

STAT3 induces anoikis resistance, promotes cell invasion and metastatic potential in pancreatic cancer cells

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Tumor cells need to attain anoikis resistance to survive prior to metastasis making it a vital trait of malignancy. The mechanism by which pancreatic cancer cells resist anoikis and metastasize is not well established. Significant proportion of pancreatic cancer cells resisted anoikis when grown under anchorage-independent conditions. The cells that resisted anoikis showed higher migratory and invasive characteristics than the cells that were cultured under anchorage-dependent condition. Interestingly, anoikis-resistant cells exhibited significantly increased expression and phosphorylation of signal transducer and activation of transcription 3 (STAT3) at Tyr 705, as compared to adherent cells. AG 490 and piplartine (PL) induced significant anoikis in anoikis-resistant pancreatic cancer cells. Silencing STAT3 not only reduced the capacity of pancreatic cancer cells to resist anoikis but also reversed its invasive characteristics. Interleukin-6 treatment and overexpression of STAT3 enhanced anoikis resistance and protected the cells from PL-induced anoikis. PL-treated cells completely failed to develop tumors when injected subcutaneously in immune-compromised mice. Moreover, these cells also failed to metastasize when injected intravenously. On the other hand, untreated anoikis-resistant cells not only formed aggressive tumors but also metastasized substantially to lungs and liver when injected intravenously. Metastatic nodules formed by untreated anoikis-resistant cells in lungs exhibited significant phosphorylation of STAT3 at Tyr705. Taken together, our results established the critical involvement of STAT3 in conferring anoikis resistance to pancreatic cancer cells and increased metastasis.

Introduction

Extracellular matrix serves as a basement membrane for the cells to grow and differentiate (1). The cells detached from extracellular matrix succumb to classical apoptosis commonly known as anoikis (2). Epithelial cells show high dependence on appropriate cell–cell and cell–matrix environment for survival (3). However, tumor cells develop ability to survive and grow under anchorage-independent conditions and are termed as anoikis-resistant cells (4). These cells invade and migrate to distant metastatic sites. Nonetheless, the mechanism of anoikis resistance remains elusive (5). It is also essential to note that there is no common mechanism of anoikis resistance in different types of cancer (5).

Several genes that play significant role in survival, proliferation, and angiogenesis are regulated by signal transducers and activators of transcription (STAT) family of transcription factors (5–13). Many studies have shown enhanced STAT3 activity in various types of human cancers (14–18). STAT3 is activated via phosphorylation at important tyrosine or serine residues by Janus-activated kinases (JAK), interleukin-6 (IL-6), epidermal growth factor receptors and Src kinases. Upon phosphorylation, STAT3 dimerizes and translocates to the nucleus where it enhances the transcription of target genes (19–21). Tyrosine (Y705), which is one of the contingent sites

of phosphorylation, enhances the expression of various proliferation and survival genes such as Mcl-1, survivin, Bcl-2 and cyclin D1 (14,22,23).

Pancreatic cancer is the fourth leading cause of cancer-related deaths in the USA (24). Most of the patients with pancreatic cancer develop metastases and die because of unrestrained growth (25). In fact, high mortality rate is associated with rapid development of metastasis in >50% of patients with pancreatic cancer (26). Most common sites of pancreatic cancer metastasis includes lymph nodes, liver and abdominal cavity (27).

Herein, role of STAT3 was established in anoikis resistance both *in vitro* and *in vivo* in pancreatic cancer. Our results validated the association of STAT3-mediated anoikis resistance with enhanced cell migration, invasion and metastasis in *in vivo* tumor models. This study provides first-hand information on the critical role of STAT3 in anoikis resistance and metastasis of pancreatic cancer.

Materials and methods

Chemicals

AG 490 was acquired from Selleck Chemicals (Houston, TX). Transfection reagent Lipofectamine 2000 was obtained from Life Technologies (Grand Island, NY). Piplartine (PL) was obtained from Cayman Chemicals (Ann Arbor, MI). G418, Mayer's hematoxylin, eosin and PermOUNT were obtained from Fisher Scientific (Houston, TX). Poly(2-hydroxyethyl) methacrylate, sulforhodamine B and antibody against actin were obtained from Sigma-Aldrich (St Louis, MO). RPMI and Dulbecco's modified Eagle's medium were purchased from Mediatech (Manassas, VA). Nucleofection kit was purchased from Lonza (Allendale, NJ). STAT3 shRNA was obtained from SA Biosciences (Frederick, MD) and STAT3 α plasmid was a generous gift from Dr J.F.Bloomberg (Rockefeller University, NY). All the antibodies were bought from Cell Signaling (Danvers, MA) unless specified. Recombinant IL-6 was purchased from Peprotech (Rocky Hill, NJ).

Cell culture

Human pancreatic cancer cell lines AsPc-1, Panc-1, HPAC, L3.6PL and COLO-357 were procured and cultured as previously described by us (28,29). Panc-1-luc cells were provided by Dr Frank Marini (MD Anderson Cancer Center, Houston, TX). All the cell lines except Panc-1-luc cells were authenticated by short tandem repeat analysis at TTUHSC core facilities (Lubbock, TX).

Migration and invasion assay

Cells were incubated either under anchorage-dependent or -independent conditions for 48 h and wound healing assay was performed according to the previously described method by us with some modifications (29–31).

The invasion assay was performed by Boyden's chamber assay. After 48 h incubation of cells under adherent or suspension conditions, cells were transferred to the upper chamber of Boyden's chamber (BD Biosciences, Bedford, MA) and the assay was performed according to the manufacturer's protocol.

Anoikis assay

To mimic anchorage-independent growth conditions, culture dishes were coated with poly(2-hydroxyethyl) methacrylate and performed anoikis assay according to the previously described protocol by us (32). Approximately 1×10^6 cells were seeded under anchorage-independent conditions with or without treatment for 48 h. Thereafter, cells were centrifuged and uniformly divided in a 24-well plated. After 8 h, cells were processed for sulforhodamine B assay (33). Anoikis resistance of control was taken as 100% and that of other groups was calculated as percentage of control.

Western blotting

Cells were incubated in either anchorage-dependent or -independent conditions, with or without any treatment for 48 h. Following these incubations, cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by immunoblotting as described previously (28). Prior to the incubation with primary antibody, membrane was cut into three portions according to the molecular weight and three different molecules were probed on the same membrane based on the molecular weight of the protein. One of the blots was stripped to probe for actin, which was used as a loading control.

Abbreviations: IL-6, interleukin 6; PL, piplartine; STAT3, signal transducer and activation of transcription 3.

The bands shown in the figure are cropped images after the developing the entire membrane.

STAT3 transient transfection

STAT3 was either knocked down using shRNA or overexpressed using a plasmid as described previously by us (34). Two shRNAs with different sequences were used to silence STAT3 and named as shRNA1 and shRNA2. Transfections in L3.6PL and COLO-357 were performed using Lipofectamine 2000 according to the manufacturer's protocol. AsPc-1 and Panc-1 cells were transfected by nucleofection as previously described by us (29). After 12 h of transfection, cells were processed for anoikis assay, invasion assay or western blotting as described above. Before the experiment, the extent of silencing or overexpression was tested by western blotting.

Activation of STAT3 by IL-6

STAT3 (Y705) activation was performed as explained previously by us (31,34). Cells were treated with 10 ng/ml IL-6 under anchorage-independent conditions 1 h before PL (5 μ M PL) treatment. After 48 h, cells were processed for anoikis assay.

Immunofluorescence

Panc-1 cells were incubated with or without anchorage for 48 h. Cells were fixed with formalin and permeabilized using 0.05% triton-X. Cells were then immunostained with anti-p-STAT3 (Y705) antibody as described previously by us (35,36).

In vivo anoikis xenograft experiment

In vivo xenograft experiment was performed as described previously, with slight modifications (32). Male SCID/NSG mice (5–7 weeks old) were obtained from TTUHSC Breeding Facility (Lubbock, TX). The use of SCID/NSG mice was approved by the Institutional Animal Care and use Committee

(IACUC) and the experiments were performed in strict compliance with the regulations. Panc-1 cells with (5 μ M PL) or without treatment with STAT3 inhibitor were cultured under anchorage-independent conditions for 48 h. Percentage of viable cells were determined by trypan blue assay and 5×10^6 live cells resuspended in 1:1 Dulbecco phosphate-buffered saline:Matrigel (BD Biosciences, Houston, TX) were injected subcutaneously in both the flanks of the mice (two groups; $n = 9$ mice per group). Tumor volume was measured using vernier calipers twice a week and the tumor volume was calculated using the formula described by us previously (37,38).

In vivo anoikis metastasis experiment

Panc-1-luc cells were used for this experiment in order to monitor the progression of metastasis. Cells either untreated or treated with STAT3 inhibitor (PL) were cultured under anchorage-independent conditions for 48 h. Cell viability was assessed by trypan blue dye exclusion assay. About 0.8×10^6 viable cells re-suspended in Dulbecco phosphate-buffered saline were injected intravenously through the lateral tail vein (two groups; $n = 10$ mice per group). Animals were imaged twice a week using IVIS Bioluminescent System equipped with Live Imaging software (Caliper Life Sciences, Hopkinton, MA). At the end 35 days, mice were killed and liver and lungs were imaged *ex vivo*. Moreover, lungs and liver were also analyzed for metastatic tumor nodules by immunohistochemistry.

Immunohistochemistry

H & E staining was performed in the paraffin-embedded sections of lungs and liver of the mice to confirm the presence of metastatic nodules according to the previously described procedure (34). Expression of STAT3 in metastatic nodules was analyzed by immunohistochemistry in paraffin sections of the lungs of four mice from each group. The sections were deparaffinized and rehydrated using decreasing concentrations of ethanol. Antigen retrieval process

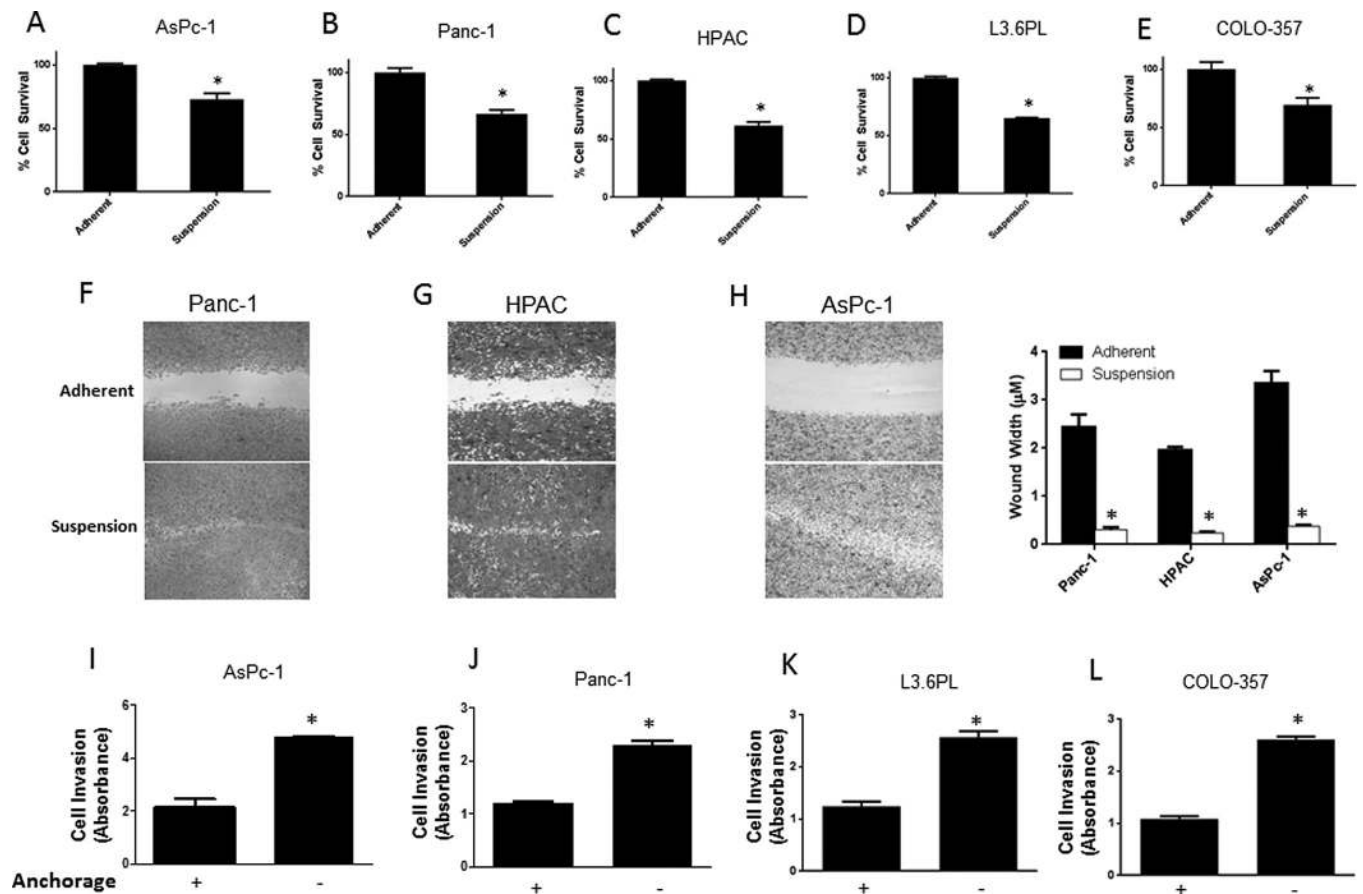


Fig. 1. Pancreatic cancer cells resist anoikis under anchorage-independent conditions. (A–E) AsPc-1, Panc-1, HPAC, L3.6PL and COLO-357 cells were cultured in poly(2-hydroxyethyl) methacrylate-coated plates under anchorage-independent conditions for 48 h following which anoikis assay was performed. The cell survival was compared with the cells cultured in adherent conditions for same time period. Anoikis-resistant cells are highly migratory and invasive. (F–H) Panc-1, HPAC and AsPc-1 cells were cultured in adherent or suspension conditions for 48 h and then replated in a 24-well plate. Following this, wound healing assay was performed on the anoikis-resistant cells and the results were compared to the adherent cells. (I–L) Invasion of AsPc-1, Panc-1, L3.6PL and COLO-357 cells was measured by Boyden's Transwell assay according to the manufacturer's instructions. Values are plotted as mean \pm SD. * $P < 0.05$ compared with adherent group. Each experiment was repeated at least three times with similar results.

was carried out by boiling the sections in citrate buffer (pH 6) for 10 min. Endogenous peroxides were quenched by incubating the sections in 3% hydrogen peroxide solution. Sections were blocked using 6% goat serum for 30 min after which they were exposed to primary antibody overnight (p-STAT3). Following the incubation, the expression was detected using Ultravision ONE detection reagent (Thermo Fisher, Houston, TX) according to the manufacturer's protocol. The sections were then counterstained with Mayer's hematoxylin and dehydrated using increasing concentrations of ethanol and xylene and observed under the microscope.

Statistical analysis

All the statistical calculations were performed using Prism 6.0 (GraphPad Software, San Diego, CA). The data were represented as mean \pm SD or SEM. Student's *t*-test was used for comparison of two groups. For experiments involving more than two groups, analysis of variance followed by Tukey's *post hoc* multiple comparison test was used. All the statistical tests were two sided. Differences were considered statistically significant when *P* value was <0.05 .

Results

Pancreatic cancer cells exhibit anoikis resistance

Why cancer cells resist anoikis and acquire metastatic potential is not known (4). We evaluated anoikis resistance in five human pancreatic cancer cell lines that were isolated from metastatic sites and were metastatic in nature. AsPc-1, Panc-1, HPAC, L3.6PL and COLO-357 cells were cultured under anchorage-independent conditions for 48 h and survival of the cells was assessed by sulforhodamine B assay and compared with the cells cultured simultaneously under adherent conditions. Even though notable anoikis was induced in all the cell lines cultured under anchorage-free conditions, there were a significant percentage of cells that resisted anoikis (Figure 1A–E). About

75% of AsPc-1 and COLO-357 cells resisted anoikis, whereas 65% of Panc-1, HPAC and L3.6PL cells resisted anoikis when cultured under anchorage-independent conditions (Figure 1A–E)

Anoikis-resistant cells have high rate of migration and invasion

In order to metastasize, cancer cells migrate and invade to the secondary organs. Migration and invasion assays were performed in anoikis-resistant cells and the results were compared with adherent cells. Wound healing assay was performed for cell migration in Panc-1, AsPc-1 and HPAC cells. Cells were incubated under anchorage-dependent or -independent conditions for 48 h and then transferred to 24-well plates and a wound was created when the cells were attached. Our results showed that the cells that resisted anoikis healed the wound at much faster rate as compared to adherent cells (Figure 1F–H). In fact, anoikis-resistant AsPc-1 cells healed the wound even before the adherent cells initiated the wound healing process (Figure 1H). Invasion assay was performed in AsPc-1, Panc-1, L3.6PL and COLO-357 cells using Boyden's chamber. Apart from being migratory in nature, cells that evaded anoikis were also highly invasive as compared to adherent cells (Figure 1I–L). Anoikis-resistant AsPc-1 and COLO-357 cells showed 2.5-fold higher rate of invasion, whereas Panc-1 and L3.6PL showed 2-fold increased rate of invasion, as compared to their adherent counterparts (Figure 1I–L). Our results thus suggested that anoikis-resistant cells acquire enhanced migratory and invasive characteristics.

Anoikis-resistant pancreatic cancer cells exhibit overexpression of STAT3

We next wanted to investigate the molecular changes that were responsible for not only inducing anoikis resistance but also

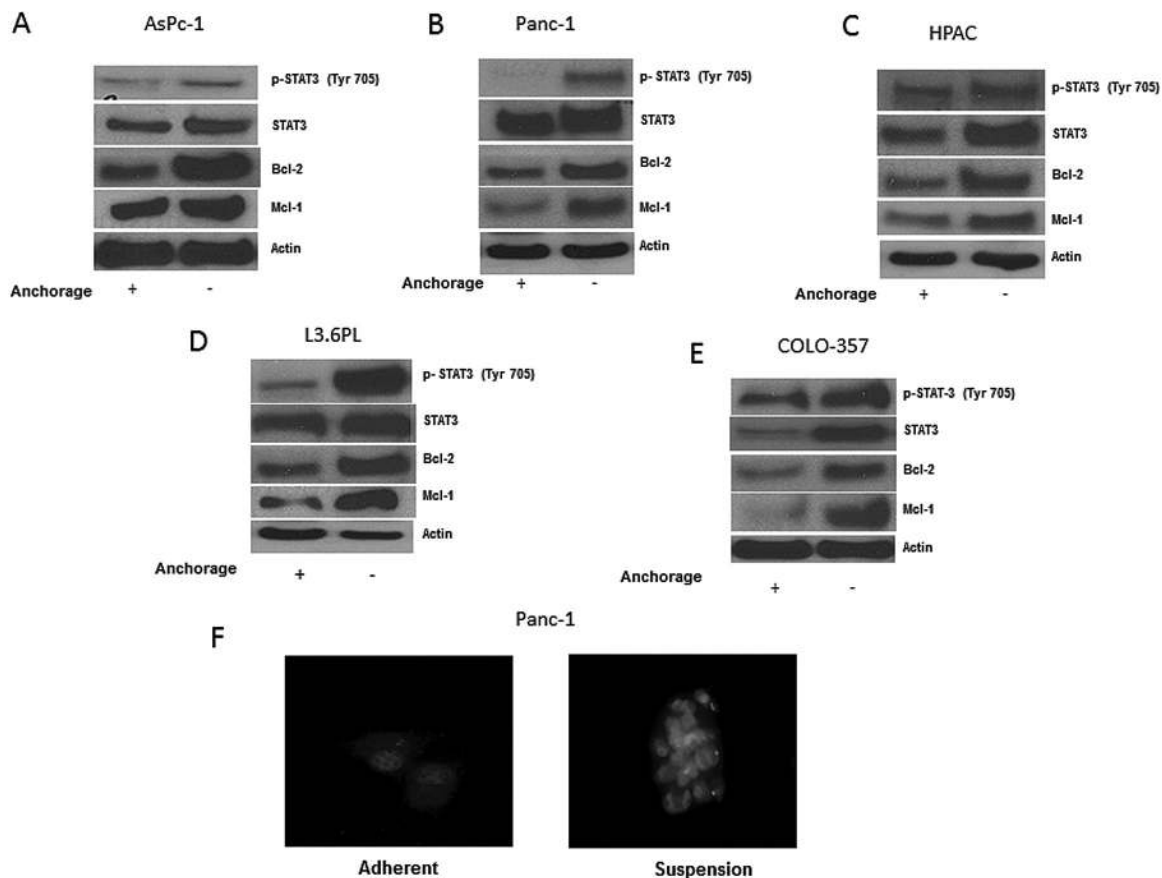


Fig. 2. Anoikis-resistant pancreatic cancer cells exhibit STAT3 overexpression. (A–E) Representative blots of pSTAT3, STAT3, Bcl-2 and Mcl-1 from lysates of adherent or anoikis-resistant AsPc-1, Panc-1, HPAC, L3.6PL and COLO-357 cells. Actin was used as loading control. The bands shown in the figure are cropped images after developing the entire membrane. (F) Panc-1 cells cultured in either adherent or anchorage-independent conditions were immunostained for pSTAT3 (Y705) (red). DAPI was used to stain nucleus. Each experiment was repeated at least three times with similar results.

transforming the cells into a highly migratory and invasive phenotype. Hence, we examined the expression of various proteins in anoikis-resistant AsPc-1, Panc-1, HPAC, L3.6PL and COLO-375 cells and compared the results with respective adherent cells. Our results showed a remarkable increase in the phosphorylation of STAT3 at Y705 in the cells that resisted anoikis as compared to adherent cells (Figure 2A–E). Significant increase in the protein expression of STAT3 was also observed in anoikis-resistant cells (Figure 2A–E). In addition, in anoikis-resistant cells, we observed an increase in the expression of antiapoptotic proteins Bcl-2 and Mcl-1, which are regulated by STAT3 transcriptionally (Figure 2A–E). Immunofluorescence results clearly showed the formation of pancreatic cancer cell spheroids under anchorage-independent conditions. Substantial red staining for p-STAT3 (Y705) was observed in Panc-1 cells that were cultured under anchorage-independent condition in comparison to adherent cells (Figure 2F). Moreover, every cell in the spheroid showed enhanced phosphorylation of STAT3 than adherent cells (Figure 2F).

STAT3 inhibitors overcome anoikis resistance in pancreatic cancer cells

Based on the observations made so far, we hypothesized that STAT3 plays significant role in imparting anoikis resistance to pancreatic cancer cells as well as promoting their migration and invasion potential leading to metastasis. We therefore evaluated the effect of AG 490, a STAT3 inhibitor, on anoikis resistance in pancreatic cancer cells.

Following the treatment with 50 or 100 μM AG 490 under suspension conditions for 48 h, AsPc-1, Panc-1, HPAC, L3.6PL and COLO-375 cells were re-cultured on adherent plates. Sulforhodamine B assay was used to assess cell survival. Substantial reduction in anoikis resistance was observed by AG 490 treatment in all the pancreatic cancer cell lines (Figure 3A–E). Treatment with 50–100 μM AG 490 reduced anoikis resistance by 70–80% in AsPc-1 and Panc-1 cells, 40–60% in HPAC cells, 40–50% in L3.6PL cells and 30–60% in COLO-375 cells (Figure 3A–E).

We further tested the effect of PL on anoikis resistance in pancreatic cancer cells. PL treatment reduced the survival of pancreatic cancer cells (Srivastava, unpublished observations). After 48 h treatment, PL (5–10 μM) significantly reduced anoikis resistance in all the pancreatic cancer cell lines (Figure 3F–J). Most of the cell lines showed ~90% reduction in anoikis resistance after treatment with 10 μM PL (Figure 3F–J).

PL overcomes anoikis resistance by STAT3 inhibition

Western blotting was used to analyze the molecular changes that occurred after PL treatment in cells cultured under anchorage-independent condition. Significant and concentration-dependent decrease in the phosphorylation of STAT3 at Y705 was observed in all the pancreatic cancer cell lines treated with PL (Figure 3K). At 10 μM PL, it suppressed almost 90% phosphorylation of STAT3 (Figure 3K). PL treatment furthermore downregulated not only the protein levels of STAT3 but also the expression of Mcl-1 and Bcl-2, antiapoptotic proteins regulated transcriptionally by STAT3 (Figure 3K).

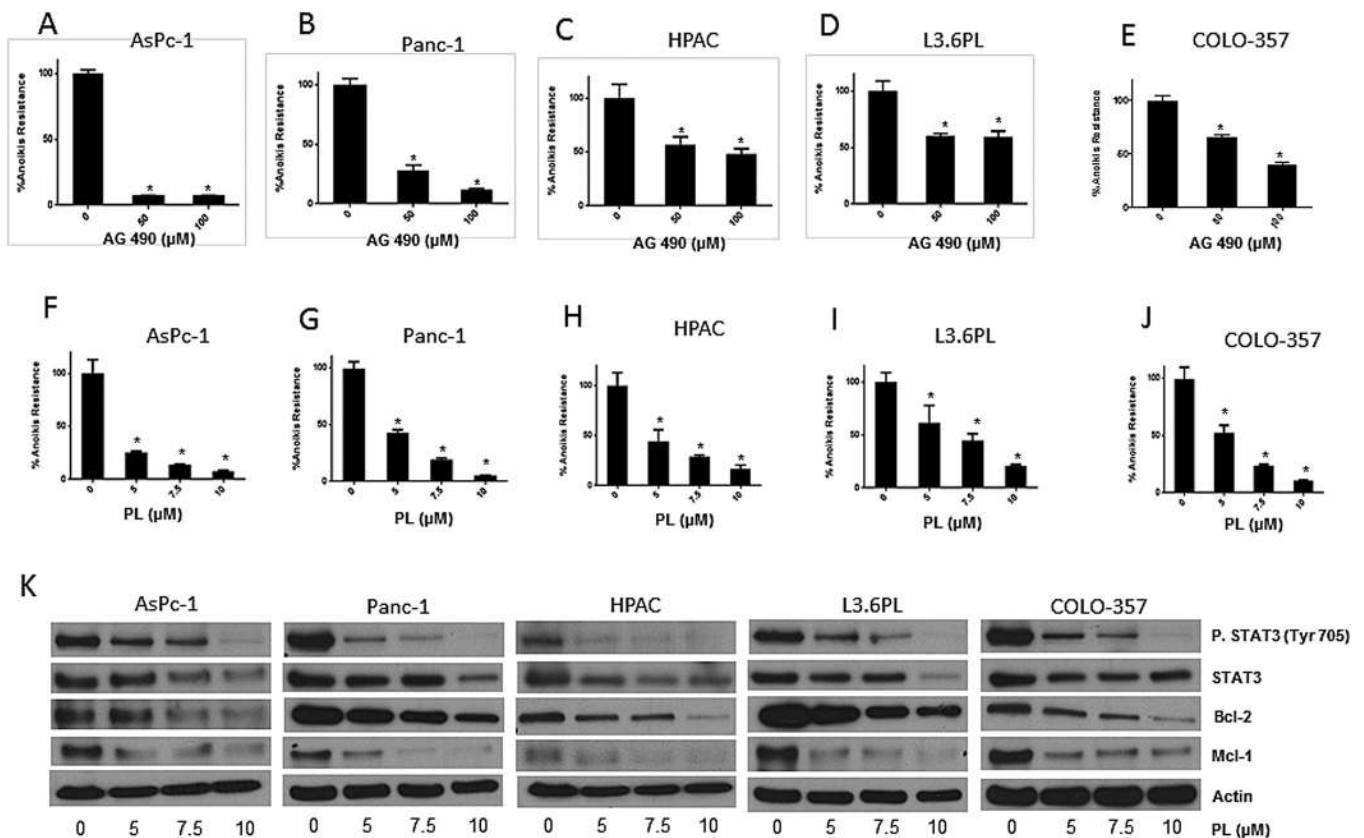


Fig. 3. STAT3 inhibitors overcome anoikis resistance in cancer cells. (A–E) AsPc-1, Panc-1, HPAC, L3.6PL and COLO-375 cells were treated with various concentrations of AG 490 for 48 h under anchorage-independent conditions. Following the treatment, anoikis assay was performed in these cells. Representative bar graphs show the percentage anoikis resistance in different treatment conditions. Values are plotted as mean \pm SD. * $P < 0.05$ compared with control group. (F–J) AsPc-1, Panc-1, HPAC, L3.6PL and COLO-375 cells were treated with various concentrations of PL under anchorage-independent for 48 h. Following the treatment, anoikis assay was performed using these cells. Representative bar graphs show the percentage anoikis resistance in different treatment conditions. Values are plotted as mean \pm SD. * $P < 0.05$ compared with control group. PL induces reverses anoikis resistance by inhibition of STAT3. (K) Blots are representative of pSTAT3 (Y705), STAT3, Bcl-2 and Mcl-1 from lysates of AsPc-1, Panc-1, HPAC, L3.6PL and COLO-375 cells treated with various concentrations of PL under anchorage-independent conditions for 48 h. Actin was used as loading control. The bands shown in the figure are cropped images after the developing the entire membrane. Each experiment was repeated at least three times with similar results.

STAT3 knockdown blocks anoikis resistance in cancer cells

STAT3 was silenced using shRNA in AsPc-1, Panc-1, L3.6PL and COLO-357 pancreatic cancer cells to confirm the role of STAT3 in anoikis resistance. Two STAT3 shRNAs (shRNA1 and shRNA2) with different sequences were used to show target specificity. Using both the shRNAs in all the four cell lines, ~65–75% STAT3 silencing was achieved (Figure 4A). Upon STAT3 silencing, anoikis assay was performed and scrambled shRNA was used as control. We observed remarkable reduction in anoikis resistance as a result of STAT3 silencing (Figure 4B–E). STAT3 silencing decreased anoikis resistance by 70–80% in all the cell lines (Figure 4B–E). In fact, reduction in anoikis resistance highly correlated with the extent of STAT3 silencing. For example, in AsPc-1 cells, ~65% STAT3 silencing was achieved by both the shRNAs, which in turn reduced the anoikis resistance by 80% (Figure 4B). Similar correlation was observed in all the other cell lines. The percent STAT3 silencing was similar in all the cell lines and so was the reduction in anoikis resistance (Figure 4A–E).

Silencing STAT3 diminishes the invasiveness of anoikis-resistant cells

Since our results showed that anoikis resistance was associated with enhanced invasiveness, we performed a Boyden’s chamber assay in four pancreatic cancer cell lines (AsPc-1, Panc-1, L3.6PL and COLO-357) after silencing STAT3. Two different shRNAs were used to silence STAT3 as described above and scrambled shRNA was used as control. There was a remarkable decrease in the rate of invasion upon STAT3 silencing in all the cell lines (Figure 4F–I) and the results correlated with the extent of silencing. For example, in AsPc-1 cells, ~65% STAT3 silencing was achieved by both the shRNAs, which in turn caused 95% decrease in the rate of invasion (Figure 4F). Similar

results were observed in other cell lines. These results established the role of STAT3 in anoikis resistance and invasion in pancreatic cancer cells.

IL-6 pretreatment reverses anoikis sensitization by PL

IL-6 was used to confirm the role of STAT3 in imparting anoikis resistance to pancreatic cancer cells. IL-6 is a cytokine that induces the phosphorylation of STAT3 at Y705 (28). Four pancreatic cancer cell lines (AsPc-1, Panc-1, L3.6PL and COLO-357) were treated with IL-6 1 h prior to PL treatment. In comparison to untreated controls, IL-6 treatment significantly augmented anoikis resistance in all the pancreatic cancer cell lines tested (Figure 5A–D). IL-6 treatment increased the anoikis resistance by 1.4-fold in AsPc-1, 1.6-fold in Panc-1, 1.5-fold in L3.6PL and 2-fold in COLO-357 cells (Figure 5A–D). Interestingly, IL-6 treatment abrogated the reduction in anoikis resistance mediated by PL treatment in all four cell lines (Figure 5A–D). For instance, PL reduced anoikis resistance in AsPc-1 cells by ~50%, however, IL-6 completely blocked PL-mediated reduction in anoikis resistance (Figure 5A). Similar observations were made in Panc-1, L3.6PL and COLO-357 cells (Figure 5A–D).

Overexpression of STAT3 enhances anoikis resistance

We finally tested our hypothesis by evaluating the effect of STAT3 overexpression on anoikis resistance. STAT3 was overexpressed by transfection with STAT3 expressing plasmid in AsPc-1, Panc-1, L3.6PL and COLO-357 pancreatic cancer cell lines. Following transfection, cells were cultured under anchorage-independent condition with or without PL treatment for 48 h and anoikis assay was performed thereafter. Approximately, 3.5- to 6.0-fold increase in the

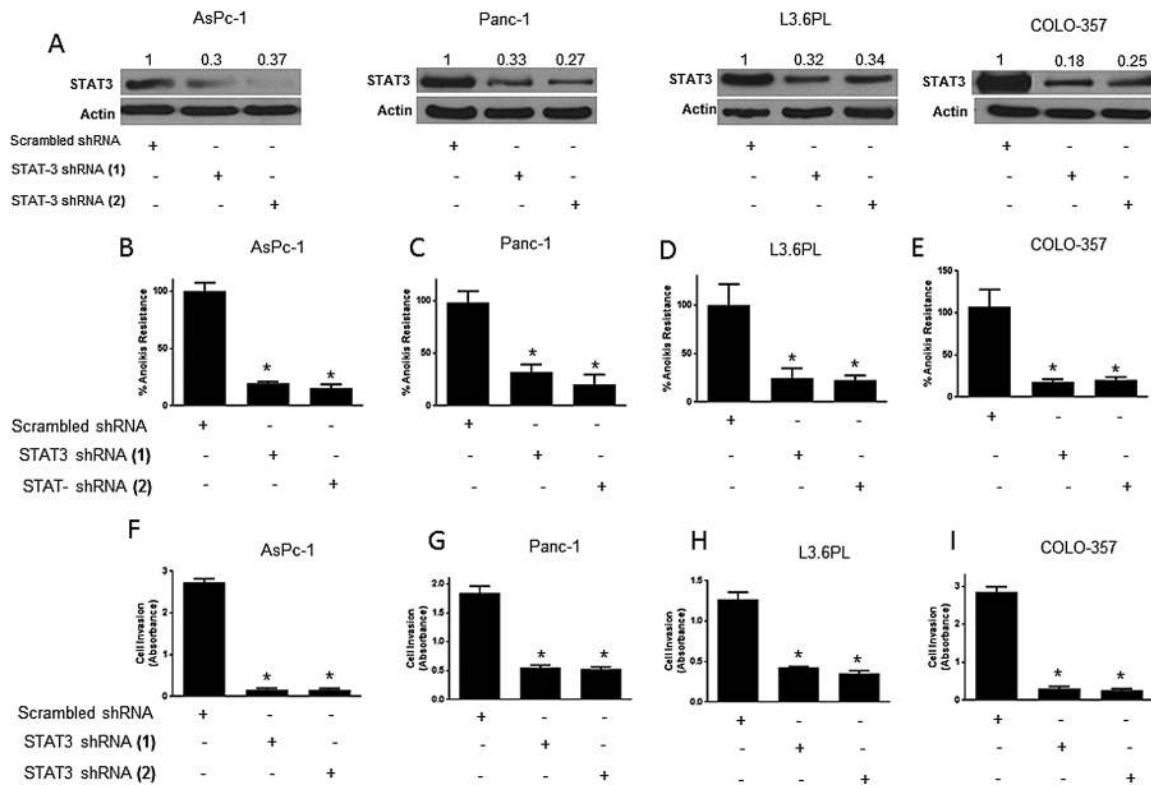


Fig. 4. Pancreatic cancer cells with silenced STAT3 are sensitive to anoikis and lose invasiveness. (A–E) AsPc-1, Panc-1, L3.6PL and COLO-357 cells were transfected with either STAT3 shRNA (1) or shRNA (2) for 12h after which they were cultured in suspension conditions for 48h. Cells transfected with scrambled shRNA and cultured under similar conditions were used as control. Western blot was used to test the extent of STAT3 silencing. The bands shown in the figure are cropped images after developing the entire membrane. Blots were quantitated by densitometric analysis as indicated by numerical values above each band. Values are plotted as mean ± SD. *P < 0.05 compared with control group. (F–I) AsPc-1, Panc-1, L3.6PL and COLO-357 cells were either transfected with STAT3 shRNA (1) or shRNA (2) for 12h after which they were cultured in suspension conditions for 48h. Following the incubation, invasion assay was performed using Boyden’s chamber assay. Cells transfected with scrambled shRNA was used as control. Each experiment was repeated at least three times with similar results. Values are plotted as mean ± SD. *P < 0.05.

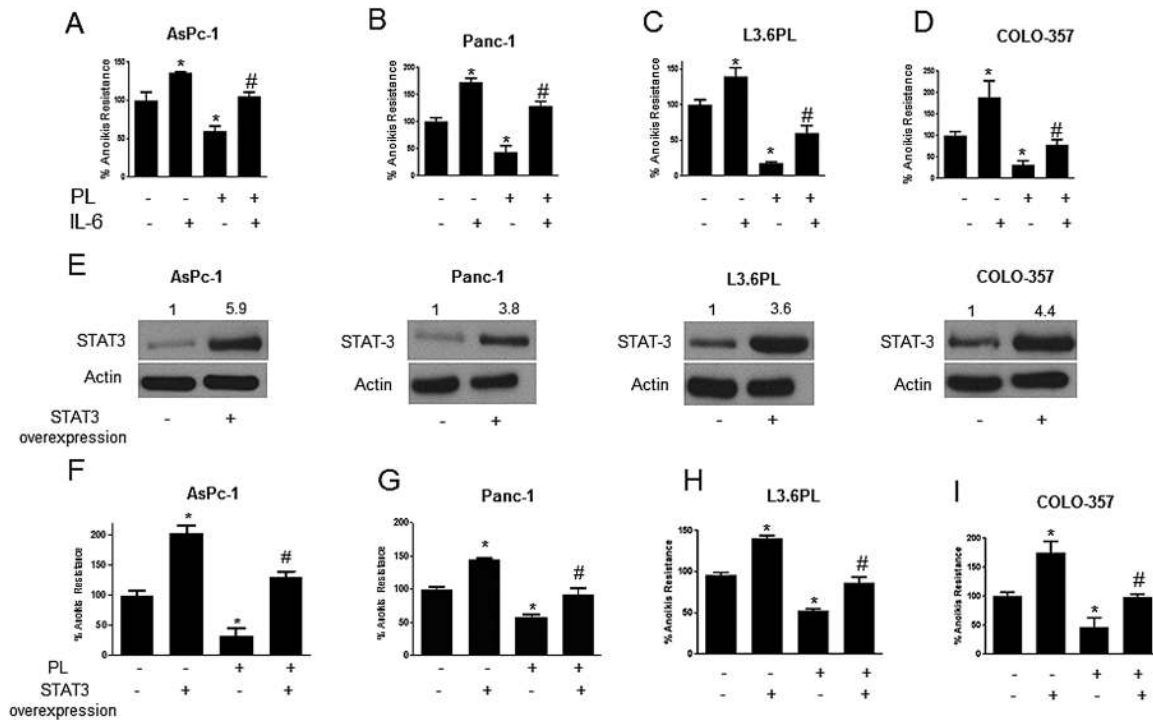


Fig. 5. STAT3 overexpression and IL-6 treatment confers anoikis resistance to pancreatic cancer cells and reverses PL-induced anoikis. (A–D) AsPc-1, Panc-1, L3.6PL and COLO-357 cells were treated with IL-6 alone or in combination with 5 μ M PL for 48 h after which anoikis assay was performed in these cells. Representative bar graph shows percent anoikis resistance of cells exposed to various suspensions under various treatments. Values are plotted as mean \pm SD. * P < 0.05 compared with control group and # P < 0.05 when compared with PL-treated cells. (E–I) AsPc-1, Panc-1, L3.6PL and COLO-357 cells were transfected with STAT3 overexpressing plasmid. Cells were then cultured under anchorage-independent conditions for 48 h with or without PL treatment. After 48 h, anoikis assay was performed on these cells. Representative bar graph shows percent anoikis resistance after various treatments. Values were plotted as mean \pm SD. * P < 0.05 compared with control cells and # P < 0.05 when compared with PL-treated cells. Western blot was used to determine fold increase in STAT3 overexpression prior to the experiment and a representative blot is shown in the figure. The bands shown in the figure are cropped images after developing the entire membrane. Blots were quantitated by densitometric analysis as indicated by numerical values above each band. Each experiment was repeated at least three times with similar results

expression of STAT3 was achieved after transfection (Figure 5E). Our results showed that there was a significant increase in anoikis resistance in AsPc-1 (2-fold), Panc-1 (1.4-fold), L3.6PL (1.4-fold) and COLO-357 (1.9-fold) cells with STAT3 overexpression (Figure 5F–I). The increase in anoikis resistance highly correlated with the extent of STAT3 overexpression. For example, in AsPc-1 cells, there was a 6-fold increase in STAT3 expression, which increased anoikis resistance by 2-fold. In L3.6PL cells, 3.6-fold increase in STAT3 overexpression resulted in 1.4-fold increase in anoikis resistance (Figure 5F and H). STAT3 overexpression also markedly blocked PL-induced anoikis establishing the involvement of STAT3 in PL-mediated inhibition of anoikis resistance (Figure 5F–I). In AsPc-1 cells, 50% anoikis resistance was reduced by PL, but this effect was completely nullified by STAT3 overexpression (Figure 5F). Similar results were obtained by STAT3 overexpression in Panc-1, L3.6PL and COLO-357 cells (Figure 5F–I). These results confirmed the role of STAT3 in anoikis resistance in pancreatic cancer.

Cells with diminished STAT3 expression fail to form tumors in vivo

We further translated our hypothesis using *in vivo* tumor model. Two groups with 10 mice ($n = 10$) in each were taken. About 5×10^6 live anoikis-resistant cells with or without treatment with STAT3 inhibitor (5 μ M PL for 48 h) were injected subcutaneously in both the flanks of SCID/NSG mice to determine the tumorigenic potential. Tumor measurement was taken twice a week using vernier calipers, once each mouse had palpable tumors. At the end of day 32, Panc-1 wild-type anchorage-independent cells significantly formed large tumors with an average volume of $325 \pm 34 \text{ mm}^3$ (Figure 6A). On the other hand, Panc-1 cells treated with PL had an average tumor volume of $12 \pm 3.6 \text{ mm}^3$ (Figure 6A). Moreover, 7 out of 10 mice in PL-treated

group were completely free of tumors, whereas there was not a single mouse in the control group without tumor (Figure 6A). These results further confirmed the role of STAT3 in anoikis resistance and tumorigenicity in pancreatic cancer cells.

Cells with reduced STAT3 expression failed to metastasize in vivo

One of the key characteristics of a metastatic cancer cell is anoikis resistance. To test our hypothesis in metastasis, $\sim 0.8 \times 10^6$ live untreated anchorage-independent control or PL-treated anchorage-independent Panc-1-luc cells were injected intravenously through tail vein in SCID/NSG mice and the animals were imaged twice a week using IVIS Bio Luminescent System equipped with Living Imaging software (Caliper Life Sciences, Hopkinton, MA) to monitor the progression of metastasis. Our results showed that the mice which were injected with untreated anchorage-independent cells had significantly higher levels of metastasis in the lungs and liver as compared to PL treated group, as observed by bioluminescence imaging (Figure 6B; data represented in log scale). At the end of day 35, the average bioluminescence in the suspension group was $2.1 \times 10^{10} \pm 9.3 \times 10^9$ photons/s/mouse, whereas in PL-treated group, bioluminescence was $0.0068 \times 10^{10} \pm 1.521 \times 10^7$ photons/s/mouse (Figure 6B). The basal luminescence without any tumor cell was $\sim 10^5$ photons/s. Six out of 10 mice in PL-treated group had no detectable bioluminescence, suggesting that 60% of mice were free of metastatic tumors (Figure 6B). At the end of the experiment, mice were euthanized and bioluminescence in individual lungs and livers was measured immediately. As shown in Figure 6C and D, lungs and livers from PL-treated mice showed modest or no luminescence, whereas those in the untreated anchorage-independent group demonstrated very strong luminescence. In fact, the entire lungs of the mice from untreated control group were

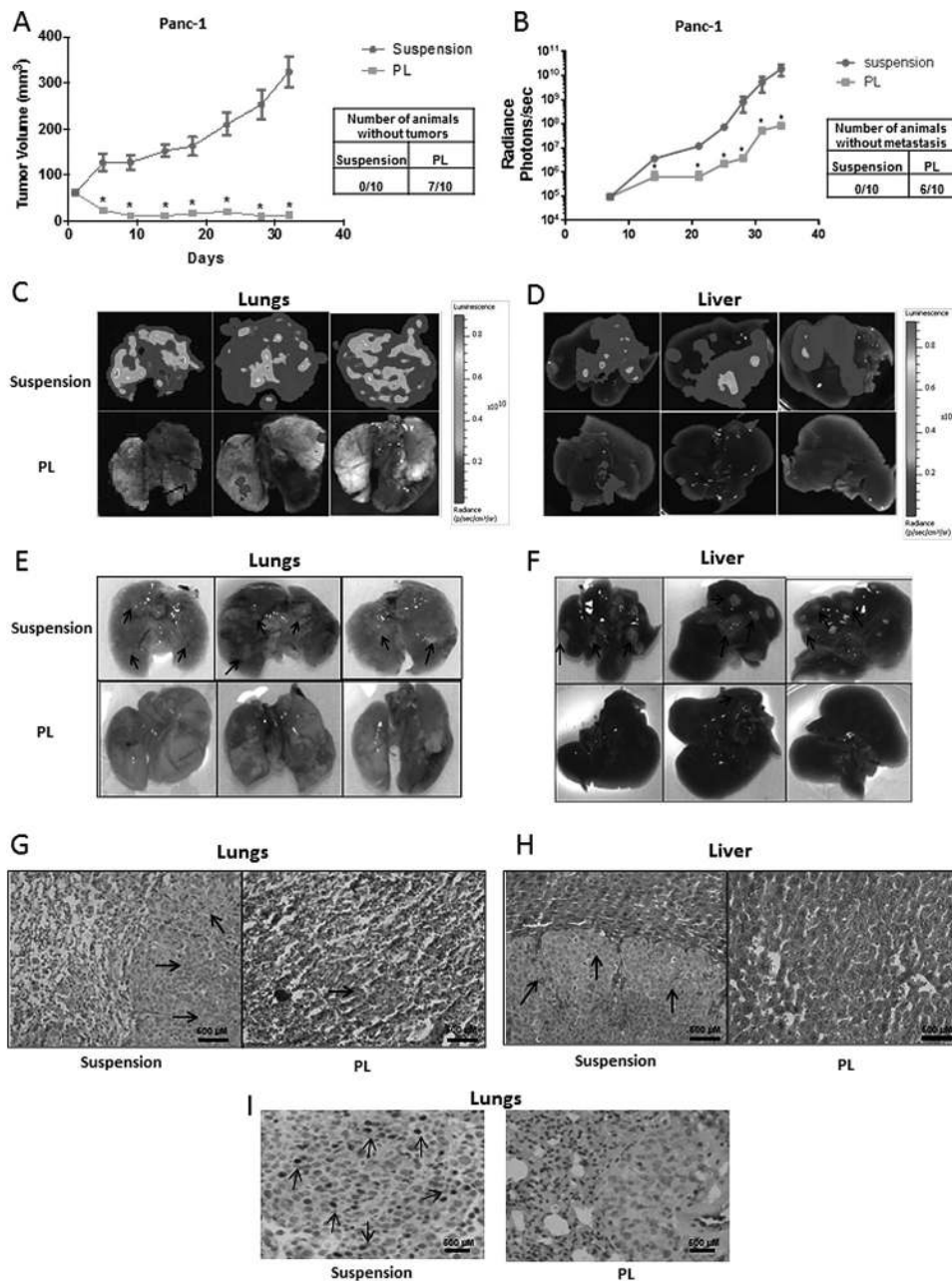


Fig. 6. Cells with diminished STAT3 expression failed to form tumor or metastasize *in vivo*. (A) Tumor volumes of the mice bearing xenografts of wild-type (suspension) and PL-treated (5 μ M) Panc-1 cells cultured in suspension conditions for 48 h. Cell viability of each group was evaluated using trypan blue assay and 5×10^6 viable cells were injected subcutaneously. Tumor dimensions were measured using vernier calipers. Mice were injected with anchorage-independent untreated or PL-treated Panc-1-luc suspension cells intravenously. Tumor luminescence was measured twice a week by IVIS imaging station. At the end of the experiment, mice were euthanized; organs were excised and imaged using camera as well as IVIS imager station. The bioluminescent images of the organs were brought to same scale. (B) Line graph showing the average bioluminescence of the mice from suspension and PL group. (C) Bioluminescent image of lungs and (D) liver of the mice from suspension and PL group. (E) Images of lungs and (F) images of liver of the mice from suspension and PL group. (G and H) are images of hematoxylin and eosin staining of lungs and liver section respective from suspension and PL groups. (I) p-STAT3 (Y705) was analyzed by immunohistochemistry in the lung sections from suspension and PL group. Images were taken at $\times 40$ using Olympus microscope. Values are plotted as mean \pm SEM. * $P < 0.05$ compared with suspension group. Arrows indicate presence of metastatic nodules.

covered with bioluminescence (Figure 6C and D). Furthermore, the organs were also examined carefully for the presence of observable metastatic modules. Numerous metastatic nodules were observed in the lungs (Figure 6E) and livers (Figure 6F) of mice from control group, whereas mice from PL-treated group did not show any visible metastatic nodules (Figure 6E and F). To confirm these observations, lungs and liver were sectioned and stained with hematoxylin and eosin. Upon staining, several metastatic nodules were detected in lungs and liver of control group as illustrated by large and irregular

nuclei (Figure 6G and H). However, in the lungs of mice from the PL-treated group, very few and relatively small metastatic nodules were observed in only 4 out of 10 mice, whereas no metastatic nodules were observed in the liver sections (Figure 6G and H).

Metastatic nodules from untreated group exhibited higher phosphorylation of STAT3

To prove that the reduced metastasis in PL-treated group was due to reduced STAT3, we examined p-STAT3 (Y705) by

immunohistochemistry in the metastatic nodules of both the groups. We observed a significant number of cell population with intense staining for p-STAT3 (Y705) (Figure 6I). Moreover, the staining was highly localized in the nucleus of the tumor cells (Figure 6I). On the other hand, cells of the small metastatic nodules from PL-treated group did not show staining for p-STAT3 (Figure 6I). The entire tissue section of the lung from anchorage-independent control group was a big tumor nodule, whereas the section from PL group showed a metastatic nodule as well as normal host lung tissue (Figure 6I). The cells that exhibited positive staining for p-STAT3 (Y705) were indicated by arrows (Figure 6I). These results clearly confirmed the role of STAT3 in anoikis resistance and metastasis.

Discussion

The take home message of our study is that STAT3 plays a critical role in anoikis resistance and that inhibition of STAT3 leads to sensitization of pancreatic cancer cells to anoikis *in vitro* and *in vivo*. Our study also established that STAT3 mediates anoikis resistance with enhanced cell migration and invasion of cancer cells *in vitro* and high metastatic potential *in vivo*.

According to our observations in all the pancreatic cancer cell lines, a significant percentage of cells were highly resistant to anoikis, whereas a small proportion of the cells were sensitive to anoikis. Since the cells that resist anoikis eventually metastasize, it was very likely that the anoikis-resistant cells would possess higher rate of migration and invasion to facilitate metastasis. This was clearly confirmed by our wound healing and Boyden's chamber assay results.

Earlier studies have shown the involvement of STAT3 in cell growth, survival, differentiation, inflammation, immune system and apoptosis. Recent study from our lab shown the role of STAT3 in anoikis resistance and metastasis in melanoma (39). Few other reports have also shown limited *in vitro* evidence of STAT3 playing a role in anoikis in squamous cell carcinoma and hepatocellular carcinoma (40–42). At the same time, none of the studies have yet shown the role of STAT3 in anoikis resistance and metastasis in pancreatic cancer. Anoikis-resistant pancreatic cancer cells exhibited significantly increased expression and phosphorylation of STAT3 at Y705 along with enhanced expression of Bcl-2 and Mcl-1 as compared to adherent cells. This led to our hypothesis that the activation and overexpression of STAT3 induces anoikis resistance in pancreatic cancer cells and promotes metastasis.

Transient silencing of STAT3 resulted in 70–80% reduction in anoikis resistance. To achieve target specificity, two different shRNAs were used to silence STAT3 and comparable level of STAT3 silencing was achieved in all the cell lines. Interestingly, reduction in anoikis resistance correlated well with the extent of STAT3 silencing indicating that all the metastatic pancreatic cancer cell lines were equally dependent on STAT3 for anoikis resistance. STAT3 silencing also reduced the invasiveness of anoikis-resistant pancreatic cancer cells. The reduction in the rate of invasion also significantly correlated with the extent of STAT3 silencing by the two shRNAs in all the cell lines tested. These results suggest that the effect of STAT3 in anoikis resistance and invasion was comparable in various pancreatic cancer cell lines. On the other hand, increase in anoikis resistance as well as protection from PL-induced anoikis was exhibited by IL-6 treatment and STAT3 overexpression. Inhibition of STAT3 by PL and its involvement in anoikis has not been reported yet. Reversion of PL-mediated anoikis by STAT3 overexpression or IL-6 treatment confirmed the role of STAT3 in anoikis resistance in pancreatic cancer cells. Moreover, there were differences in the genetic profile of the cell lines used in this study. Although all the cell lines harbored mutation in KRAS gene, there were differences in TP53, CDKN2A/p16 and SMAD4 genes. HPAC and COLO-357 had wild-type TP53, whereas Panc-1, AsPc-1 and L3.6PL had mutant TP53. Panc-1 and AsPc-1 had homozygous deletions in CDKN2A gene, HPAC had wild-type CDKN2A, whereas COLO-357 had methylation of p16 promoter region leading to its inactivation. AsPc-1, Panc-1 and HPAC had wild-type SMAD4, whereas COLO-357 and L3.6PL exhibited

homozygous deletion of SMAD4 (43,44). Even though these cell lines had differences in their genetic profiles, their dependence on STAT3 for anoikis resistance was very similar.

Panc-1 cells when treated with PL under anchorage-independent conditions completely failed to form tumors when injected subcutaneously in SCID/NSG mice, whereas wild-type untreated Panc-1 cells cultured under similar conditions formed aggressive tumors. This can be attributed to enhanced expression of STAT3 in these cells. PL-treated cells failed to form tumors due to inhibition of STAT3, which consequently sensitized the cells to anoikis.

It is also important to note that PL-treated Panc-1-luc cells completely failed to metastasize to lungs and liver when injected intravenously in SCID/NSG mice, in contrast to untreated cells, which extensively metastasized to lungs and liver. Once again, this can be attributed to the differential levels of STAT3 in untreated and PL-treated Panc-1-luc cells. Immunohistochemical analysis revealed substantial phosphorylation of STAT3 in the metastatic tumor nodules of the lungs of control mice, whereas modest or no staining for STAT3 was observed in the lungs of PL-treated group. These results clearly indicate the role of STAT3 in anoikis resistance and metastasis in pancreatic cancer cells. Our study is in agreement with earlier reports, which suggests the role of STAT3 in the aggressiveness of various cancers (14,15).

Taken together, our current study established the critical role of STAT3 in conferring resistance to anoikis and promoting metastasis in pancreatic cancer. Therefore, targeting STAT3 as a strategy to prevent or inhibit metastasis in pancreatic cancer patients could be a therapeutic approach in near future and warrants further clinical investigation.

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