukey test)

2) PMT suppressed glutamine-induced multiple cancer hallmarks in pancreatic cancer cells

In YI report, we demonstrated that PMT suppressed glutamine-induced proliferation in multiple pancreatic cancer cells (MIA PaCa-2, BxPC-3, PANC-1, Capan-2). PMT also reduced anchorage-independent growth in MIA PaCa-2 cells (Figure 4C) but had no effect in colony formation capacity in PANC-1 cells (Figure 4B). This year, we explored the role of PMT on colony formation, migration and invasion capacities in pancreatic cancer cells MIA PaCa-2. Logarithmically growing MIA PaCa-2 (n=3) 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). Cells were then trypsinized and viable cell concentration was determined by trypan blue assay. Methods for colony formation, migration and invasion assay were essentially the same as g/ml) described before in “2) Glutamine-stimulation increased multiple cancer hallmarks in pancreatic cancer cells.” Clonogenic assay showed that PMT significantly (p=0.0029) reduced glutamine-induced colony formation in MIA PaCa-2 at 25 μg/ml (Figure 4A), which is consistent with our data from YI showing that PMT significantly reduced anchorage-independent growth in MIA PaCa-2 cells (Figure 4C). In addition, PMT significantly reduced migration at 25 μg/ml (p<0.0001; Figure 4D) and invasion (Figure 4E) 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. The differences between MIA PaCa-2 and PANC-1 cells may be explained by their different KRAS-mutation status, metabolic subtype, doubling time, and other cell line specific signaling pathways (Table 1).


A
MIA PaCa-2
-Q
+Q
0
0
6.25
25
PMT (μg/ml)
Relative Colony Formation (%)
250
200
150
100
50
0
***
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 (%)
400
300
200
100
0
*
-Q
+Q
+Q+P6.25
+Q+P12.5
+Q+P25
+Q+P50
+Q+P75
+Q+100
C
MIA PaCa-2
Percent Colony Formation
100
80
60
40
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0
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*
**
****
-Q
+Q
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0
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12.5
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μg/ml PMT
D
MIA PaCa-2
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+Q
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0
6.25
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PMT (μg/ml)
Migration (%)
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100
50
0
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ns
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-Q
+Q
+Q+P6.25
+Q+P25
E
MIA PaCa-2
-Q
+Q
0
0
6.25
25
PMT (μg/ml)
Invasion (%)
200
150
100
50
0
**
*
*
-Q
+Q
+Q+P6.25
+Q+P25

Figure 4 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 YI report, we established that glutamine-stimulation increased pSTAT3 and survivin 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 5A). 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 5B). 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 5C). 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 5 A-C).

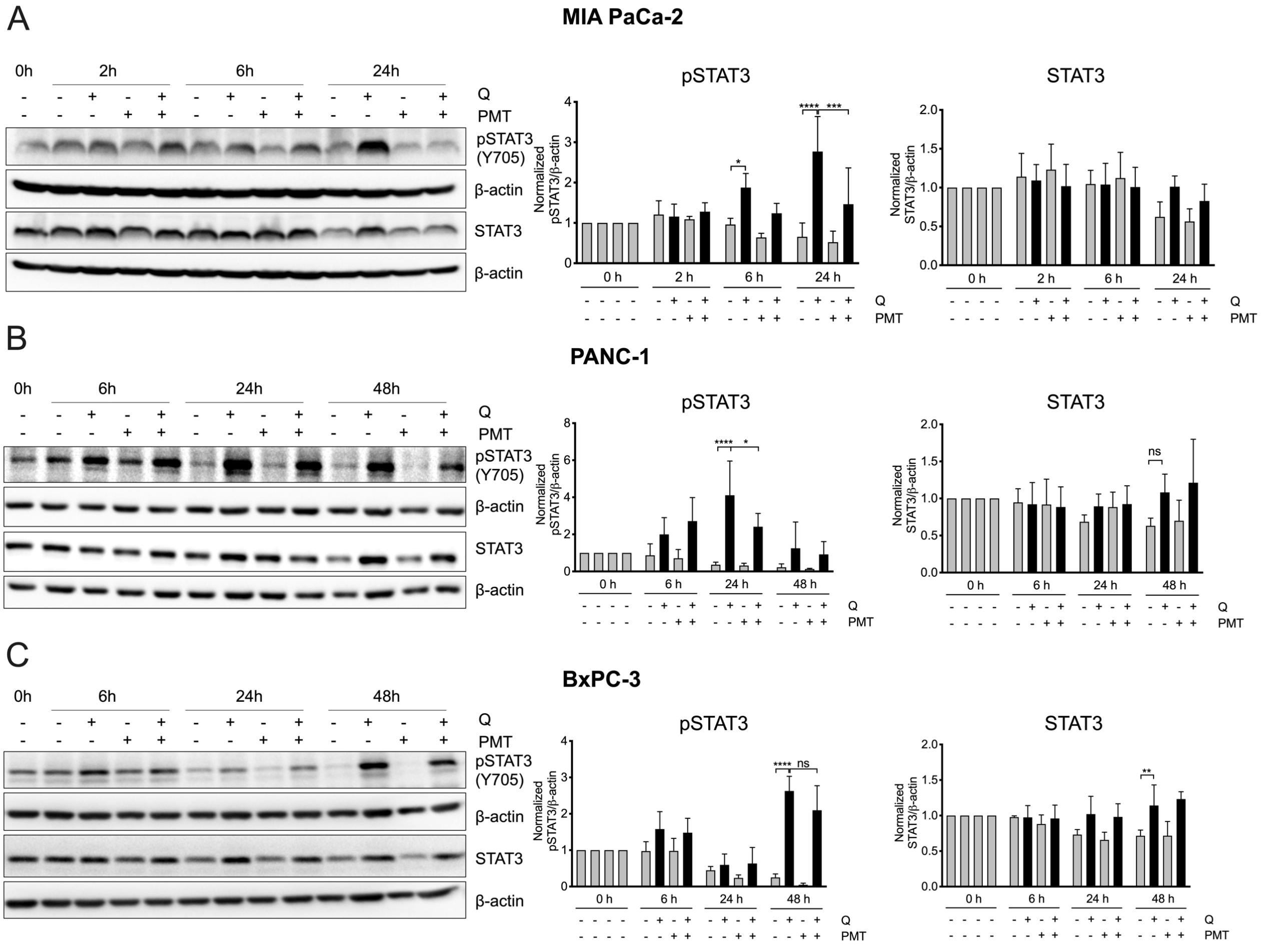
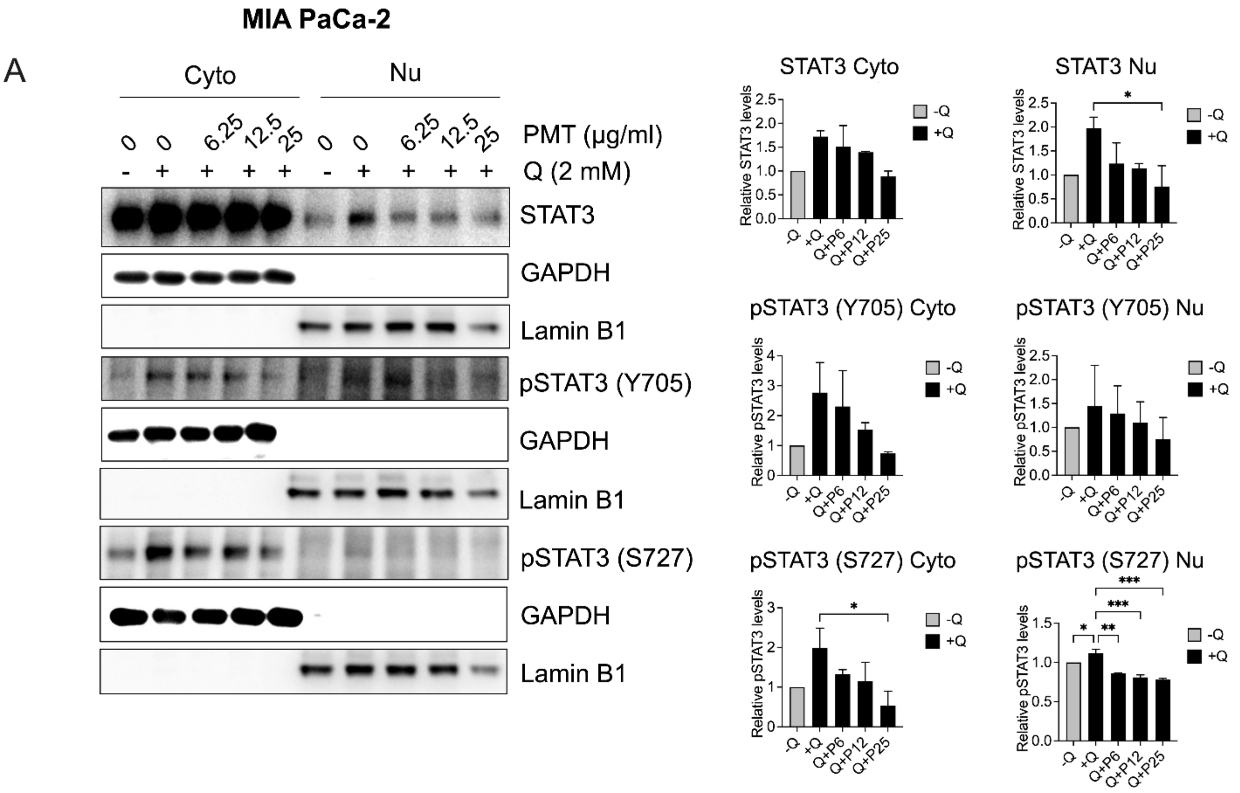


Figure 5 PMT Treatment Reduces Glutamine–induced pSTAT3 Levels in Pancreatic Cancer Cells in a Cell Line Specific Manner. [A-C] MIA PaCa-2 (n=3) (A), PANC-1 (n=4) (B) and BxPC-3 (n=3) (C) cells were starved in glutamine-deficient media overnight, and stimulated with 2 mM glutamine in the presence and 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 reduced 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 6 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 6 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 6 B). These data suggest that PMT treatment reduced glutamine-induced STAT3 signaling in MIA PaCa-2 cells.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Nu
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GAPDH
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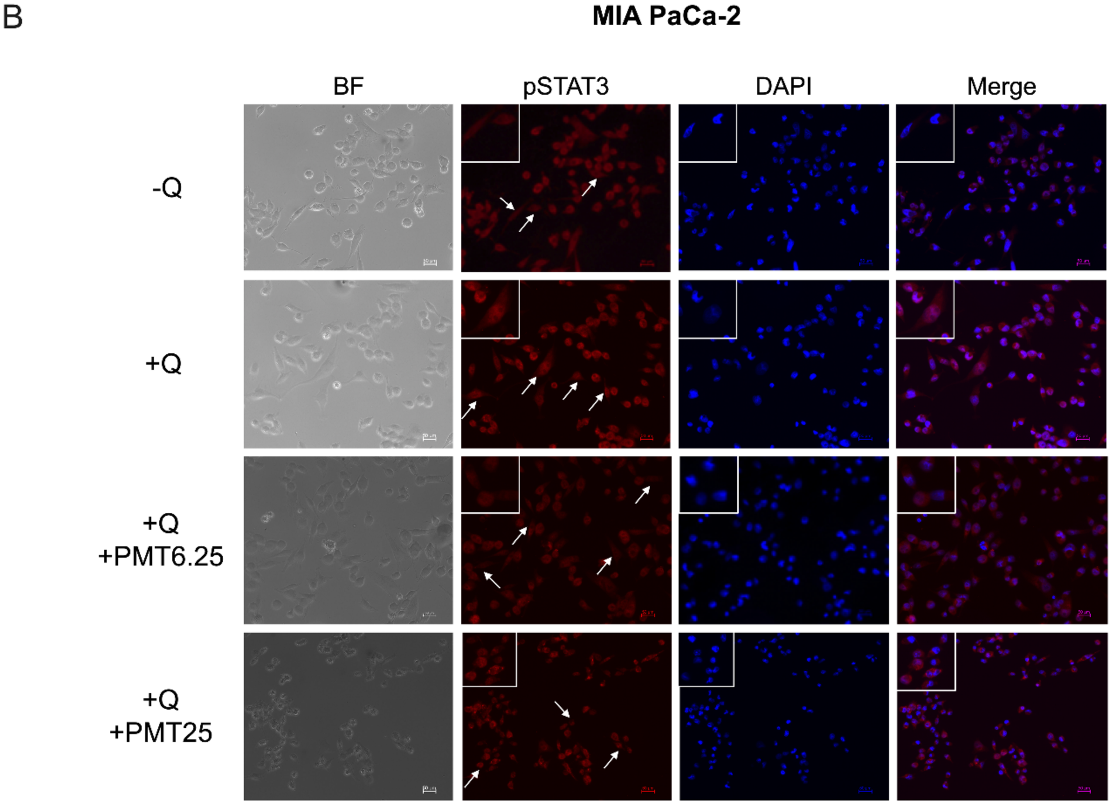
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+Q +PMT6.25
+Q +PMT25

Figure 6 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

In YI report, we showed that PMT reduced survivin (a direct down-stream target of STAT3 that is associated with poor prognosis of pancreatic cancer) levels in a dose-dependent manner (Figure 7A-C) and that glutamine stimulation enhance survivin levels in multiple pancreatic cancer cells. Here we tested 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 7 D-E). However, PMT treatment showed no effect on glutamine-induced survivin levels in BxPC-3 cells (Figure 7 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 increased survivin promoter activity in MIA PaCa-2 using 6.25 ($p=0.0144$) and 25 µg/ml ($p=0.0023$) (Figure 7 G). However, PMT showed no effect on inhibiting glutamine-induced survivin's promoter activity in PANC-1 cells (Figure 7 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 (knockdown validation in YI report). 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 7 I).

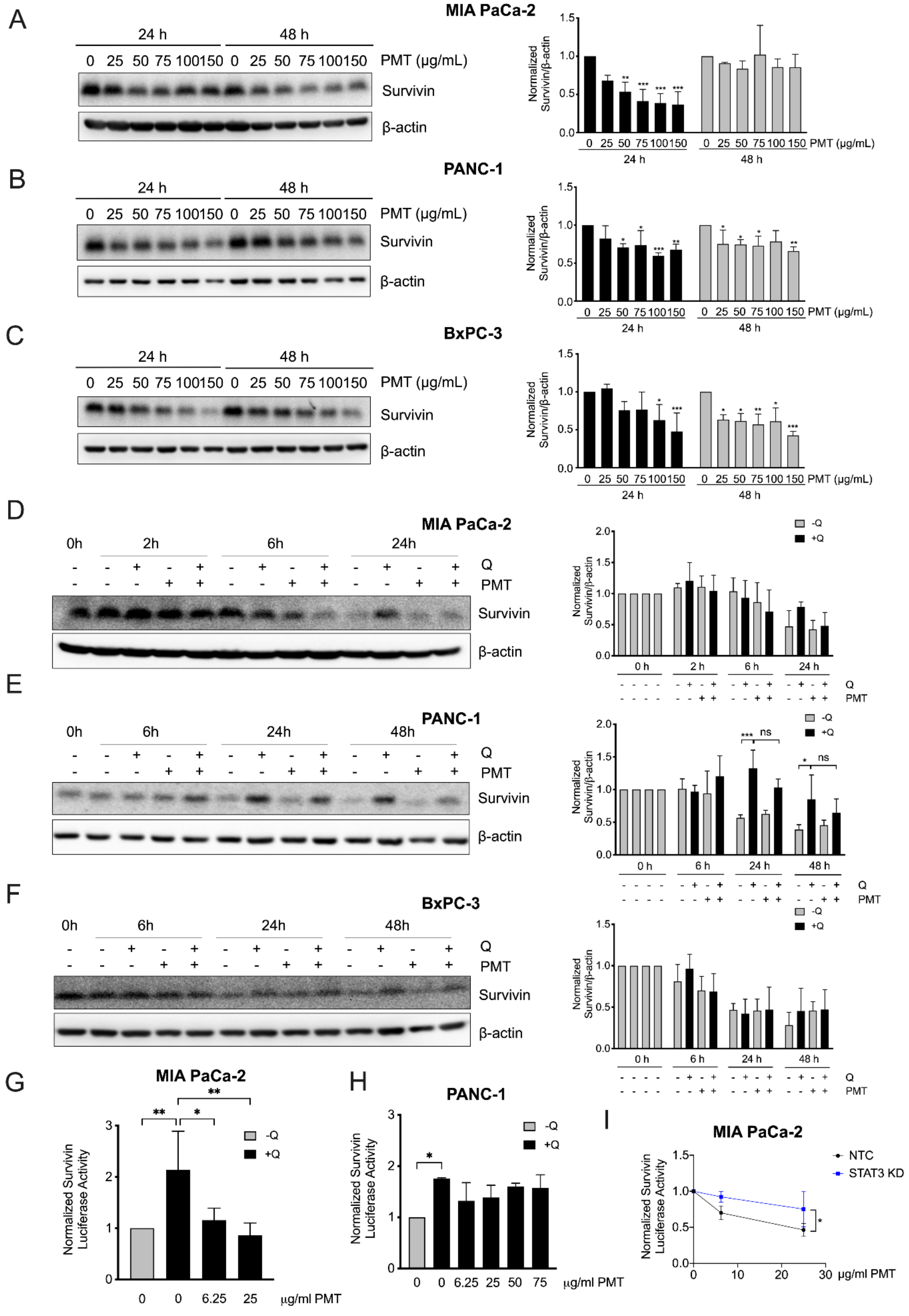


Figure 7 PMT Suppresses Glutamine–induced Survivin's Promoter Activity in Pancreatic Cancer Cells. **[A-C]** MIA PaCa-2 (n=3) **(A)**, PANC-1 (n=3) **(B)** and BxPC-3 (n=3) **(C)** cells were treated with increasing concentrations of PMT (0-150 µg/ml) for 24 h and 48 h. Whole cell lysates were collected 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 8 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 (Figure 8 B). In addition, 343 DEGs were identified in STAT3 KD versus NTC cells (Figure 8 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 8 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 8 D).

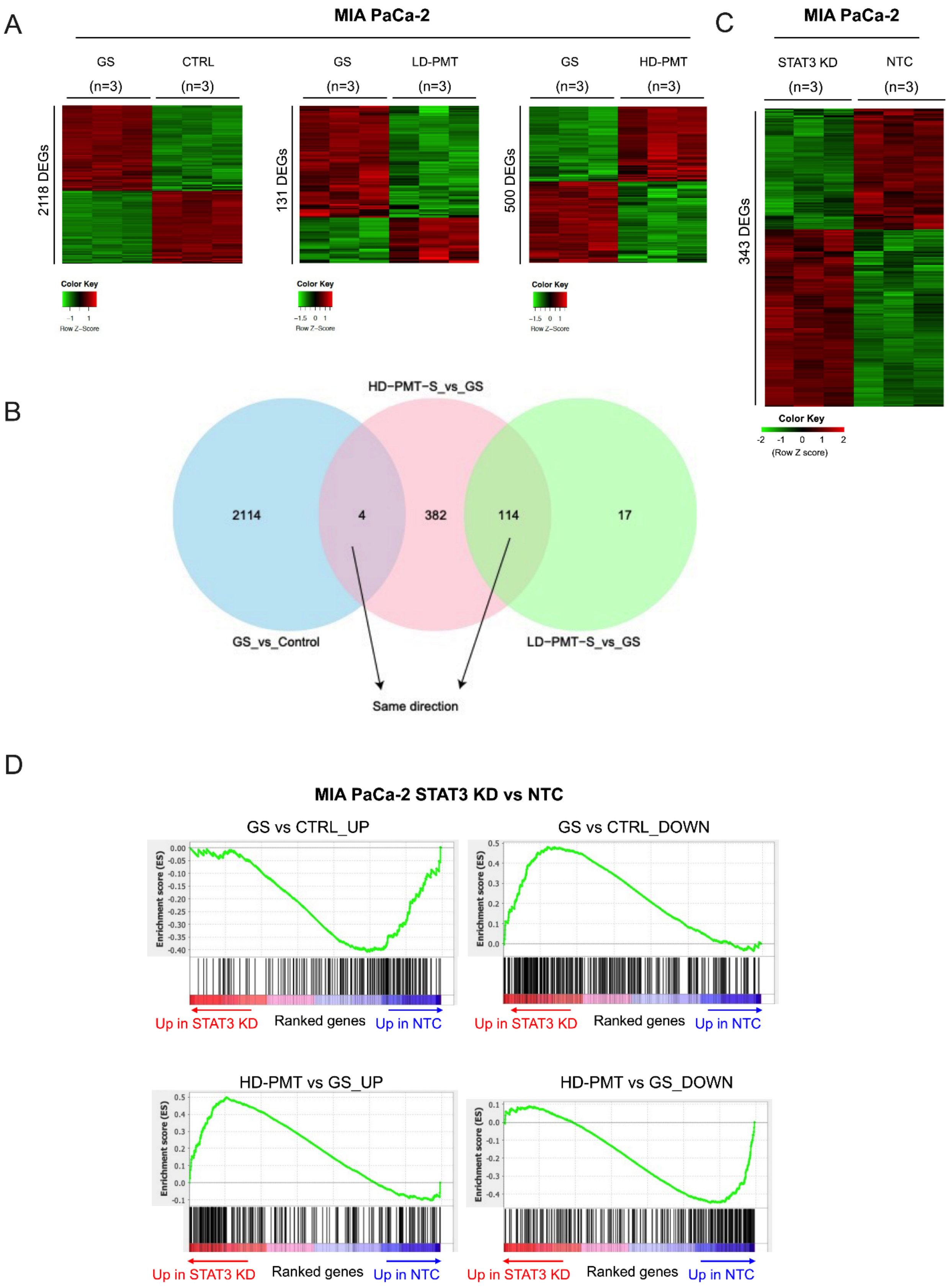


Figure 8 Gene Expression Profile of PMT Treatment Mimics STAT3 KD. [A] RNA-seq analysis on MIA PaCa-2 cells under the conditions of control (CTRL; glutamine starvation), glutamine stimulation (GS; 2mM), glutamine stimulation plus low dose PMT (LD-PMT; 6.25 µg/ml) and glutamine stimulation plus high dose

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

In YI report, we established dose-response studies with PMT, Gemcitabine (GEM) and 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. In YI report, we demonstrate the combinatorial benefits of PMT plus GEM and Abr in PSCs and MIA PaCa-2 cells. Our result showed that PMT+GEM+Abr triple combination is better than GEM plus Abr combination in terms of proliferation inhibition in PSCs, while in MIA PaCa-2 cells combination of PMT plus Abr appears to be the best relative to triple or GEM plus Abr combination (Figure 9 A-B). This year, we tested the effect of PMT plus GEM and Abr on a GEM-resistant cell line, PANC-1 (Figure 9C). Our result showed that, while GEM alone had minimal anti-proliferative impact on PANC-1 cells, PMT alone was able to significantly reduce proliferation in PANC-1 cells, suggesting that PMT showed anti-proliferative effect in GEM-resistant cell lines.

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). Surprisingly, GEM alone treatment appears to enhance the anchorage-independent growth by 36% (non-significant), suggesting the activation of resistant signaling pathways. 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 (Figure 9D).

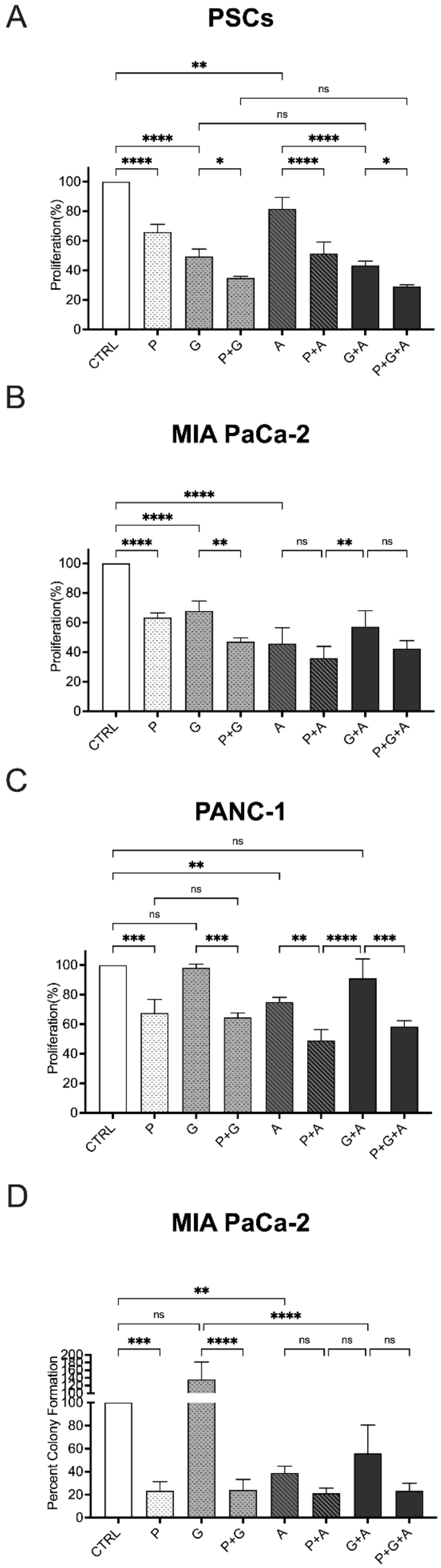


Figure 9 PMT Potentiates Growth Inhibitory Effect of GEM and Abr in Pancreatic Stellate and Cancer Cells. [A-C] PSC (n=3) **(A)**, MIA PaCa-2 (n=4) **(B)** and PANC-1 (n=3) **(C)** cells were treated with PMT, GEM and Abr or combinations or PMT+GEM, PMT+Abr, GEM+Abr, PMT+GEM+Abr for 48h. Doses selections 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

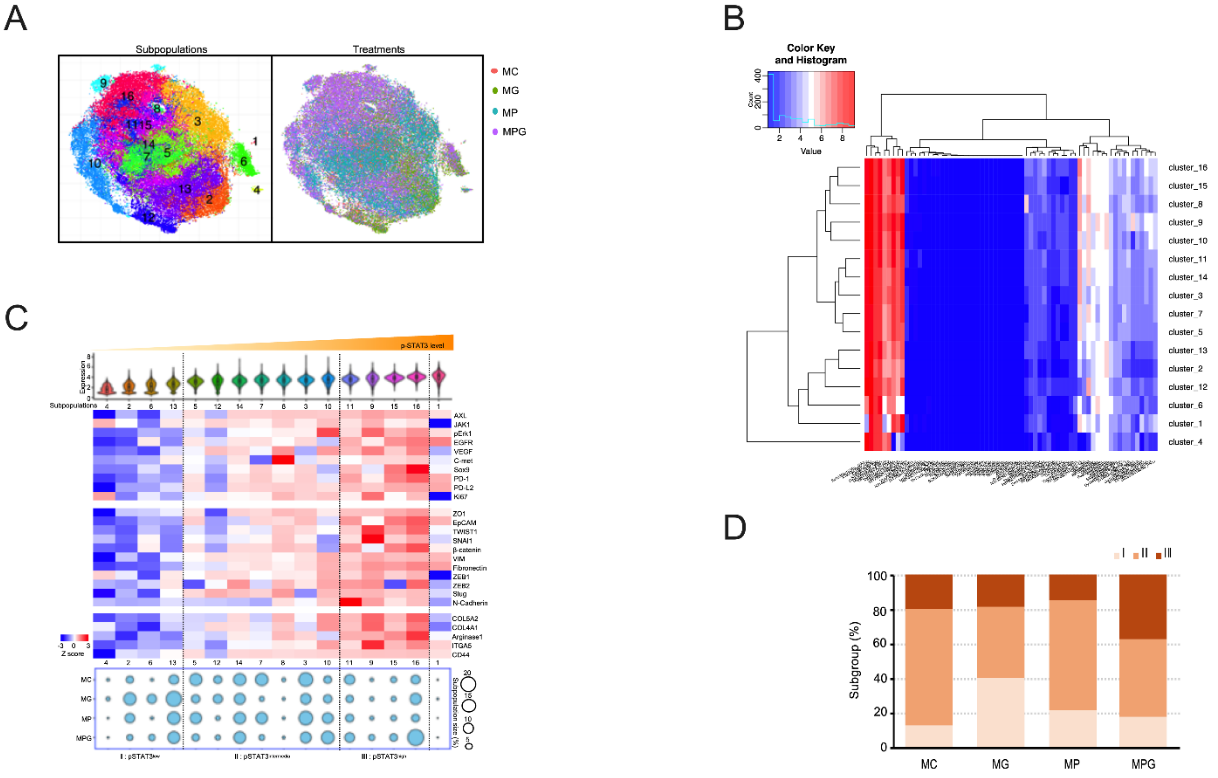
assay (which measures intracellular metabolic activity). Data presented is an average ± SD of three to four independent experiments. ns: not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, *** $P < 0.0001$ (One-way ANOVA followed by post-hoc Tukey test). **[D]** MIA PaCa-2 cells were treated 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).

2) 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 10 A and E). A heatmap was generated showing the changes of all the proteins analyzed among different clusters (Figure 10 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 10 C and G). The percentage of each sub-population of cells was calculated and plotted (Figure 10 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 10 D 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



PANC-1

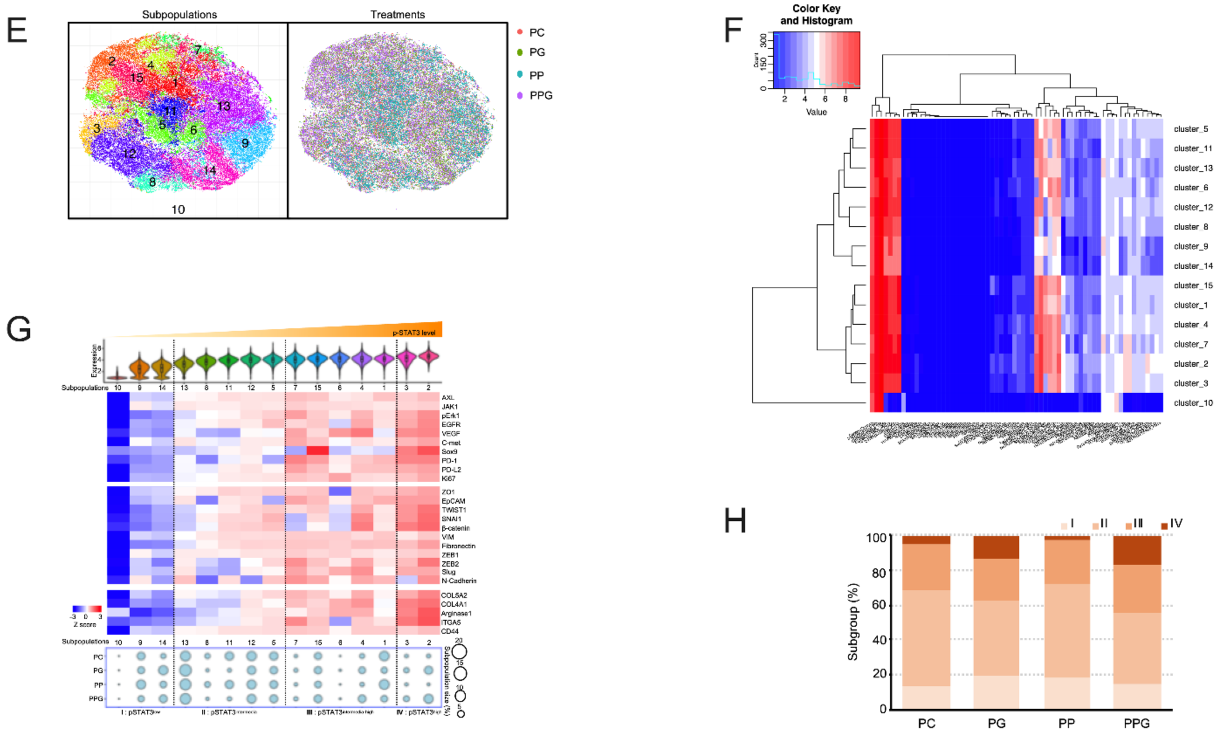


Figure 10 CyTOF Analysis Revealed Heterogeneous pSTAT3 Expression in Pancreatic Cancer Cells and Association with Response to Treatment. **[A-D]** MIA PaCa-2 and **[E-H]** PANC-1 cells were treated with PMT, GEM, or combination for 48h. Dose selections are MIA PaCa-2 (50 µg/ml PMT, 50 nM GEM) and PANC-1 (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.

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:** this year's training has provided me great opportunities to gain and sharpened my technical skills (such as CyTOF, immunofluorescence and subcellular fractionation). I have also participated in preclinical studies in other ongoing projects in Dr. Kumar's lab, which are valuable exercises that prepare me for my own preclinical study (which I will initiate soon). My collaborative work has resulted in 1 publication in the year of 2021 and a 2nd co-author publication is currently under revision.
- **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 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?

During the next 6 months, I plan to initiate and complete preclinical studies to determine the efficacy of the combination treatment PMT, gemcitabine (GEM) and Abraxane (Abr) (Major Goal 5). I also plan to compile my data for a manuscript for publication.

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

COVID-19 related hurdles including procuring necessary reagents in a timely fashion delayed some of the preclinical studies. We have requested a no-cost extension to finish these experiments.

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

Nothing to Report.

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.

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

  Nothing to Report.

  Other publications, conference papers and presentations.

Presentations:

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

- **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) Award Chart

W81XWH-19-1-0596 (CA181275) : Glutamine-Mediated Tumor-Stromal Interaction: A Novel Target for Pancreatic Cancer Treatment

PI: Xiaoyu Yang, UT Health San Antonio, TX **Budget:** $228,750.00
Topic Area: FY19 Peer Reviewed Cancer Research Program **Mechanism:** Horizon Award

Research Area(s): 0804 **Award Status: 08/15/2019 – 08/14/2021**

Study Goals:

The goal of this project is to investigate the role of glutamine in the communication between pancreatic stellate cells and pancreatic cancer cells to promote pancreatic cancer progression and to clarify how palmatine can disrupt this communication to potentiate response to conventional therapeutics

Specific Aims:

Specific aim 1: To determine the mechanism through which glutamine mediates pancreatic stellate cells - pancreatic cancer cells communication to promote cancer survival hallmarks (proliferation, invasion, metastasis and cell death) and the ability of palmatine to inhibit this process.
Specific aim 2: To determine the effectiveness of palmatine-mediated glutamine inhibition to improve response to conventional therapeutic agents.

Key Accomplishments and Outcomes:

Publications: none to date
Patents: none to date
Funding Obtained: none to date