UC San Diego

UC San Diego Electronic Theses and Dissertations

Title

The Role of Interleukin-11 in Pancreatic Cancer Progression /

Permalink

https://escholarship.org/uc/item/9nc4z8zm

Author

Miyamoto, Jaclyn Kuniko

Publication Date

2014

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of Interleukin-11 in Pancreatic Cancer Progression

A thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in

Biology

by

Jaclyn Kuniko Miyamoto

Committee in charge:

Professor Andrew Lowy, Chair
Professor Li-Fan Lu, Co-Chair
Professor Dong-Er Zhang

2014

Copyright

Jaclyn Kuniko Miyamoto, 2014

All rights reserved.

The thesis of Jaclyn Kuniko Miyamoto is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2014

Table of Contents

List of Figures

ABSTRACT OF THE THESIS

The Role of Interleukin-11 in Pancreatic Cancer Progression

by

Jaclyn Kuniko Miyamoto

Master of Science in Biology

University of California, San Diego, 2014

Professor Andrew Lowy, Chair

Professor Li-Fan Lu, Co-Chair

Pancreatic cancer patients have a five-year survival rate of approximately 6%, far worse than any other common malignancy. Clearly a greater understanding of the multiple signaling pathways involved in pancreatic cancer progression is needed to develop more effective treatment for this devastating disease. Interleukin-11 (IL-11) is a member of the IL-6 class of cytokines and has been implicated in the development and/or progression of multiple human cancers. IL-11 promotes inflammation involved

in tumor progression, cell migration and proliferation, differentiation of tumor cells in the tumor microenvironment, invasiveness, and survival. However, the role of IL-11 in pancreatic cancer is largely unknown. In this study, we show that the IL-11 ligand is overexpressed in pancreatic cancer. In cultured pancreatic cancer cells, IL-11 signaling activates the Akt, ERK, and Stat3 signaling pathways, yet IL-11 has no effect on cell proliferation or migration. We demonstrate that TGF-β increases IL-11 expression by pancreatic cancer associated fibroblasts (CAFs), an effect mediated by the ERK signaling pathway. Our results thus far show IL-11 activates core oncogenic signaling pathways in pancreatic cancer cells and thus IL-11 signaling may help promote pancreatic cancer progression. Further studies are necessary to better define the oncogenic phenotypes impacted by IL-11 in the pancreatic cancer microenvironment.

I:

Introduction

Pancreatic Cancer

Pancreatic cancer is the fourth leading cause of cancer related deaths in the United States, with a five-year survival rate of 6% (Siegel et al., 2014). Most pancreatic cancers are diagnosed at advanced stage, resulting in poor prognosis. Currently the only cure requires surgical resection, but less than 20% of patients present with localized, operable tumors (Hidalgo, 2010). The current standard of care for advanced pancreatic cancer involves single agent gemcitabine, or multi-agent chemotherapy using gemcitabine or fluorouracil combinations. In locally advanced cancers, chemotherapy is often followed by chemoradiation. Despite some improvements in the activity of chemotherapy regimens, overall survival rates for pancreatic cancer patients have shown little improvement in the past 30 years, indicating the critical need for novel therapies (Siegel et al., 2012).

Pancreatic cancer is marked by the accumulation of numerous gene mutations. Four genes are most commonly mutated in pancreatic cancers, including KRAS2, p16/CDKN2A, TP53, and DPC4/SMAD4 (Maitra et al., 2008). KRAS2 is an oncogene, and is mutated in 90-95% of pancreatic cancers (Caldas et al., 1995). Mutated KRAS results in constitutively active Ras, which activates proliferative and survival pathways. In contrast, CDKN2A, TP53, and DPC4 are tumor suppressor genes. Abnormal inactivation of CDKN2A promotes cell proliferation. TP53 mutation promotes cell survival and cell division. Lastly, DPC4 deletion results in aberrant transforming growth factor-β (TGF-β) signaling. In addition to the genes described, many other genes may be mutated in pancreatic cancer. Because the average

pancreatic cancer has 63 somatic mutations, pancreatic cancer is an extremely complex and heterogeneous disease. A greater understanding of the multiple signaling pathways involved in pancreatic cancer progression is needed to develop more effective treatment.

A hallmark feature of pancreatic cancer is an immense desmoplastic response in the tumor microenvironment. The dynamic stroma is an important component of the tumor microenvironment and surrounds and interacts with the cancer cells. In addition to serving as a mechanical barrier that protects the tumor from chemotherapy, it is intimately involved in tumor formation, progression, invasion, and metastasis (Chu et al., 2007). Components of the tumor stroma include mesenchymal cells, vascular cells, and immune cells. Fibroblasts, which are a type of mesenchymal cell, can be activated to become cancer associated fibroblasts (CAFs). CAFs are a source of cytokines and other soluble factors that contribute to tumor cell growth and proliferation (Bhowmick et al., 2004). The crosstalk between pancreatic ductal adenocarcinoma (PDAC) cells and the stroma contributes to the cancer phenotype and the unique clinical behavior of this disease. Understanding this relationship may allow us to develop better methods for early detection and treatment of pancreatic cancer.

TGF-β is a regulatory cytokine that has a key role in tumor biology. TGF-β normally has tumor suppressive functions, such as inhibition of cell proliferation; other biological functions include extracellular matrix (ECM) remodeling and wound healing, immunosuppression, apoptosis, angiogenesis, differentiation, and embryogenesis (Truty et al., 2007). The TGF-β signaling pathway involves ligands

TGF-β1, TGF-β2, and TGF-β3, and receptors TGF-βRI, TGF-βRII, and TGF-βRIII. TGF-β signals by binding and activating its receptors, which then phosphorylate Smad transcription factors. Smads are translocated to the nucleus where they bind to target genes. Disruption of TGF-β signaling promotes tumor development and progression. Cancer cells acquire the ability to evade host immune defenses, and in turn they can use TGF-β to express genes that promote tumor growth and invasion, evasion of tumor suppression by the immune system, and metastasis (Massague, 2008). In the tumor there are multiple sources of TGF-β, including the cancer cells, stromal cells, and tumor infiltrates such as leukocytes and macrophages. TGF-β is frequently overexpressed in both the serum and tissues of patients with pancreatic cancer (Bellone et al., 2006).

IL-11 AND IL-11Rα

Interleukin-11 is a cytokine that was first cloned based on its similarity to IL-6 bioactivity (Du et al., 1997). The human IL-11 protein is composed of 178 amino acids and has a molecular mass of 19kDa (Schwertschlag et al., 1999). IL-11 gene expression is observed in multiple tissues, including CNS, thymus, lung, bone, connective tissues, uterus, skin, and testes (Du et al., 1997). A pleiotropic cytokine, IL-11 acts on many tissues and cell types. The hematopoietic effects of IL-11 include proliferation of primitive stem cells, hematopoiesis, megakaryocytopoiesis, and thrombopoiesis. Additionally, IL-11 has many non-hematopoietic effects including mediating pulmonary inflammation, regulating growth of gastrointestinal epithelial cells, and regulating bone metabolism (Du et al., 1997).

IL-11 is a member of the IL-6 class of cytokines and has been implicated in the development of gastric (Nakayama et al., 2007), colorectal (Calon et al., 2012), prostate (Zurita et al., 2004), breast (McCoy et al., 2013), gastrointestinal (Putoczki et al., 2013), and endometrial (Lay et al., 2012) carcinomas. The IL-6 family of cytokines signal by binding to their specific receptors, and subsequently coupling with the common signaling receptor subunit glycoprotein 130 (gp130). This begins a signaling cascade that may activate signaling from the JAK/Stat3, PI3K/mTOR, and/or the Ras/ERK pathways. Stat3 is a transcription factor that is associated with cancer-promoting inflammatory responses. Activated Stat3 promotes survival, proliferation, and angiogenesis. Unregulated Stat3 activation is highly associated with human epithelial cancers, and has been correlated with poor outcomes. PI3Ks are a lipid kinase family, which upon activation, result in Akt translocation to the inner membrane and activation of target genes that promote cell survival, cell cycle progression, and cellular growth. Furthermore, the PI3K pathway is commonly altered in human cancers, and promotes tumor development and resistance to cancer treatment. Lastly, the ERK pathway is a kinase cascade that regulates cell cycle progression and promotes cell survival. This cascade regulates many proteins that are involved in resistance to apoptosis. Mutations in this pathway are frequently observed in many human cancers, and aberrant activation is associated with poor prognosis.

In cancer, IL-11 has been shown to promote inflammation involved in tumor progression (Putoczki et al., 2010), cancer cell migration and proliferation (Lay et al.,

2012), differentiation of progenitor cells in the tumor microenvironment (McCoy et al., 2013), invasiveness (Shin et al., 2012), and survival (Kiessling et al., 2004). In colorectal carcinoma, elevated IL-11Rα expression is associated with increased tumor invasion and lymphatic infiltration (Yoshizaki et al., 2006). A recent study in gastrointestinal cancer demonstrated that inhibition of IL-11 signaling reduces Stat3 activation, tumor cell proliferation, and invasiveness (Putoczki et al., 2013). Therefore IL-11 represents a rationale targeted for therapy in pancreatic and other cancers that are associated with Stat3 induced inflammation.

The role of IL-11 in pancreatic cancer is largely unknown. A study by Bellone et al. evaluated expression profiles of pro-inflammatory and anti-inflammatory cytokines using pancreatic carcinoma cell lines and specimens from 65 patients. They found that IL-11 mRNA was overexpressed in tumor versus non-tumor tissues (Bellone et al., 2006). Moreover, they observed that increased IL-11 expression was associated with later stage disease, however there was no data on IL-11 protein expression and no exploration of IL-11 function was undertaken. Thus a greater understanding of both IL-11 expression and signaling is needed as are studies that begin to dissect the ways in which IL-11 may affect tumor progression. Such knowledge may enable us to evaluate the potential for IL-11 directed therapy in pancreatic cancer.

II:

Results

IL-11 is overexpressed in pancreatic cancer

To evaluate IL-11 protein expression in pancreatic cancer, we initially evaluated six human pancreatic ductal adenocarcinoma (PDAC) cell lines and three cancer associated fibroblast (CAF) lines using an ELISA assay. We found that IL-11 was expressed and secreted in both the epithelial and fibroblast cell lines in the range of 10-40 pg/ml. FG and CAF 1444 cells expressed higher levels of IL-11, at 80-90 pg/ml and 1400 pg/ml, respectively. Because IL-11 expression may be induced by cellular stress factors, we compared expression levels 24 hours and 72 hours after the cells reached 100% confluence. Concordant with this we observed increases in IL-11 expression in the range of 9.9% to 75.6% at 72 hours compared to 24 hours for all cell lines used.

To evaluate IL-11 expression in human tissue, we compared normal human pancreas to primary human pancreas cancers by immunohistochemistry. We found that IL-11 is not expressed in normal tissues. In contrast, IL-11 is highly expressed in tumors, predominantly in the stroma. Additionally we observed that IL-11 is expressed in some epithelial cells in the lining of malignant ducts. Collectively, these observations suggest that IL-11 is overexpressed in pancreatic cancer as compared to normal pancreas.

IL-11Rα is expressed in pancreatic cancer

To determine if pancreatic cancer cell lines express the IL-11 specific receptor IL-11Rα, we evaluated six human PDAC lines and four human CAF lines by Western blot. IL-11Rα is expressed in all cell lines.

We then examined human tissue to compare IL-11Rα expression in normal pancreas and in primary pancreatic cancers. IL-11Rα is expressed in both normal pancreas and in primary tumors. We observed localization of IL-11Rα expression to the cytoplasm of epithelial cells in both normal and tumor tissue. In sum, these results demonstrate that while the IL-11Rα is expressed in the epithelial cells of both normal pancreas and pancreatic cancer, expression of the IL-11 ligand is uniquely expressed at high levels within the tumor microenvironment.

IL-11 signaling activates the Akt, ERK, and Stat3 pathways

To evaluate the consequences of IL-11 secretion in pancreatic cancer, we stimulated pancreatic cancer cell lines with recombinant human IL-11 (rhIL-11) and performed Western blots to evaluate activation of the Akt, ERK, and Stat3 signaling pathways. The cell lines examined included AsPC-1, BxPC3, FG, MIAPaCa-2, CAF 1299, CAF 1316, and CAF 1444.

We observed constitutively active Akt in BxPC3, FG, and CAF 1316 cells, and in these cell lines, IL-11 stimulation results in further increased levels of pAkt. IL-11 stimulation activates Akt in CAF 1299 and CAF 1444 cells. We conclude that IL-11 stimulation activates Akt in both pancreatic cancer cells and CAFs.

Six out of seven cell lines examined exhibit constitutively active ERK. In BxPC3, FG, MIAPaCa, and CAF 1444 cells, IL-11 stimulation activates ERK signaling. In contrast, IL-11 had no effect on ERK signaling in AsPC-1, CAF 1299, and CAF 1316 cells. In CAF 1444 cells there is no basal ERK signaling, and ERK is activated by IL-11 stimulation. From our data we conclude that IL-11 stimulation can

activate ERK in some pancreatic cancer cells and CAFs, and the level of activation is dependent on the cell line being examined.

We observed constitutive Stat3 activation in five of seven cell lines examined. IL-11 stimulation activates the Stat3 pathway in AsPC-1, CAF 1299, and CAF 1316 cells. In contrast, Stat3 activation is reduced with IL-11 stimulation in MIAPaCa and CAF 1444 cells. We conclude that IL-11 stimulation likely has a context-specific effect on Stat3 activation, whereby IL-11 may result in either an increase or a decrease in the level of Stat3 activation.

Overall, we show that IL-11 stimulation results in differential pathway activation, which is cell-line specific, but has the most uniform effects on Akt signaling. Furthermore, PDAC cells and CAF cells have similar responses to IL-11 stimulation with respect to the signaling pathways described.

IL-11 signaling has no effect on proliferation in pancreatic cancer

To evaluate the effects of IL-11 on pancreatic cancer cell proliferation, we performed an Alamar Blue assay on five PDAC cell lines. We stimulated AsPC-1, BxPC3, Capan2, FG, and MIAPaCa cells with rhIL-11 for 24 hours, 48 hours, and 72 hours. We observed no effect of IL-11 stimulation on pancreatic cancer cell proliferation.

Additionally, four human CAF cell lines were stimulated with rhIL-11 and an MTT cell viability assay was performed to evaluate the effects of IL-11 in CAF 1299, CAF 1316, CAF 1424, and CAF 1444 cells. We observed no differences in response to IL-11 stimulation in all cell lines evaluated.

Our initial ELISA assay revealed significant IL-11 overexpression at 1400 pg/ml in CAF 1444 cells. We hypothesized that at these levels, IL-11Rα is saturated by IL-11 ligand and may enhance proliferation on the basis of autocrine signaling. To evaluate the validity of our hypothesis, we used an IL-11 neutralizing antibody to block IL-11 signaling in CAF 1444 cells. After 24 hours, 48 hours, and 72 hours of treatment, we saw no difference in cell proliferation using the MTT cell viability assay. We conclude that inhibition of IL-11 signaling in CAF 1444 cells does not affect cell proliferation.

IL-11 signaling has no effect on migration in pancreatic cancer

The effect of IL-11 stimulation on cell migration in pancreatic cancer is not known. To evaluate if IL-11 promotes migration, we stimulated BxPC3, FG, and MIAPaCa cells with rhIL-11 and performed a scratch wound healing assay. We observed no significant differences in percent wound closure between untreated and IL-11 treated cells. We conclude from our results that IL-11 has no effect on cell migration in the three cell lines studied.

IL-11 is upregulated by TGF-β in pancreatic fibroblast cells

TGF-β upregulates IL-11 gene expression in colon-derived fibroblasts (Calon et al., 2012). To explore the mechanism regulating IL-11 expression in pancreatic cancer, we examined the effects of TGF-β stimulation on IL-11 expression in pancreatic CAF cells. We stimulated CAF 1299 and CAF 1444 cells with recombinant human TGF-β and then evaluated IL-11 expression using an ELISA assay. Following

exposure to TGF-β, we observed a four-fold increase in IL-11 expression in CAF 1299 cells, and a ten-fold increase in IL-11 expression in CAF 1444 cells.

We confirmed the contribution of TGF-β stimulation to increased IL-11 expression by treating CAF 1444 cells with TGF-β inhibitor LY2157299. Stimulation with TGF-β resulted in increased IL-11 expression, consistent with our previous data. Simultaneous treatment with TGF-β + LY2157299 resulted in no change in IL-11 expression compared to control. From these studies we conclude that TGF-β stimulation increases IL-11 expression in pancreatic CAF cells.

To determine the mechanism by which TGF-β mediates increased IL-11 expression, we treated CAF 1316 and CAF 1444 cells with the MEK/ERK inhibitor UO126 or the PI3K/Akt inhibitor LY294002. As we have previously shown, TGF-β stimulation results in increased IL-11 expression, and LY2157299 inhibits TGF-β induced IL-11 expression. In CAF 1316 and CAF 1444 cells, treatment with UO126 + TGF-β resulted in decreased IL-11 expression compared to control; the same effect was observed with UO126 inhibitor alone. Because inhibiting ERK signaling reduces IL-11 expression, we conclude that basal ERK signaling mediates IL-11 expression. When cells were treated with PI3K inhibitor LY294002 + TGF-β, we observed an increase in IL-11 expression compared to control. Treatment with LY294002 inhibitor alone resulted in no change in IL-11 expression compared to control in CAF 1316 cells, and increased IL-11 expression in CAF 1444 cells. Our results indicate that TGF-β stimulation mediates IL-11 expression by activation of the ERK signaling pathway.

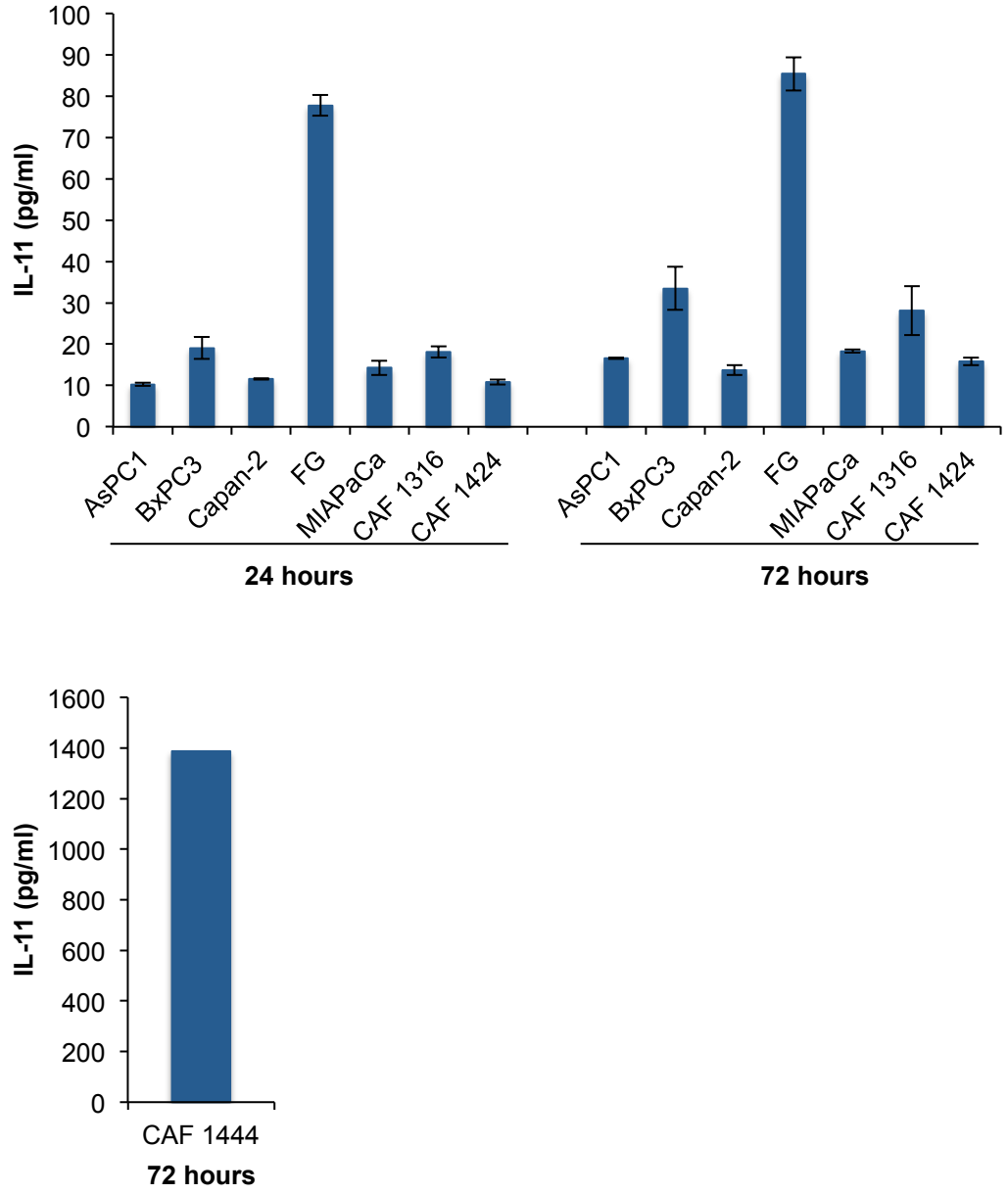


Figure 1. IL-11 expression in human PDAC and CAF cells
ELISA assay for IL-11 expression in human PDAC and pancreatic CAF cells 24 hours and 48 hours after reaching 100% confluence. Values are mean protein concentration ± SD (n=3).

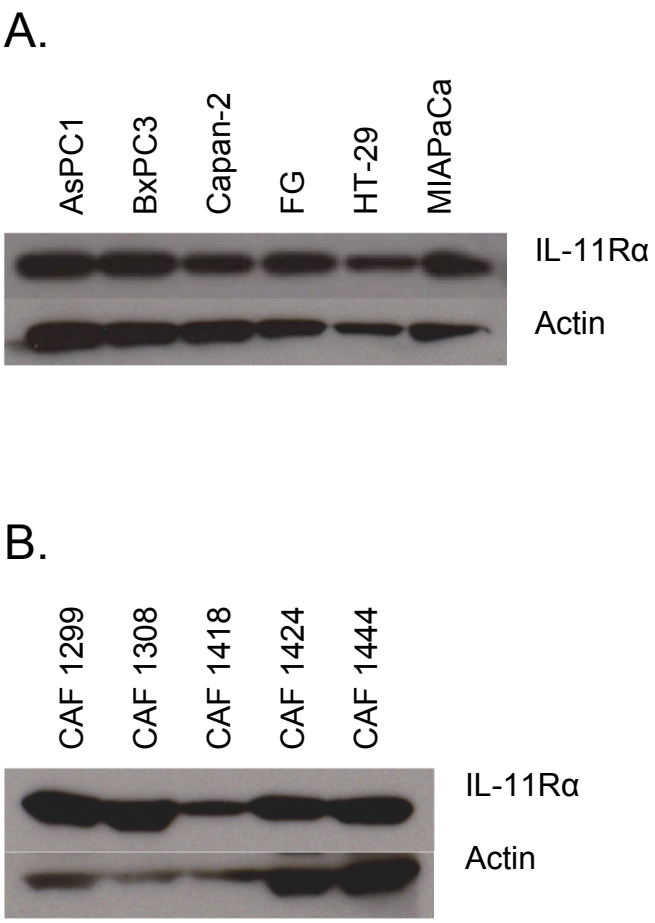


Figure 2. IL-11Rα expression in human PDAC and CAF cells
Western blot analysis for IL-11Rα in human PDAC (A) and CAF (B) cells.

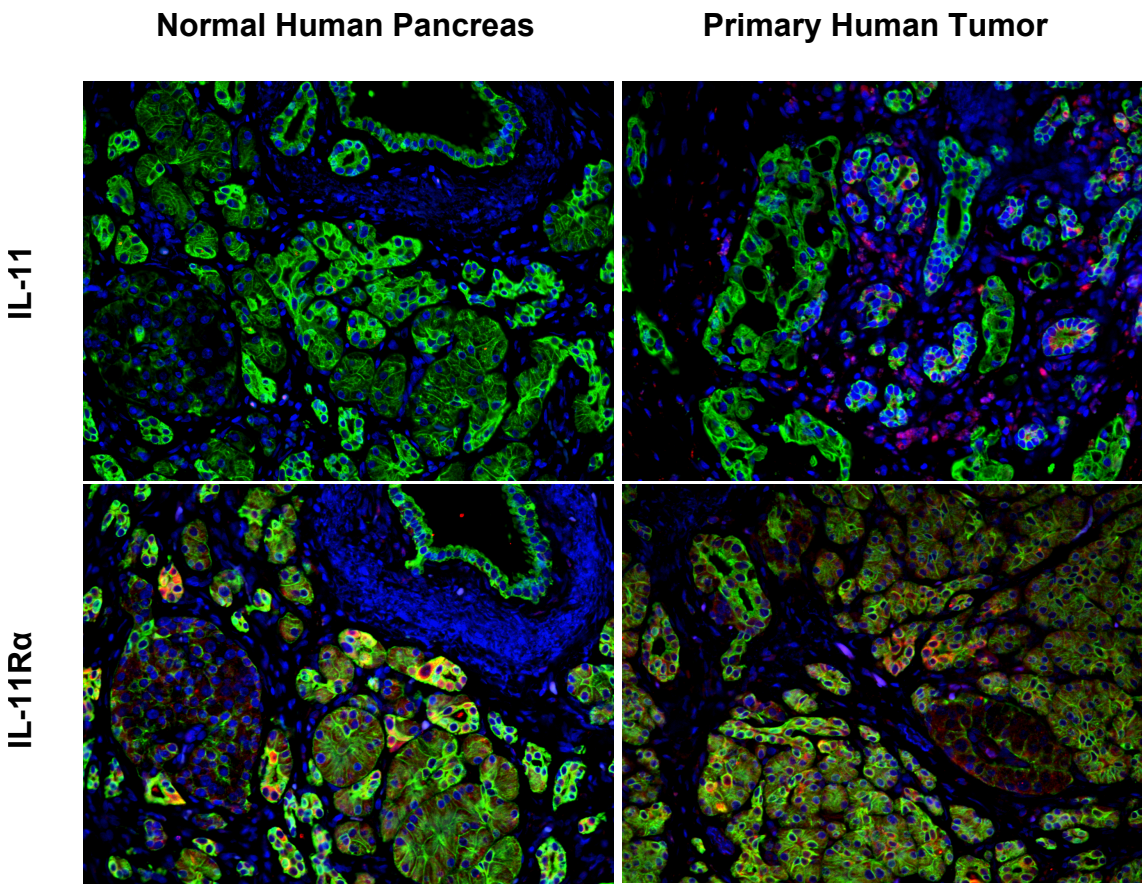


Figure 3. IL-11 expression in human pancreatic cancer tumors
Immunofluorescence detection of IL-11 (red) or IL-11Rα (red) in normal human pancreas and in primary human tumor. Epithelial cells (green) labeled with pan-keratin and nuclei (blue) labeled with DAPI.

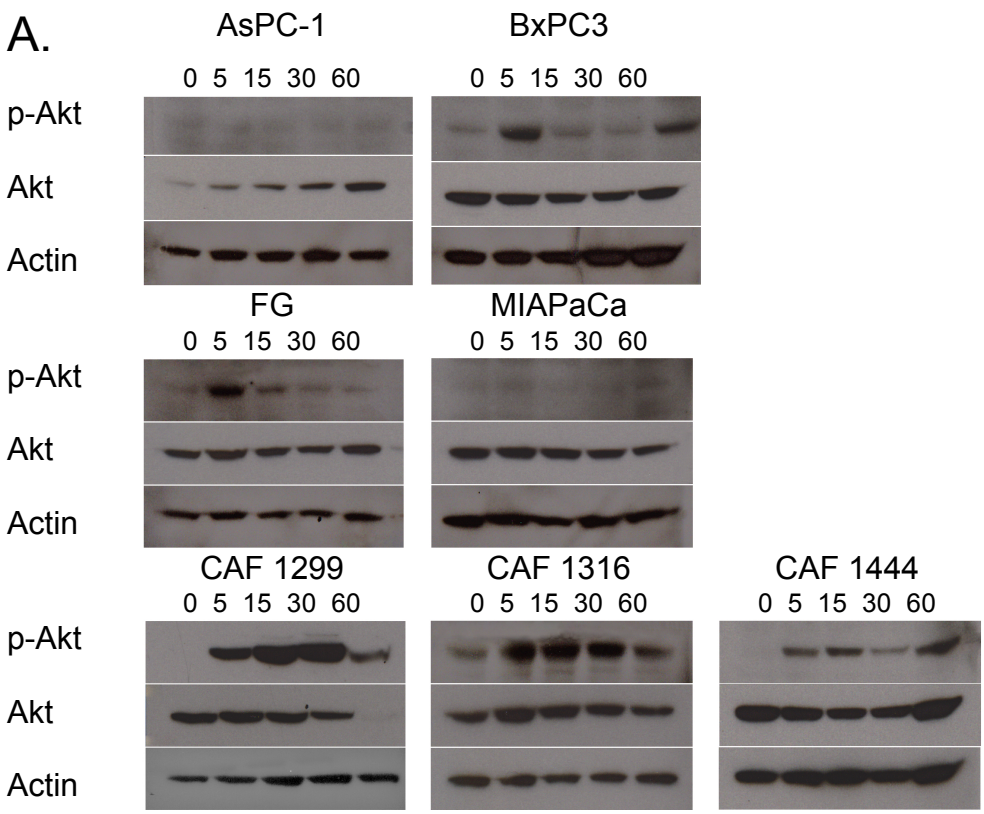


Figure 4. IL-11 stimulation activates Akt, ERK, and Stat3 signaling pathways
A. Activated Akt was detected by Western blot with an antibody specific to phosphorylated Akt (p-Akt) at serine residue 473. To confirm equal protein loading, each blot was reprobed with anti-Akt Ab (Akt) and anti-β-Actin (β-Actin). Time-dependent effects on the phosphorylation of Akt induced by IL-11 (50 ng/mL) stimulation for 5-60 min in PDAC and CAF cells are shown.
B. Activated ERK was detected by Western blotting with an antibody specific to phosphorylated p44/42 MAPK (p-ERK1/2) at threonine residue 202 and tyrosine residue 204. To confirm equal protein loading, each blot was reprobed with anti-ERK Ab (ERK) and anti-β-Actin (β-Actin). Time-dependent effects on the phosphorylation of ERK induced by IL-11 (50 ng/mL) stimulation for 5-60 min in PDAC and CAF cells are shown.
C. Activated Stat3 was detected by Western blotting with an antibody specific to phosphorylated Stat3 (Stat3) at tyrisine residue 705. To confirm equal protein loading, each blot was reprobed with anti-Stat3 Ab (Stat) and anti-β-Actin (β-Actin). Time-dependent effects on the phosphorylation of Stat3 induced by IL-11 (50 ng/mL) stimulation for 5-60 min in PDAC and CAF cells are shown.

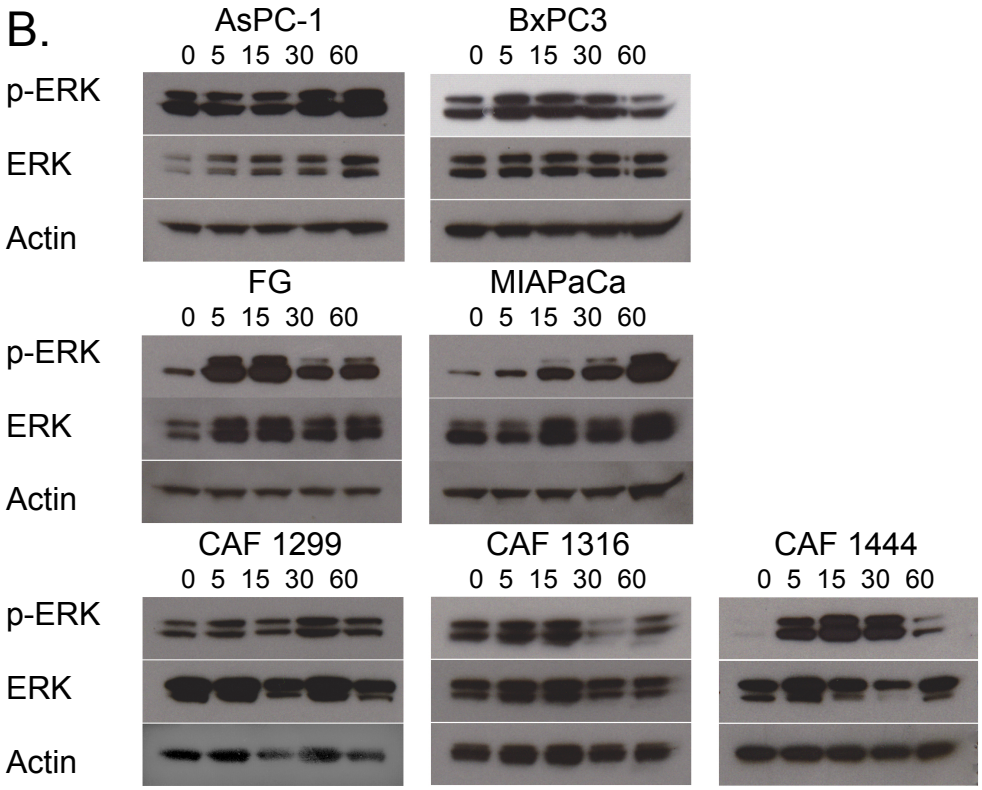


Figure 4. continued

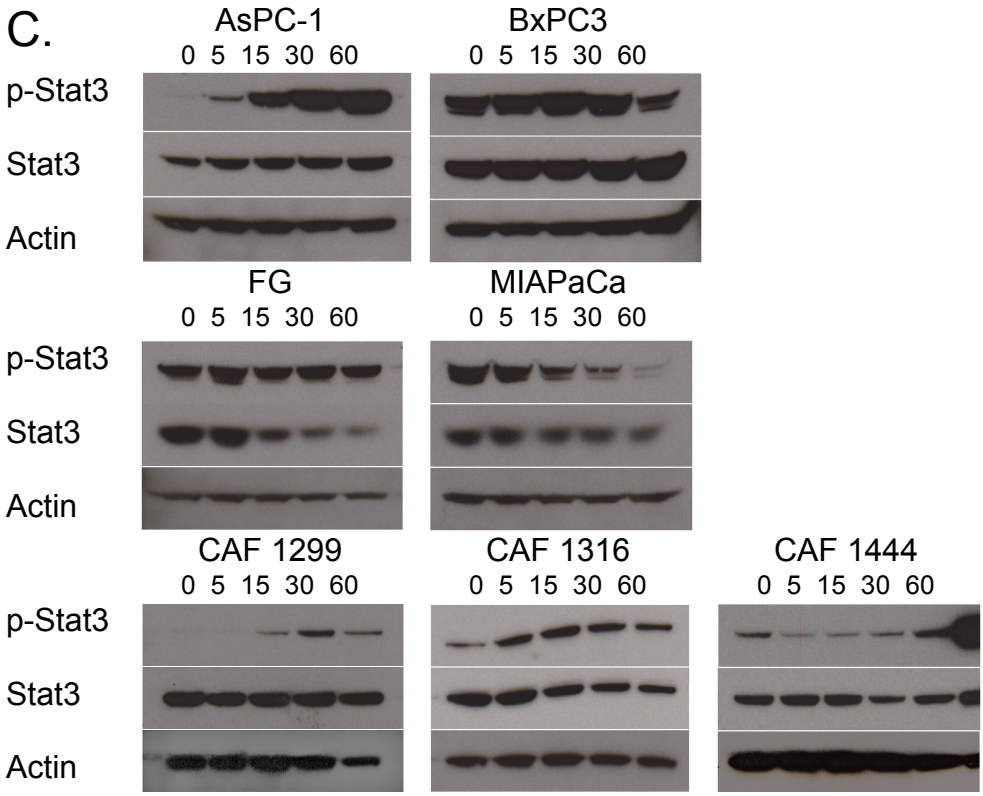


Figure 4. continued

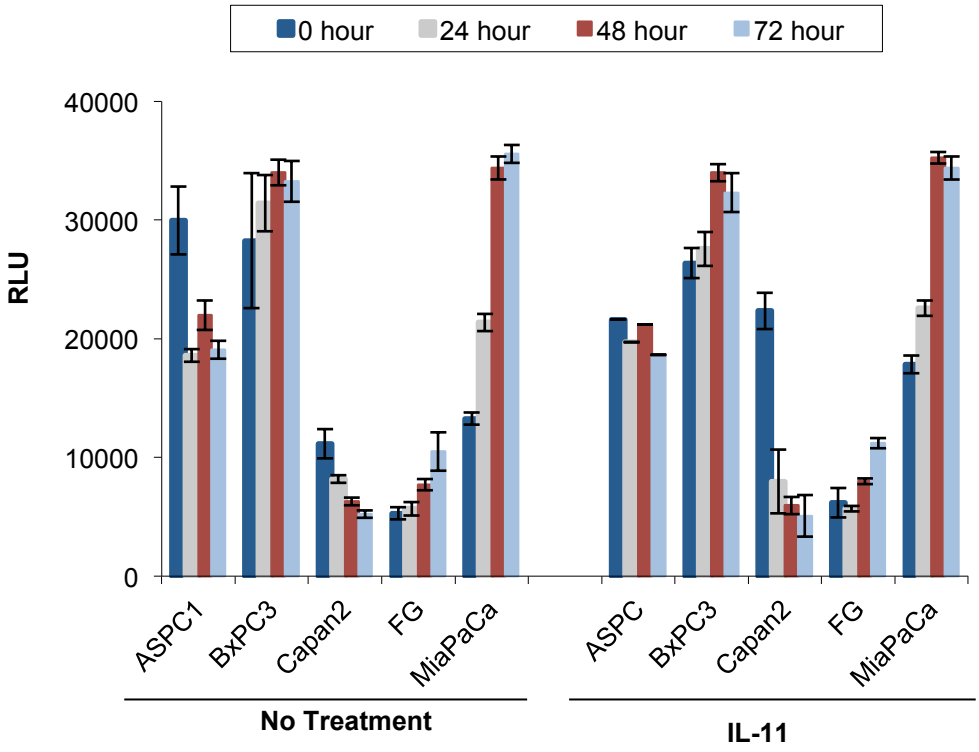
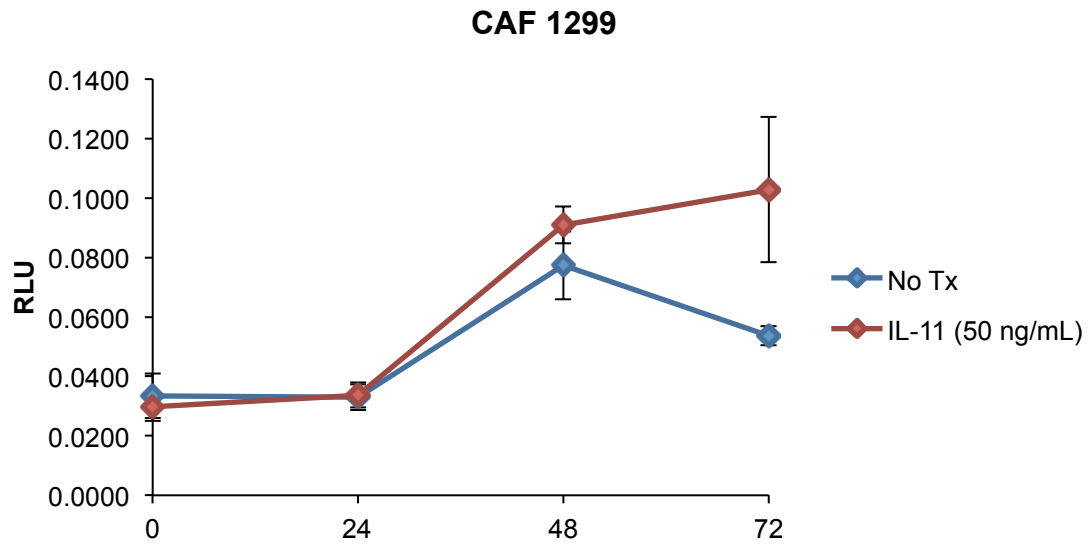


Figure 5. Effect of IL-11 stimulation on cell proliferation in human PDAC cells
Cell proliferation was observed using Alamar blue proliferation assay. PDAC cells were treated with IL-11 (50 ng/mL) for 24 hours, 48 hours, and 72 hours. Values are mean relative absorbance (RLU) over time ± SD (n=3)

A.



B.

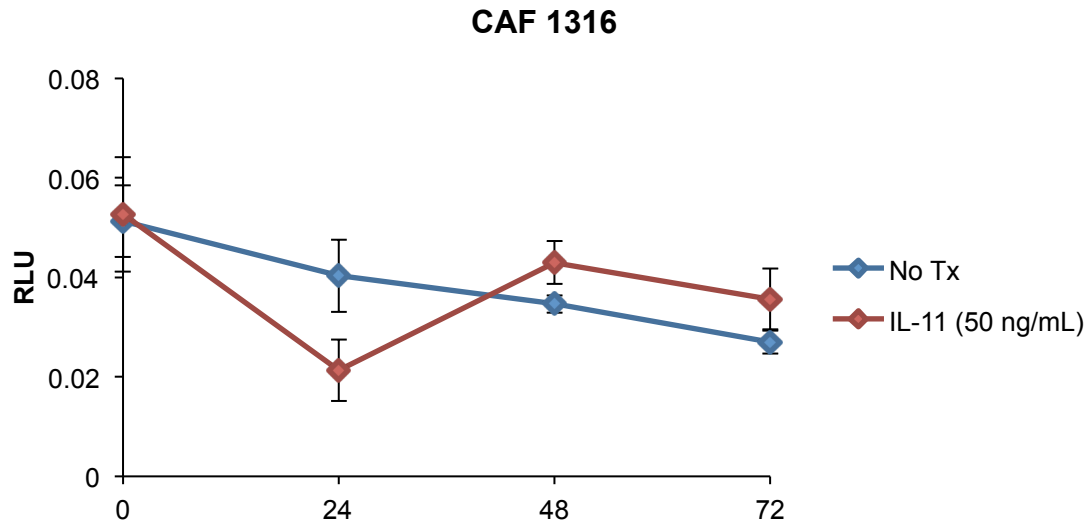
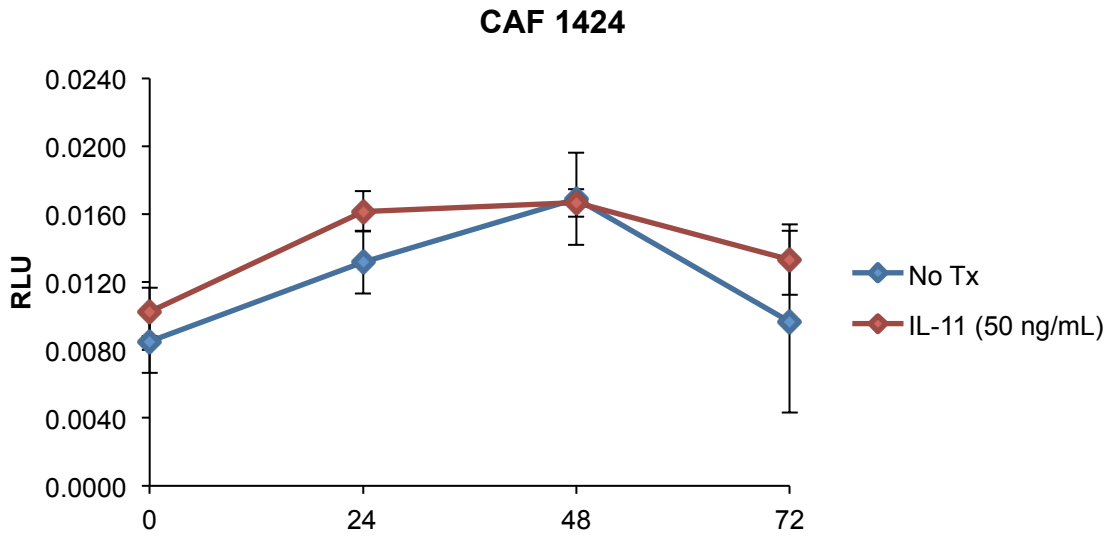


Figure 6. Effect of IL-11 stimulation on cell proliferation in human pancreatic CAF cells

Cell proliferation was observed using MTT proliferation assay. CAF 1299 (A), CAF 1316 (B), CAF 1424 (C), and CAF 1444 (D) cells were treated with IL-11 (50 ng/mL) for 24 hours, 48 hours, and 72 hours. Values are mean relative absorbance (RLU) over time ± SD (n=3).

C.



D.

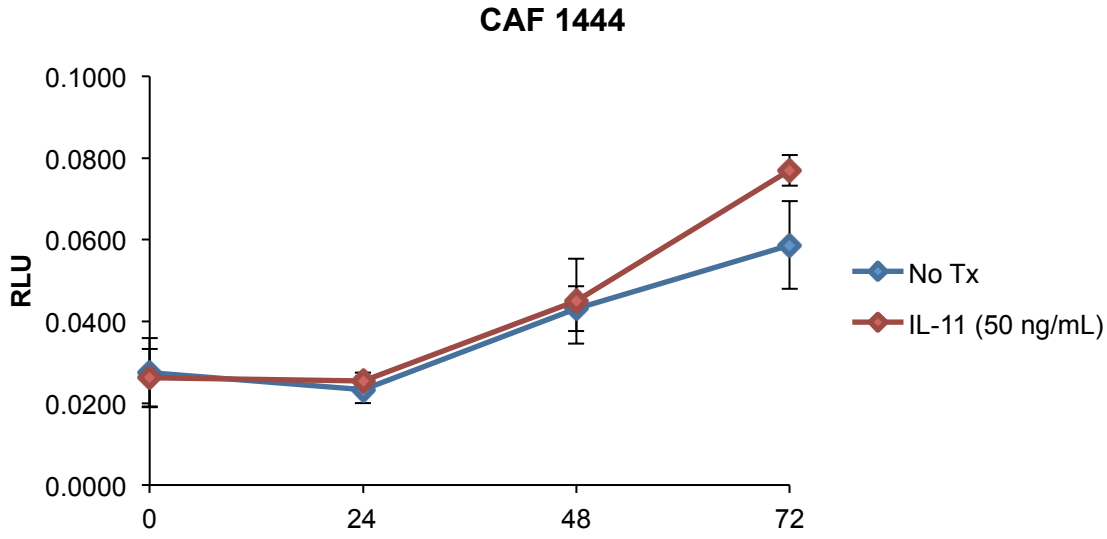


Figure 6. continued

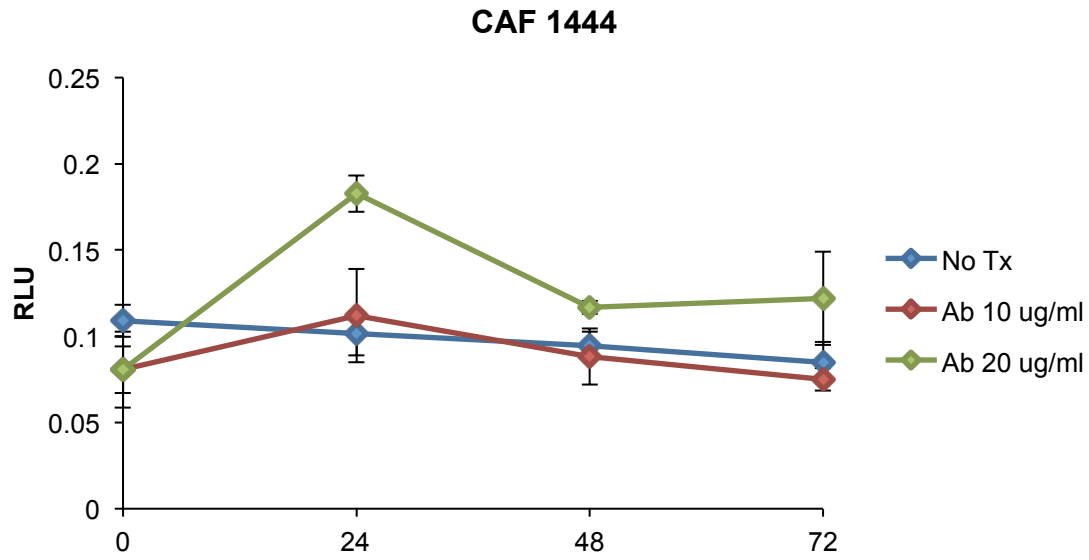


Figure 7. Effect of blocking IL-11 signaling on cell proliferation in IL-11 overexpressing CAF 1444 cells
CAF 1444 cells were treated with IL-11 neutralizing antibody (10 ug/mL or 20 ug/mL) for 24 hours, 48 hours, and 72 hours. Cell proliferation was observed using MTT proliferation assay. Values are mean relative absorbance (RLU) over time ± SD (n=3).

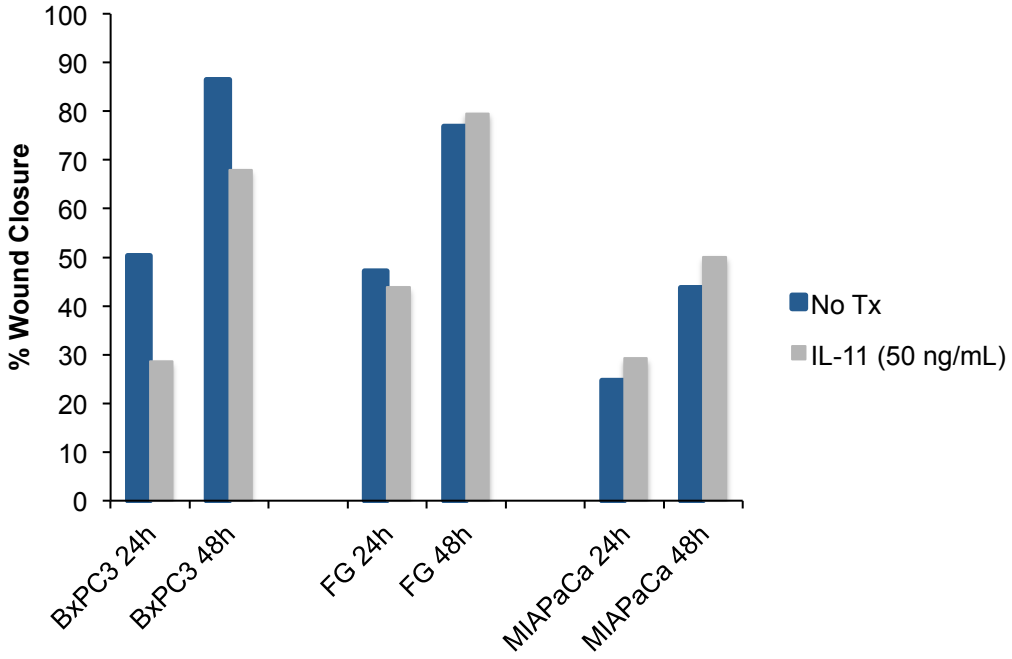


Figure 8. Effect of IL-11 stimulation on cell migration in pancreatic cancer cells
A scratch wound healing assay was used to evaluate the effects of IL-11 stimulation on cell migration in BxPC3, FG, and MIAPaCa cells. The distance covered by cells in the initial scratch area was measured and plotted in %.

A.

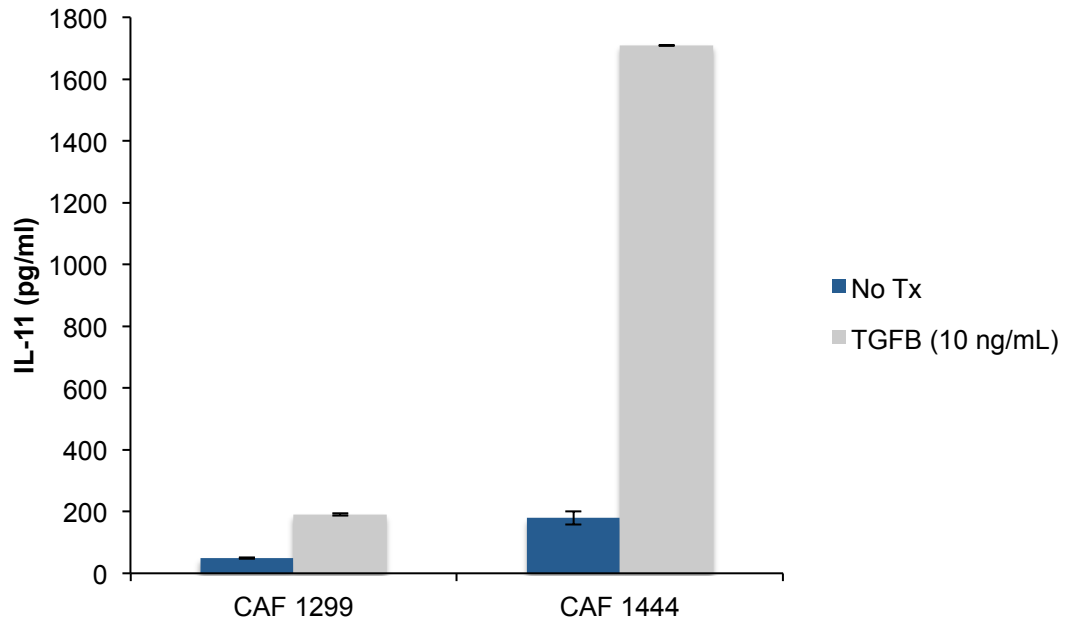
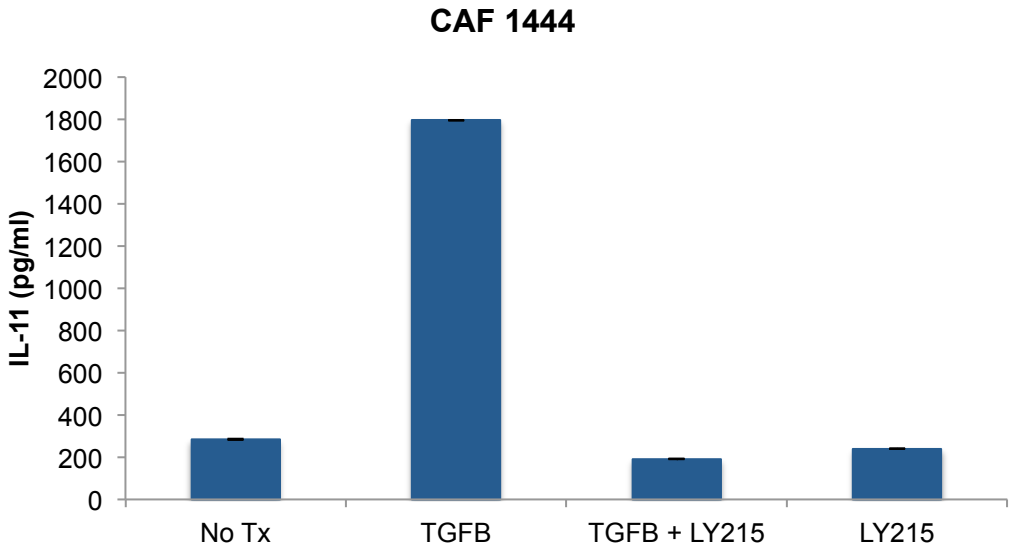


Figure 9. Effect of TGF-β stimulation on IL-11 expression in CAF cells
A. CAF cells were incubated in medium alone (control) or in medium containing TGF-β (10 ng/mL). Conditioned media was collected after 24 hours and ELISA assay was used to determine IL-11 expression. Values are mean protein concentration ± SD (n=3).
B. CAF 1444 cells were stimulated with TGF-β (10 ng/mL) or TGF-β inhibitor LY2157299 (10 μM). IL-11 expression was determined by ELISA assay.
C, D. CAF 1444 (C) and CAF 1316 (D) cells were stimulated with TGF-β (10 ng/mL), TGF-β inhibitor LY2157299 (10 μM), ERK inhibitor UO126 (10μM), or Akt inhibitor LY294002 (20μM). IL-11 expression was determined by ELISA assay. Values are mean protein concentration ± SD (n=3).

B.



C.

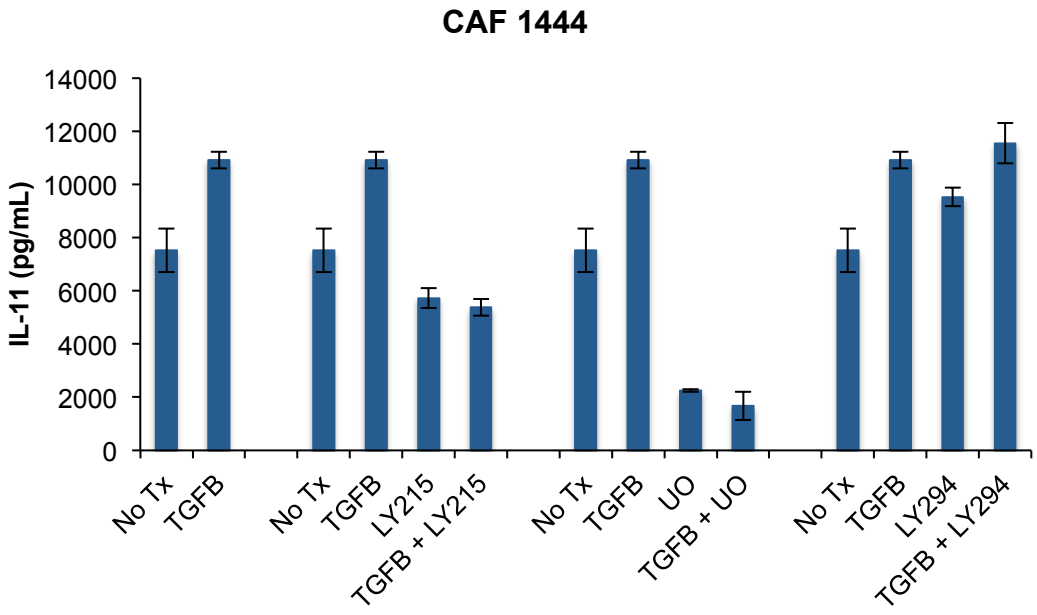


Figure 9. continued

D.

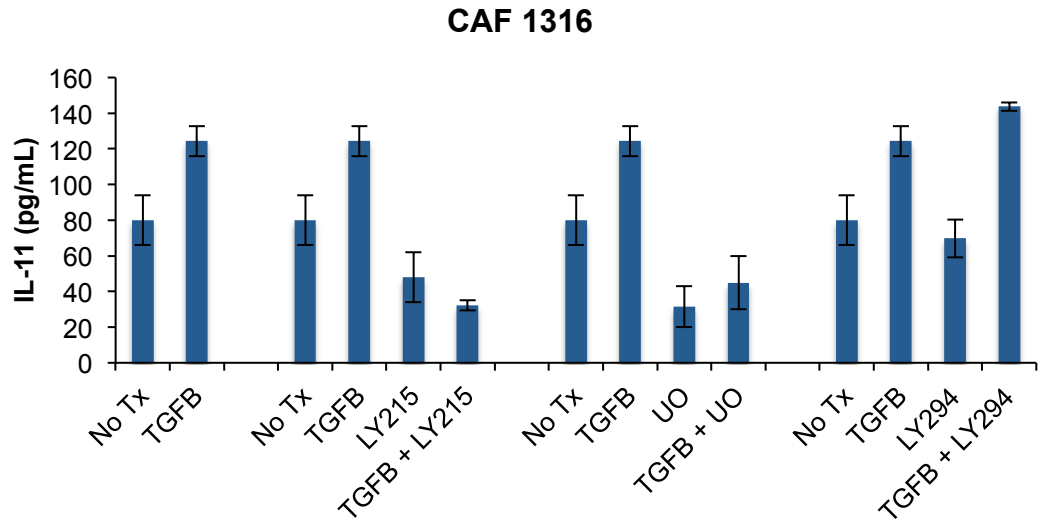


Figure 9. continued

III:

Discussion

Cytokine-induced signaling has an important role in cancer progression, and thus inhibition of cytokine signaling is attracting interest as a therapeutic strategy. IL-11 has been implicated in multiple cancers, making it a potentially attractive therapeutic target. The aims of this study were to: 1) characterize the expression of IL-11 and the IL-11Rα in pancreatic cancer, 2) determine the role of IL-11 in pancreatic cancer progression, and 3) determine if IL-11 mediates signaling in both stromal and tumor cells. Therefore, in our experiments we examined both PDAC cells and pancreatic CAFs. We demonstrate that the IL-11 ligand is overexpressed in pancreatic cancer, and that the IL-11Rα is expressed on normal pancreas, pancreatic cancer cells, and pancreatic CAFs. We further show that IL-11 signals through the Akt, ERK, Stat3, and TGF-β pathways, which are known to promote pancreatic cancer progression.

IL-11 may be associated with pancreatic cancer progression, as later stage tumors exhibit elevated IL-11 expression (Bellone et al., 2005). Here we show that, in vitro, IL-11 expression increases as cell density increases. IL-11 expression is induced in PDAC cells and CAFs incubated past confluence for 24 hours and 72 hours. Interestingly, it has been shown that IL-11 is a hypoxia-inducible gene upregulated through HIF-1 signaling (Onnis et al., 2013). Another study by Shen et al. revealed that in gastric cancer cells, IL-11 expression can be upregulated in tumor associated macrophages during hypoxic conditions (Shen et al., 2012). Pancreatic cancers are known to maintain among the most hypoxic tumor microenvironments that have been evaluated. Because our study did not evaluate the effects of hypoxia on IL-11

expression, it would be useful to perform additional experiments that examine if IL-11 expression is upregulated by pancreatic cancer cells cultured in in hypoxic conditions.

Because the Akt, ERK, and Stat3 pathways promote survival and proliferation of cancer cells, we hypothesize that IL-11 signaling promotes the cancer phenotype by signaling through these pathways. We found IL-11 stimulation has the greatest effect on Akt activation in both PDAC cells and CAFs, indicating that IL-11 signaling may predominantly promote cell survival, and related processes such as therapeutic resistance and metastasis. A study by Kiessling et al. found that IL-11 stimulation decreases caspase-9 activity in human colonic epithelial cells cultured in apoptosis-promoting conditions. Furthermore, they evaluated the effects of IL-11 on apoptosis in vivo by treating mice with colitis with rhIL-11 for two weeks. They found that the mice treated with IL-11 had a significantly reduced number of apoptotic cells compared to untreated mice (Kiessling et al., 2004).

A study by Calon et al. show that colorectal cancer cells that overexpress IL-11 promote metastasis when injected into mice. To study the relationship between metastasis and survival, they demonstrated that IL-11 overexpression decreases caspase-9 activity in tumor cells after six hours of liver colonization (Calon et al., 2012). Ren et al. found that IL-11 serum and mRNA expression levels were higher in patients whose breast cancer metastasized to bone compared to patients with primary breast cancer only. They also found that IL-11 serum levels are correlated with tumor size and number of metastatic sites (Ren et al., 2013). IL-11 expression is correlated with bone metastasis in hepatocellular carcinoma (Gao et al., 2013) and IL-11

stimulation increases ICAM-1 expression, which is associated with metastasis, by signaling through the Akt pathway in chondrosarcoma cells (Li et al., 2012). Because metastasis requires robust survival mechanisms, it is possible that IL-11 may function to promote survival and thereby facilitate metastasis of pancreatic cancer. Since we also found that IL-11 signals through the Akt pathway in pancreatic cancer cells, future studies will evaluate the effects of IL-11 on ICAM-1 expression and metastasis in pancreatic cancer.

ERK is constitutively active in nearly all pancreatic cancer cell lines used in this study, including PDAC cells and CAFs. We show that ERK activation is not greatly affected by IL-11 signaling in cells that express moderate levels of IL-11. However, in cells that significantly overexpress IL-11, including FG and CAF 1444 cells, IL-11 stimulation results in an immediate and dramatic increase in ERK activation. We hypothesize that ERK activation is dependent on basal IL-11 expression levels. Further experiments to evaluate our hypothesis could be undertaken to knock down IL-11 expression in FG and CAF 1444 cells, followed by stimulating with IL-11 to examine if ERK is no longer activated in the knockdown lines. Additionally, we show that inhibition of ERK signaling in CAF cells results in decreased IL-11 expression. Therefore, basal ERK signaling mediates IL-11 expression in pancreatic CAF cells.

Stat3 is an important signaling pathway in pancreatic cancer. In cancer cells Stat3 is often constitutively activated, promoting tumor cell proliferation and survival, angiogenesis, immunosuppression, and metastasis (Yu et al., 2007). We show that

Stat3 activation is often constitutive in PDAC cells and pancreatic CAFs, and Stat3 activation may increase in response to IL-11 stimulation. A study by Ernst et al. showed that inhibiting Stat3 activity in *gp130*$^{F/F}$ mutant mice, which exhibit hyperactivation of Stat3 by IL-6 family cytokines, reduces both tumor mass and IL-11 expression in gastric cancer (Ernst et al., 2008). Therefore future studies could investigate if inhibition of IL-11 signaling decreases Stat3 activation in pancreatic cancer. Further research is necessary to determine if inhibition of Stat3 mediated IL-11 signaling can suppress any of the hallmark characteristics of pancreatic cancer that are associated with Stat3.

Our results indicate that IL-11 stimulation does not have an effect on pancreatic cancer cell proliferation or migration in vitro. Because our study examined nine different cell lines, including PDAC cells and CAFs, we can be confident that IL-11 does not affect proliferation in pancreatic cancer. Our migration assays examined only PDAC cells, as such assays are technically challenging with cultured CAFs. As such we cannot rule out that IL-11 signaling may influence CAF migration in pancreatic cancer. Also, further studies are needed to determine if IL-11 affects invasion in pancreatic cancer, since our study did not address this possibility. Certainly another explanation for our results is that although cell proliferation and migration were not affected in vitro, IL-11 overexpression may have differential effects in vivo. The tumor microenvironment is very complex and cannot be entirely replicated by cells in vitro. To determine if IL-11 affects cancer cell proliferation in pancreatic cancer, additional studies performed in vivo are necessary.

In this study we found that IL-11 expression is upregulated by TGF-β in pancreatic CAFs. We show that ERK signaling mediates TGF-β stimulation of IL-11 expression. TGF-β signaling is an important contributor to progression of pancreatic cancer and long-term exposure to TGF-β promotes a tumorigenic phenotype in pancreatic cells (Ito et al., 2004). Additionally, TGF-β overexpression is associated with advanced stage and decreased survival in pancreatic cancer (Friess et al., 1993). Clearly, TGF-β is linked to pancreatic cancer progression, and our study links TGF-β signaling to IL-11. A recent study by Yuan et al. shows that TGF-β regulates long noncoding RNA lncRNA-ATB, which binds to IL-11 mRNA and stimulates IL-11 expression. They hypothesize that lncRNA-ATB enhances the prometastatic effects of TGF-β by mediating IL-11 signaling (Li et al., 2014). As previously discussed, we hypothesize that IL-11 has a role in pancreatic cancer metastasis, and it is possible that this is mediated by TGF-β signaling. Further research is necessary to determine if inhibiting IL-11 attenuates pancreatic cancer progression and/or metastasis. Additionally, inhibiting TGF-β signaling alone results in decreased IL-11 expression, indicating that basal TGF-β signaling mediates IL-11 expression in CAF cells. Therapeutic inhibition of TGF-β may be clinically beneficial in tumors that overexpress IL-11.

This preliminary study demonstrates that IL-11 signaling components are present in pancreatic cancer cells and their associated stromal cells, and suggests a potential role for IL-11 signaling in pancreatic cancer progression via signaling through multiple oncogenic pathways. To our knowledge, this is the first study that

has aimed to evaluate the role of IL-11 specifically in pancreatic cancer. However, all experiments performed in this study were done in vitro, and parallel experiments in vivo are still necessary. Our current work involves experiments inhibiting IL-11 or IL-11Rα expression. A study by Lee et al. in 2008 developed IL-11 mutein, which inhibits IL-11 from binding to IL-11Rα with high specificity (Lee et al., 2008). A later study by Putoczki et al. treated *gp130*$^{F/F}$ mutant mice with IL-11 mutein. They observed decreased activation of Stat3 in tumors (Putoczki et al., 2013). Additional IL-11 signaling inhibition studies could involve knocking down IL-11Rα in vivo. These experiments are highly relevant, as knocking down IL-11Rα has already been shown to reduce tumor burden in other gastrointestinal cancers (Putoczki et al., 2013). We conclude that inhibiting IL-11 and IL-11Rα signaling experiments are necessary in order to determine if IL-11 has a role in pancreatic cancer progression.

A study by Nakayama et al. studied 73 cases of surgically resected human gastric tumors and found that IL-11Rα expression is correlated with tumor invasion and vessel infiltration (Nakayama et al., 2007). However there have been no studies that specifically aim to determine if IL-11 and IL-11Rα expression correlate with prognosis and/or clinicopathologic characteristics in surgically resected pancreatic cancer. This knowledge would be useful in suggesting whether or not IL-11 has a role in pancreatic cancer progression.

Our study shows that IL-11 signaling components are present at significant levels within the tumor microenvironment in pancreatic cancer. IL-11 activates oncogenic signaling pathways in both PDAC and CAF cells further suggesting a

potential relevance to pancreatic cancer biology. Further work is required to develop a better understanding if/how IL-11 promotes tumor progression in pancreatic cancer and subsequently to determine whether IL-11 may be targeted therapeutically for the treatment of pancreatic cancer.

IV:

Materials and Methods

Human pancreatic cell lines

ASPC-1 and BxPC3 cells were grown in RPMI medium supplemented with 10% fetal bovine serum (FBS). Capan-2 cells were grown in McCoy's medium supplemented with 10% FBS. FG and MIAPaCa cells were grown in DMEM medium supplemented with 10% FBS. All CAF lines: 50, 64, 1299, 1308, 1316, 1424, and 1444 were grown in DMEM medium supplemented with 30% FBS.

ELISA

Cells were seeded at 80% confluence. Total levels of IL-11 protein were measured on cell supernatants using the human IL-11 ELISA kit (RayBiotech) according to manufacturer's instructions.

Immunofluorescence

Tissues were fixed in formalin and paraffin-embedded for analysis. Anti-IL-11 (Santa Cruz #7924), anti-IL-11Rα (Abcam #125015), and anti-pan-keratin (Abcam #8068) antibodies were incubated overnight at 4°C and detected by fluorescence using secondary Alexa antibodies coupled to fluorochromes (1/500) for 1 hour in the dark. Cell nuclei were stained with DAPI and slides were observed using Nikon upright fluorescence microscope.

Western Blot

Protein extracts were obtained using RIPA buffer (20mM Tris pH 8, 5mM EDTA pH 8, 150 mM NaCl, 1% Triton, cOmplete tablet (Roche), PhosStop tablet (Roche)). Protein concentration was determined using Micro BCA Protein Assay Kit (Thermo Scientific). Proteins were separated by SDS gel electrophoresis and

transferred to PVDF membrane. Blots were blocked in 5% milk or 5% BSA for phosphorylated proteins. The following primary antibodies (1/1000) were used: anti-IL-11Rα (Abcam #125015), anti-phosphorylated PI3K (Cell Signaling #9271), anti-PI3K (Cell Signaling #9272), anti-phosphorylated p44/42 MAPK (Cell Signaling #9101), anti-p44/42 MAPK (Cell Signaling #9102), anti-phosphorylated Stat3 (Cell Signaling #9145), anti-Stat3 (Cell Signaling #4904). The secondary antibody used was goat anti-rabbit secondary antibody (1/7500) (Santa Cruz #2004). To confirm equal protein loading, each blot was reprobed with anti-β-Actin (Sigma #A2066). Signals were detected using Pierce ECL Western Blotting Substrate (Thermo Scientific) according to the manufactuerer's protocol.

Proliferation Assay

PDAC cells were grown in culture medium with 10% FBS and seeded at 50% confluence. CAF cells were grown in culture medium with 30% FBS and seeded at 50% confluence. After 24 hours, media with 10% FBS and IL-11 (50 ng/mL) (R&D Systems) was added to the cells. Media with FBS was used as control. Cell viability was analyzed using an AlamarBlue cell proliferation assay (Invitrogen) or an MTT cell viability assay (Sigma) according to the manufacturer's protocol.

IL-11 neutralizing antibody

Cells were plated at 80% confluence in media with 30% FBS and treated with IL-11 neutralizing antibody (10 μg/mL, 20ug/mL) (R&D Systems) for 24 hours, 48 hours, and 72 hours. Media with 30% FBS was used as control.

Scratch assays for cell migration

Cell migration was determined by scratch assays. The cells were grown in culture medium with 10% FBS to 90% confluence in 6-well tissue culture plates. Once confluence was achieved, media was changed to 5% FBS for the remainder of the experiment to inhibit cell proliferation. A wound was made by scratching the dish uniformly with a pipette tip. Cells were washed twice, then media with 5% FBS and IL-11 (50 ng/mL) (R&D Systems) was added to the wells. Cells treated with media with 5% FBS were used as control. Cell migration was monitored by capturing images at 24 hours and 48 hours after the scratch was made. The distance covered by cells in the empty area was calculated in % using the following equation: $[1-(T_n/T_0)]*100$.

TGF-β treatment

Pancreatic CAF cells were plated at 100% confluence and treated with TGF-β1 (10 ng/mL) (Peprotech) for 24 hours. For inhibitor studies, cells were pre-incubated with inhibitor for 2.5 hours prior to TGF-β1 incubation. The following inhibitors were used: LY2157299 (10μM) (Selleck USA), UO126 (10μM) (Selleck USA), and LY294002 (20 μM) (Selleck USA).

References

Bellone G, Smirne C, Mauri FA, Tonel E, Carbone A, Buffolino A, Dughera L, Robecchi A, Pirisi M, Emanuelli G. Cytokine expression profile in human pancreatic carcinoma cells and in surgical specimens: implications for survival. *Cancer Immunol. Immunother*. 2006; 55:684-698.

Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. *Nature*. 2004;432:332–7.

Caldas C, Kern SE. K-ras mutation and pancreatic adenocarcinoma. *Int J Pancreatol*. 1995;18:1-6.

Calon A, Espinet E, Palomo-Ponce S, Tauriello DV, Iglesias M, Cespedes MV, Sevillano M, Nadal C, Jung P, Zhang XH, Byrom D, Riera A, Rossell D, Mangues R, Massague J, Sancho E, Batlle E. Dependency of colorectal cancer on a TGF-β driven program in stromal cells for metastasis initiation. *Cancer Cell*. 2012;22,571-584.

Chu GC, Kimmelman AC, Hezel AF, DePinho RA. Stromal biology of pancreatic cancer. *J Cell Biochem*. 2007;101:887-907.

Du X, Williams DA. Interleukin-11: review of molecular, cell biology, and clinical use. *Blood*. 1997;89:3897-3908.

Ernst M, Najdovska M, Grail D, Lundgren-May T, Buchert M, Tye H, Matthews VB, Armes J, Bhathal PS, Hughes NR, Marcusson EG, Karras JG, Na S, Sedgwick JD, Hertzog PJ, Jenkins BJ. STAT3 and STAT1 mediate IL-11 dependent and inflammation-associated gastric tumorigenesis in gp130 receptor mutant mice. *J Clin Invest*. 2008;118:1727-1738.

Friess H, Yamanaka Y, Buchler M, Ebert M, Beger HG, Gold LI, Korc M. Enhanced expression of transforming growth factor beta isoforms in pancreatic cancer correlates with decreased survival. *Gastroenterology*. 1993;105:1846-1856.

Gao YB, Xiang ZL, Zhou LY, Wu ZF, Fan J, Zeng HY, Zeng ZH. Enhanced production of CTGF and IL-11 from highly metastatic hepatoma cells under hypoxic conditions: an implication of hepatocellular carcinoma metastasis to bone. *J Cancer Res Clin Oncol.* 2013;139:669-679.

Hidalgo M. Pancreatic cancer. *N Engl J Med*. 2010;36:1605-1617.

Howlett M, Menheniott TR, Judd LM, Giraud AS. Cytokine signaling via gp130 in gastric cancer. *Biochimica et Biophysica Acta*. 2009;1793:1623-1633.

Ito D, Fujimoto K, Doi R, Koizumi M, Toyoda E, Mori T, Kami K, Kawaguchi Y, Whitehead R, Imamura M. Chronic exposure of transforming growth factor beta 1

confers a more aggressive tumor phenotype through downregulation of p21(WAF1/ CIP1) in conditionally immortalized pancreatic epithelial cells. *Surgery.* 2004;136:364-374.

Kiessling S, Muller-Newen G, Leeb SN, Hausmann M, Rath HC, Strater J, Spottl T, Schlottmann K, Grossmann J, Montero-Julian FA, Scholmerich J, Andus T, Buschauer A, Heinrich PC, Rogler G. Functional expression of the interleukin-11 receptor alpha-chain and evidence of antiapoptotic effects in human colonic epithelial cells. *J Biol Chem*. 2004;279:10304-10315.

Lay V, Yap J, Sonderegger S, Dimitriadis E. Interleukin 11 regulates endometrial cancer cell adhesion and migration via STAT3. *Int J Oncol*. 2012;41:759-764.

Lee CG, Hartl D, Matsuura H, Dunlop FM, Scotney PD, Fabri LJ, Nash AD, Chen NY, Tang CY, Chen Q, Homer RJ, Baca M, Elias JA. Endogenous IL-11 signaling is essential in Th2- and IL-13-induced inflammation and mucus production. *Am J Respir Cell Mol Biol*. 2008;39:739-746.

Li TM, Wu CM, Huang HC, Chou PC, Fong YC, Tang CH. Interleukin-11 increases cell motility and up-regulates intercellular adhesion molecule-1 expression in human chondrosarcoma cells. *J Cell Biochem.* 2012;113:3353-3362.

Li W, Kang Y. A new lnc in metastasis: long noncoding RNA mediates the prometastatic functions of TGF-b. *Cancer Cell*. 2014;25:557-559.

Massague J. TGF-B in cancer. *Cell*. 2008;134:215-230.

Maitra A, Hruban RH. Pancreatic cancer. *Annu Rev Pathol*. 2008;3:157-88.

McCoy EM, Hong HX, Pruittetal HC. IL-11 produced by breast cancer cells augments osteoclastogenesis by sustaining the pool of osteoclast progenitor cells. *BMC Cancer*. 2013;13:1-11

Nakayama T, Yoshizaki A, Izumida S, Suehiro T, Miura S, Uemura T. Expression of interleukin-11 (IL-11) and IL-11 receptor alpha in human gastric carcinoma and IL-11 upregulates the invasive activity of human gastric carcinoma cells. *Int J Oncol*. 2007;30:825-33.

Onnis B, Fer N, Rapisarda A, Perez VS, Melillo G. Autocrine production of IL-11 mediates tumorigenicity in hypoxic cancer cells. *J Clin Invest.* 2013;123:1615-1629.

Putoczki T, Ernst M. More than a sidekick: the IL-6 family cytokine IL-11 links inflammation to cancer. *J Leukoc Biol*. 2010;88:1109-1117.

Putoczki TL, Thiem S, Loving A, Busuttil RA, Wilson NJ, Ziegler PK, Nguyen PM,

Preaudet A, Farid R, Edwards KM, Boglev Y, Luwor RB, Jarnicki A, Horst D, Boussioutas A, Heath JK, Sieber O, Pleines I, Kile BT, Nash A, Greten FR, McKenzie BS, Ernst M. Interleukin-11 is the dominant IL-6 family cytokine during gastrointestinal tumorigenesis and can be targeted therapeutically. *Cancer Cell*. 2013;24,257-271.

Ren L, Wang X, Dong Z, Liu J, Zhang S. Bone metastasis from breast cancer involves elevated IL-11 expression and the gp130/STAT3 pathway. *Med Oncol*. 2013;30(3):634.

Schwertschlag US, Trepicchio WL, Dykstra KH, Keith JC, Turner KJ, Dorner AJ. Hematopoietic, immunomodulatory and epithelial effects of interleukin-11. *Leukemia*. 1999;13:1307-1315.

Shen Z, Seppanen H, Vainionpaa S, Ye Y, Wang S, Mustonen H, Puolakkainen P. IL-10, IL-11, IL-18 are differently expressed in CD14+ TAMs and play different role in regulating the invasion of gastric cancer cells under hypoxia. *Cytokine*. 2012;59:352-357.

Shin SY, Choi C, Lee HG, Lim Y, Lee YH. Transcriptional regulation of the interleukin-11 gene by oncogenic Ras. *Carcinogenesis*. 2012;33:2467-2476.

Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin*. 2012;62:10-29.

Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin*. 2014;64:9-29.

Truty MJ, Urrutia R. Basics of TGF-B and pancreatic cancer. *Pancreatology*. 2007;7:423-435.

Yoshizaki A, Nakayama T, Yamazumi K, Yakata Y, Taba M, Sekine I. Expression of interleukin (IL)-11 and IL-11 receptor in human colorectal adenocarcinoma: IL-11 up-regulation of the invasive and proliferative activity of human colorectal carcinoma cells. *Int J Onc*. 2006;29:869-876.

Yu H, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. *Nat Rev Immunol*. 2007;7:41-51.

Zurita AJ, Troncoso P, Cardo-Vila M, Logothetis CJ, Pasqualini R, Arap W. Combinatorial screenings in patients: the interleukin-11 receptor as a candidate target in the progression of human prostate cancer. *Cancer Res*. 2004;64:435-439.