UNIVERSITY OF CALIFORNIA SAN DIEGO

The combined role of stress and lysophosphatidic acid in pancreatic ductal adenocarcinoma progression

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by

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The Dissertation of Taha Rakhshandehroo is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California San Diego

2019

DEDICATION

I dedicate this dissertation to those who stood by me and supported me throughout this difficult yet satisfying endeavor. My parents, my in-laws, my wife, my friends.

EPIGRAPH

Thus, tumors appear to the host in the guise of wounds or, more correctly, of an unending series of wounds that continually initiate healing but never heal completely.

Harold F. Dvorak, M.D.

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Chapter 2 is coauthored with Tami Von Schalscha and David A. Cheresh. The dissertation author is the primary author of this chapter.

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TR, SMW, and DAC designed research studies and wrote the manuscript. TR, HIW, TVS, MM, MA, and JT conducted experiments and acquired data. BC and AML contributed to experimental designs. All authors contributed to analyzing data and reviewing the manuscript.

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ABSTRACT OF THE DISSERTATION

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by

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Doctor of Philosophy in Biology

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Professor David Cheresh, Chair

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Pancreatic cancers are notoriously aggressive and drug-resistant, and surgical resection is complicated by widespread intraperitoneal dissemination and ascites. One component of malignant ascites is lysophosphatidic acid (LPA), a bioactive lipid that promotes multiple aspects of tumor progression. Here, I report that expression of the cancer stem cell marker αvβ3 integrin is necessary and

sufficient for the production of LPA. In turn, LPA was shown to induce the expression of stem genes OCT4 and NANOG and promote anchorage-independent growth. Upon interrogating the LPA metabolic pathway, I found that expression of αvβ3 integrin is necessary and sufficient to promote the expression of cytoplasmic phospholipase A2 (cPLA2), an important enzyme in the production of LPA. Furthermore, αvβ3 integrin forms a physical complex with cPLA2 to stabilize and stimulate its activity. Subsequently, KRAS mutant pancreatic cancer cells utilize LPA to overcome the effects of cellular stress, including cancer therapy. A variety of stress, including nutrient deprivation, oxidative stress, and treatment with standard-of-care chemotherapies, sensitizes cells to LPA-induced migration as well as KRAS-driven advantages such as macropinocytosis in response to nutrient stress and ROS elimination in response to oxidative stress. In order to cope with these stresses, cancer cells selectively upregulate the expression of LPA Receptor 4 (LPAR4). I illustrate that this receptor is necessary and sufficient for adaptation to stress *in vitro* and tumor progression *in vivo*. I showed that LPAR4 is necessary and sufficient to activate the $G_{\alpha s}$/cAMP/PKA signaling pathway and that this pathway is required for LPA-mediated protection from stress. This is accomplished through activation of Vasodilator Stimulated Phosphoprotein (VASP), an enzyme involved in actin polymerization. Ultimately, I found that VASP is necessary for stress-mediated and LPAR4-mediate migration and macropinocytosis. My work highlights how stress changes the repertoire of LPA receptors, leading to dependence on LPAR4 by virtue of its stress mitigating

functions, such as macropinocytosis and ROS elimination. Furthermore, my findings illustrate that αvβ3 integrin expressing cancer stem-like cells can secrete the bioactive lipid LPA through cPLA2 upregulation. Subsequently, cellular stress drives a more aggressive cancer phenotype by priming pancreatic cells to be highly responsive to LPA.

Chapter 1 – Introduction

Pancreatic ductal adenocarcinoma (PDAC), although rare, is a type of aggressive exocrine cancer that is estimated to be the second highest cause of cancer deaths by 2030 [1]. PDAC commonly presents a striking desmoplastic reaction, which includes infiltration of fibroblasts, immune cells, and a dense extracellular matrix [2]. The profound matrix deposition in the PDAC microenvironment presents a major physical barrier that impedes the vasculature, leading to hypoxia, and blocks delivery of chemotherapeutic agents [3-5], leading to progression after therapy. This characteristic raises the possibility that PDAC cells undergo a great deal of stress in the tumor microenvironment, such as nutrient deprivation, hypoxia, and buildup of reactive oxygen species (ROS). A recent study by Rabinowitz and colleagues using metabolomic analysis of surgically resected human PDAC illustrated that these tumors are nutrient-poor [6], raising the question of how PDAC cells can survive and proliferate under such conditions.

One method that PDAC cells utilize to overcome cellular stress is the adaptive gain of stem-like qualities, thus becoming “cancer stem cells” (CSC). According to the CSC theory, a hierarchal system exists in which only a few undifferentiated tumor cells can self-renew and asymmetrically divide in order to populate the tumor, similar to how normal stem cells renew and sustain organs and tissues. According to this theory, CSC can initiate tumors, cause failure of

therapy, and lead to metastasis. Studies conducted by John Dick and colleagues were the first to establish that a few rare cells of acute myeloid leukemia (AML) from mice were able to recapitulate the cancer in other mice, indicating the existence of a subpopulation of cells capable of initiating this cancer [7, 8]. Indeed, the presence of CSC was confirmed in solid tumors by Clarke and colleagues when a few hundred cells from a subpopulation of a breast cancer tumor were sufficient to form tumors in mice [9]. Subsequent studies confirmed the presence of subpopulations of CSC in glioblastoma [10], colon [11], and pancreatic cancer [12].

However, the CSC theory was flawed, especially in regards to it hierarchal structure. For example, CSC were thought to only comprise of 0.1–0.0001% of the tumor population. However, Morrison and colleagues demonstrated that 25% of unselected melanoma cells from 12 separate patients were able to form tumors in a limiting dilution assay with severely immunocompromised NOD/SCID/Il2rg$^{-/-}$ mice [13]. Use of this mouse model increased tumor initiating capacity by several orders of magnitude when compared to NOD/SCID mice. This study confirmed cells that are apparently non-tumorigenic can become tumorigenic in the presence of an appropriate microenvironment. Thus, being too rigid in the way of defining CSC hierarchy is not appropriate. The latest versions of the CSC theory refer to a “tumor reprogramming” event involving major epigenetic and expression changes leading to dynamic stem-like state [14]. Cancer cells can acquire this state in response to their environment. Our lab recently discovered that non-small cell lung

cancer (NSCLC) cells that have acquired resistance against RTK inhibitors have much higher tumor initiating capacity. Through screening of cell surface markers known to be associated with CSC, we determined that RTK-resistant cells have increased expression of cell surface αvβ3 integrin. This leads to a RalB-TBK1-NFκB signaling pathway that promotes drug resistance and stem-like characteristics, such as increased tumor initiating capacity and anchorage independent growth [15].

Integrins are the main cell adhesion receptors for components of the extracellular matrix (ECM). Integrins are heterodimers composed of an α and a β subunit. There are a total of 24 integrins generated from a combination of 18 α and 8 β subunits. These integrins can be classified into receptors recognizing Arg-Gly-Asp (RGD) peptide motifs (i.e. αvβ3 integrin), leukocyte-specific, laminin, and collagen receptors [16]. Some integrins bind to only specific ECM ligands (i.e. α5β1 integrin to fibronectin) while others exhibit a broader ligand-binding repertoire. For example, αvβ3 integrin can bind to vitronectin, fibrinogen, fibronectin, thrombospondin, von Willebrand's factor, and others. Integrin binding to the ECM and subsequent activation can trigger the recruitment of the so-called adhesome: a complex array of cytoskeletal, signaling, and scaffolding proteins engaging with the integrin cytoplasmic tails [16-18]. Therefore, integrins that are responsible for adhesion to a cellular matrix represent a complex and highly dynamic machinery responsible for regulating aspects of cell fate such as migration, polarity, differentiation, metastasis, and survival [19].

Surprisingly, our studies indicate a new ligand-independent role for integrin αvβ3. We found that PDAC cells expressing αvβ3 integrin have a much higher ability to grow in soft agar, an anchorage-independent 3D environment with no available ligand or ECM to bind to, compared to cells lacking expression of αvβ3 [20]. These PDAC cells expressing αvβ3 integrin are also highly metastatic and depend on Src signaling. This presents a new ligand-independent role for αvβ3 integrin that promotes stem-like properties.

The integrin αvβ3 was first termed as the “vitronectin receptor” due to being the first protein discovered to bind to the plasma protein vitronectin [21]. However, other integrins (i.e. αvβ1 and αvβ5) are now known to bind to vitronectin while integrin αvβ3 is now known to bind a wide range of ECM molecules with an Arg-Gly-Asp (RGD) triple-peptide motif, including fibronectin, fibrinogen, von Willebrand factor, vitronectin, and heat denatured forms of collagen and laminin [22-24]. Integrin αvβ3 has many roles in distinct biological processes, including bone resorption, angiogenesis, and tumor metastasis [25]. Interestingly, integrin αvβ3 also appears to be the most important integrin involved in angiogenesis. A study conducted by our lab illustrated that αvβ3 integrin was expressed on blood vessels in wound granulation tissue but not on intact blood vessels. Furthermore, expression of αvβ3 integrin was increased during angiogenesis and functional blockage of αvβ3 integrin using a monoclonal antibody ablated the formation of new blood vessels, demonstrating that αvβ3 is a functional mediator of angiogenesis [26]. The crucial roles of integrin αvβ3 in tumor angiogenesis led to

a promising strategy to block its signaling by antagonists in order to inhibit angiogenesis and disrupt access of nutrients to the tumor. Although promising, certain αvβ3 antagonist like Cilengitide did not improve overall survival of glioblastoma patients [27, 28]. However, studies from our lab have demonstrated that only glioblastoma cells addicted to glutamine through the Glut3 transporter are sensitive to αvβ3 inhibition [29], indicating that a specific subset of glioblastoma cells are sensitive to Cilengitide. Apart from integrin αvβ3's well-defined role in angiogenesis, our lab has established integrin αvβ3 as a marker of stemness and drug resistance [15]. We also illustrated that histologically distinct cancer cells, including PDAC cells, induce the expression of αvβ3 at the cell surface upon acquired drug resistance *in vitro*.

As aforementioned, one method of cells gaining resistance to conditions of cellular stress, like hypoxia, nutrient deprivation, or ROS buildup, is through an adaptive gain of cancer stem qualities. Conversely, our lab has recently illustrated that αvβ3 integrin can mitigate nutrient deprivation through increased macropinocytotic uptake through oncogenic KRAS [30]. Macropinocytosis is an evolutionarily conserved form of bulk endocytosis by which cells can engulf extracellular nutrients through large, irregularly shaped vesicles that protrude from the cell membrane called macropinosomes [31]. These macropinosomes can engulf, digest, and utilize extracellular albumin or other proteins in order to alleviate the lack of nutrient and amino acids. Many studies, including our own, have illustrated that KRAS is essential for macropinocytosis in the absence of nutrients

[6, 30, 32]. Interestingly, PDAC cells were shown to scavenge lipid to support proliferation under hypoxic stress [33]. Under the hypovascular and nutrient-poor conditions of the PDAC tumor microenvironment, I reasoned that PDAC cells that are more stem-like and stress tolerant, like those that express αvβ3 integrin, may stimulate the same stem-like and stress tolerant phenotype on neighboring cells through a paracrine mechanism. Studies have illustrated that neighboring nonmalignant stromal cells can support stemness [34], drug resistance [35], and tumor progression [36]. Furthermore, tumor stem-like subpopulations have been shown to secrete cytokines that promote anchorage independent growth, an *in vitro* surrogate assay for assessing stemness, in non-stem like cells [37]. Recent studies have even shown lipids secreted by PDAC stromal cells can facilitate tumor progression [38].

Given that we have determined αvβ3 integrin as a marker of stemness, drug resistance, tumor progression, and metastasis, I hypothesized that αvβ3 expressing stem-like cells can secrete factors that promote stem-like qualities, such as anchorage independent growth, stress tolerance, tumor initiation, and metastasis.

Chapter 2 – αvβ3 integrin increases the production of LPA through a cPLA2 dependent mechanism

Chapter 2.1 Abstract

Soluble factors secreted by cancer associated fibroblasts, immune cells, or cancer cells themselves promote stress tolerance and a more stem-like phenotype. Within a tumor, cancer stem cells (CSCs) represent a small yet highly aggressive, drug resistant, and metastatic subpopulation. We recently showed that integrin αvβ3 is necessary and sufficient to promote the reprogramming of epithelial cancers to a CSC and drug-resistant fate. Therefore, I considered whether CSCs impact the phenotype of non-CSCs within the tumor mass resulting in their reprogramming to a stem cell fate. Here, I report that tumor cells expressing αvβ3 secrete factor(s) that reprogram non-CSCs to acquire stem cell properties such as anchorage independent growth and expression of pluripotent genes. I conducted multiple fractionation techniques, including heat denaturation, exosome depletion, and charcoal stripping, to determine that the stem reprogramming factor secreted by αvβ3 expressing cells is Lysophosphatidic Acid (LPA). I found that LPA promotes anchorage-independent growth and stem gene induction. Gain-of-function and loss-of-function studies indicated that integrin αvβ3 is necessary and sufficient to promote the expression of cytosolic Phospholipase A2 (cPLA2), a major enzyme involved in the production of LPA.

Furthermore, I found that cPLA2 forms a physical complex with αvβ3 integrin, leading to its stabilization and activation required for LPA production. These finding indicate that αvβ3-expressing CSCs can secrete LPA which, in turn, reprograms non-CSCs within their tumor environment to a stem-like fate.

Chapter 2.2 Introduction

Cancer stem cells (CSCs) are defined as tumor-initiating cells with a self-renewal capacity similar to that of normal stem cells [39] and are at the top of the tumor cell hierarchy. Their defining molecular characteristics as well as whether they emerge from transformed stem/progenitor cells, dormant tumor cells or via epithelial–mesenchymal transition (EMT) are still a matter of intense debate [40, 41]. CSCs are known to be particularly resistant to standard chemotherapeutic and targeted agents and are considered to be responsible for relapse after therapy [42, 43]. Therefore, understanding how CSCs arise and develop drug resistance have become major areas of interest to produce more effective therapeutic anti-cancer strategies.

We have identified a pathway that converts cancer cells from multiple organ sites towards a cancer stem cell fate. Specifically, exposing cancer cell lines both *in vivo* and *in vitro* to sub-lethal doses of Erlotinib, an EGFR small molecule inhibitor, over the course of weeks expands a subpopulation that is resistant to Erlotinib and highly expresses integrin αvβ3. Further experiments indicate that

αvβ3 is necessary and sufficient for drug resistance, anchorage independence, tumor initiation, and contributes to metastasis, indicating αvβ3 as a cancer “stem-like” marker with clinical relevance [15, 44]. The mechanism of this phenotype has been dissected in Seguin *et al.*, in which αvβ3 follows a KRAS-RalB-TBK1-NF-κB signaling axis.

A major question that remains is how such cancer stem cell populations arise. The dogma suggested a hierarchical tumor organization in which cancer stem cells are initially a small subpopulation that becomes selected for upon exposure to cancer therapy [39]. However, new evidence implicates that cancer cells comprise a level of plasticity where they can be induced, through accumulating mutations or drug resistance, towards a more “stem-like” phenotype [45]. Evidence from our lab indicates that cancer cells can adopt a stem-like fate when exposed to chronic cellular stress. We found that when cancer cells are exposed to different forms of stress (i.e. hypoxia, oxidative stress, and serum deprivation), αvβ3 integrin is induced through a chromatin remodeling mechanism. Given that we established αvβ3 integrin as a marker and driver of stemness, this new mechanism sheds light on how stem-like attributes can be gained through an aptive manner.

Paracrine signaling from a small population of cancer cells may also contribute to the tumor expansion. Brady *et al.* defined a highly metastatic Arntl2+ lung cancer subpopulation that secretes factors, including Smoc2, which promote

metastasis and anchorage-independent growth [37]. Also, cancer stem cells can educate the tumor stroma to secrete factors that promote stemness of the tumor itself [34, 46-48]. A growth factor screen conducted by Wilson *et al.* identified a number of growth hormones that promote resistance against certain RTK inhibitors, indicating that growth factors have the potential to contribute towards drug resistance [49]. According to the cancer stem cell model, CSCs represent only a small subpopulation of the tumor. If factors secreted by CSCs can have such a drastic paracrine effect on tumor progression, resistance, and stemness, it becomes important to study and identify how such factors may lead to the expansion of the cancer stem cell population. Therefore, I hypothesized that the small subpopulation of αvβ3(+) cells in a tumor can secrete factors that contribute to the progression of the tumor as a whole.

Chapter 2.3 αvβ3 integrin is necessary and sufficient for secretion of factors that lead to a stem-like phenotype

Previous studies have illustrated that αvβ3 integrin is a marker of stem-like characteristics in cancer cells, like anchorage-independent growth [44], increased tumor initiation, drug resistance [15], and a stem-like expression signature [29]. These characteristics can also be amplified by factors secreted by surrounding cancer cells [37, 46] or by cells in the tumor microenvironment [34, 47-50]. Therefore, I asked whether αvβ3 expressing cancer stem cells secrete factors that

promotes stem-like phenotypes. In order to test this hypothesis, I cultured cells ectopically expressing β3 integrin and GFP control cells and harvested serum-free conditioned media after 72 hours. I also harvested conditioned media from cells expressing the β3 759x dominant negative mutant integrin that is deficient in binding to Src [51]. Desgrosellier *et al.* illustrated that αvβ3 integrin promotes anchorage independent growth in a ligand independent manner. This advantage is dependent on Src activation through the β3 integrin cytoplasmic tail. A four amino acid C-terminal truncation (β3 integrin 759x mutant) prevents Src binding and negates the αvβ3 anchorage independent growth advantage and stem-like phenotype [20]. I found that αvβ3 integrin is necessary for the conditioned media effect, i.e. the promotion of anchorage independent growth (**Figure 2.1**). Furthermore, conditioned media collected from the αvβ3 dominant negative mutant expressing cells did not increase anchorage-independent growth, indicating that αvβ3 activity through Src is imperative for the conditioned media effect. In conjunction with my findings, conditioned media from αvβ3 knockout cells decreased anchorage independent growth compared to control cells (**Figure 2.2**). This finding indicates that αvβ3 is necessary for conditioned media to promote anchorage independent growth.

Pluripotency of human embryonic stem (hES) cells have been maintained without the use of a feeder layer, instead using specific soluble ligands like Activin A and bFGF [52, 53]. Pluripotency is directly related to the constant expression of Oct4, Nanog, and Sox2, well-characterized markers of pluripotency. Therefore, I

asked whether conditioned media from αvβ3 expressing cells can induce the expression of these stem markers. To our surprise, conditioned media from αvβ3 integrin expressing cells induced the expression of *OCT4*, *NANOG*, and, interestingly, *ITGB3* (**Figure 2.3**). These findings indicate that conditioned media from αvβ3 expressing cells promotes an expression profile similar to cancer stem-like cells.

Chapter 2.4 – Activity of conditioned media from αvβ3 expressing cells is derived from a small bioactive lipid

There have been numerous studies illustrating the paracrine properties of exosomes secreted by cancer cells [54]. Therefore, I postulated that αvβ3 expressing cells may secrete exosomes that transfer stem-like characteristics. I fractionated conditioned media using ultracentrifugation and treated cells lacking expression of αvβ3 integrin with each fraction. Interestingly, I found that the exosome fraction did not induce the expression of Oct4, Nanog, or ITGB3, indicating that exosomes are not involved (**Figure 2.4**).

Aforementioned proteins such as cytokines can be secreted, leading to tumor progression [37, 47, 50]. Proteins are heat-sensitive and can denature at high temperatures. To test whether αvβ3 expressing cells secrete cytokines, I heat-treated conditioned media at 80°C for 60 minutes. I found that the heat-

denatured conditioned media from αvβ3 expressing cells maintains its ability to induce the expression of stem genes (**Figure 2.5**).

This led us to hypothesize that the secreted factor is a small bioactive lipid, because it is heat-resistant. Studies have illustrated that eicosanoids [55] and ceramides [56] play a major role in cancer. To test this, I subjected conditioned media to activated charcoal, which sequesters slightly non-polar molecules. To our surprise, I found that activated charcoal treatment of conditioned media significantly diminishes the induction of stem genes (**Figure 2.6**). Therefore, I concluded that the factor secreted by αvβ3 expressing cells is a small bioactive lipid (**Figure 2.7**)

Chapter 2.5 Tumor cell expression of αvβ3 generates LPA through cPLA2

Although many lipids have been shown to be involved in inflammation and cancer, recently studies have implicated both lysophosphatidic acid (LPA) and Autotaxin, a secreted enzyme that produces LPA, in pancreatic cancer progression [38]. Therefore, I asked whether αvβ3 expressing cells secreted LPA. Using a competitive ELISA colorimetric assay, I found that, using both gain-of-function and loss-of-function approaches, αvβ3 integrin is necessary and sufficient to produce LPA (**Figure 2.8**).

To further understand how αvβ3 promotes the production of LPA, I decided to test whether αvβ3 can influence the expression of lipases involved in the LPA

metabolism. There are two different metabolic pathways that can produce LPA (**Figure 2.9**). One pathway utilizes Phospholipase D (PLD) and Phospholipase A2 (PLA2) to produce LPA from phospholipids. The second pathways uses PLA2 and Autotaxin. Studies have shown that Autotaxin is involved in tumor progression [38, 57] and can bind to αvβ3 integrin [58]. I therefore assessed the expression of Autotaxin from the conditioned media of αvβ3 expressing and non-expressing cells. To our surprise, I found that αvβ3 integrin does not affect the expression of Autotaxin (**Figure S2.1**). I decided to assess the expression of PLA2, another enzyme that is essential to the production of LPA. Interestingly, I found that αvβ3 integrin is necessary and sufficient for the expression of cytosolic PLA2 (cPLA2), a Ca^{2+} dependent phospholipase A2 isoform (**Figure 2.10**). A recent study illustrated that a cPLA2 can bind to $\alpha_{IIB}\beta3$ integrin in platelets, leading to its stabilization [59]. I therefore tested whether cPLA2 can bind to αvβ3 integrin in cancer cells. As expected, immunoprecipitation experiments illustrated that cPLA2 complexed with αvβ3 integrin in both cells endogenously expressing and ectopically expressing αvβ3 (**Figure 2.11**). These experiments indicate that αvβ3 expressing cells support the production of LPA through the expression and stabilization of cPLA2.

Chapter 2.6 LPA promotes anchorage independent growth and stem gene induction

LPA has been known in the field to promote tumor progression and metastasis [60, 61]. It has also been shown that LPA can promote migration [62] and lamellipodia formation [63] all of which are phenotypes of a stem-like cell. We, therefore, hypothesized that LPA will increase anchorage independent growth, similar to conditioned media from αvβ3 expressing cells.

First, we determined that LPA has no effect on growth in adherent (2D) conditions. However, when cells negative for αvβ3 expression are grown in soft agar (3D), we found that LPA increases colony formation (**Figure 2.12**). Given that a recent study determined that cancer associated fibroblasts can secrete factors that promote stemness in NSCLC cells through expression of *OCT4* and *NANOG* [34], we asked if LPA can confer the same phenotype. Upon addition of LPA, I found that LPA, in a dose-dependent manner, induced the expression of *OCT4* and *NANOG* (**Figure 2.13**). These findings reveal that LPA phenocopies conditioned media from αvβ3 expressing cells, indicating that LPA is indeed a functional secreted factor from αvβ3 expressing cells.

Chapter 2.7 Discussion

Our lab has previously illustrated that αvβ3 integrin is a marker and driver of cancer stem cells [15]. Integrin αvβ3 can promote cancer stemness, drug

resistance, tumor initiation, and metastasis [20]. Considering previous studies that demonstrated cancer stem cells can secrete factors that promote tumor progression and stemness, I asked whether αvβ3 expressing cancer stem cells can secrete factors that promote those stem-like characteristics. Interestingly, I found that αvβ3 integrin expressing cells, in both gain-of-function and loss-of-function models, produce factors that promote anchorage-independent growth, an *in vitro* hallmark of stemness. I also discovered conditioned media from αvβ3 expressing cells promote the expression of the stem genes *OCT4* and *NANOG*. Through exosome depletion, heat denaturation, and charcoal stripping of the conditioned media, I deduced that the factor involved in inducing the expression of *OCT4* and *NANOG* was a small bioactive lipid. Through a competitive ELISA assay, I found that αvβ3 expressing cells, in both gain-of-function and loss-of-function experiments, secrete LPA. Surprisingly, I found that Autotaxin, a major LPA-producing enzyme, expression is not affected by αvβ3. However, cPLA2, a metabolic enzyme upstream of Autotaxin, is dependent on αvβ3 expression. Additionally, cPLA2 is recruited by αvβ3 integrin to the cell membrane, which is the cellular location of the substrate of cPLA2, phospholipids. In conclusion, αvβ3 integrin promotes the secretion of LPA through the expression and recruitment of cPLA2.

Current studies, especially in pancreatic cancer, have tried to understand the symbiosis between PDAC cells and the surrounding stroma. PDAC is characterized by abundant desmoplasia that constitutes up to 90% of the total

tumor volume and contains extracellular matrix (ECM), immune cells, vasculature, and cancer-associated fibroblasts (CAF) [64]. Tuveson and colleagues [65] found that co-culture of pancreatic stellate cells (PSC) and pancreatic cancer organoids recapitulated properties of PDAC desmoplasia and tumor progression through the secretion of inflammatory cytokines by fibroblasts. Furthermore, Hunter and colleagues [36] discovered that these PSCs secrete leukemia inhibitory factor (LIF) leading to tumor progression and chemotherapy resistance. However, our work illustrates how PDAC cells themselves secretes a factor that can have effects on tumor cells and the surrounding tumor microenvironment. Interestingly, Chun and colleagues [66] found that LPA buildup can lead to fibroblast recruitment and pulmonary fibrosis upon lung injury. Consistent with this study, Sherman and colleagues [67] uncovered that PDAC cells secrete Autotaxin while PSC secrete lysophosphatidylcholine (LPC), the substrate of Autotaxin, leading to further desmoplasia and progression. Therefore, pancreatic cancer cells can utilize LPA and its mitogenic and chemotactic properties to promote an aggressive phenotype. Thus, this study complements previous work by defining a new mechanism for LPA production in pancreatic cancer cells.

Chapter 2.8 Figures

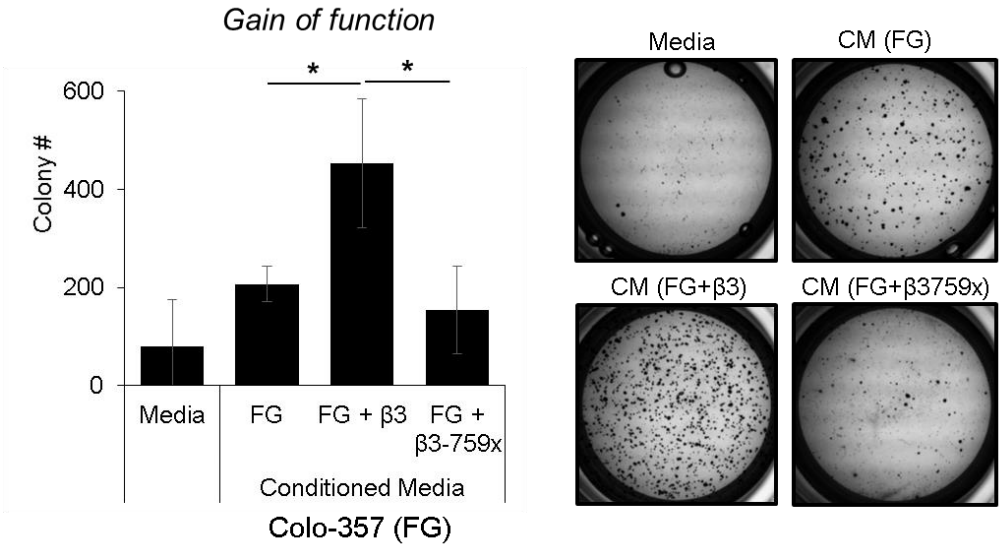


Figure 2.1 Conditioned media from αvβ3 integrin ectopically expressing cells is sufficient to increase anchorage independent growth. Soft Agar assay was performed on FG cells with conditioned media harvested from FG cells ectopically expressing empty vector, αvβ3 integrin, and αvβ3 759x truncated mutant, a Src binding deficient dominant negative mutant.

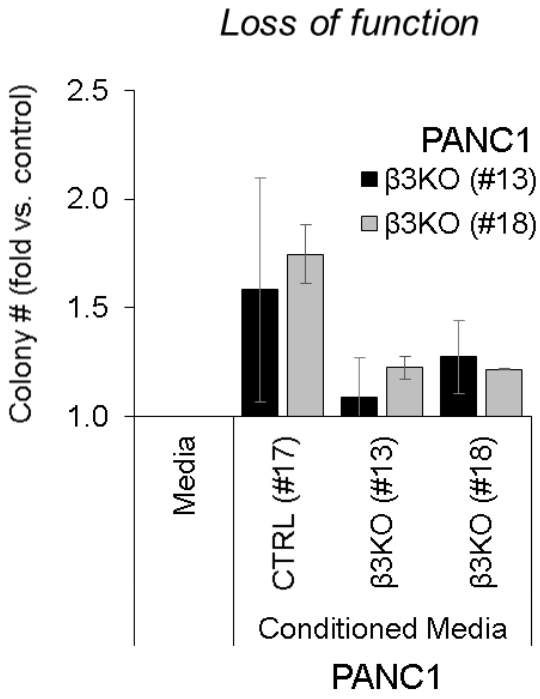


Figure 2.2 Conditioned media from αvβ3 cells is necessary for anchorage independent growth. Soft Agar assay was performed on PANC1 αvβ3 KO cells with conditioned media harvested from PANC1 (endogenously αvβ3+) and PANC1 αvβ3 KO cells (#13 & #18). Bar graph represents mean±SD of n = 3 independent experiments.

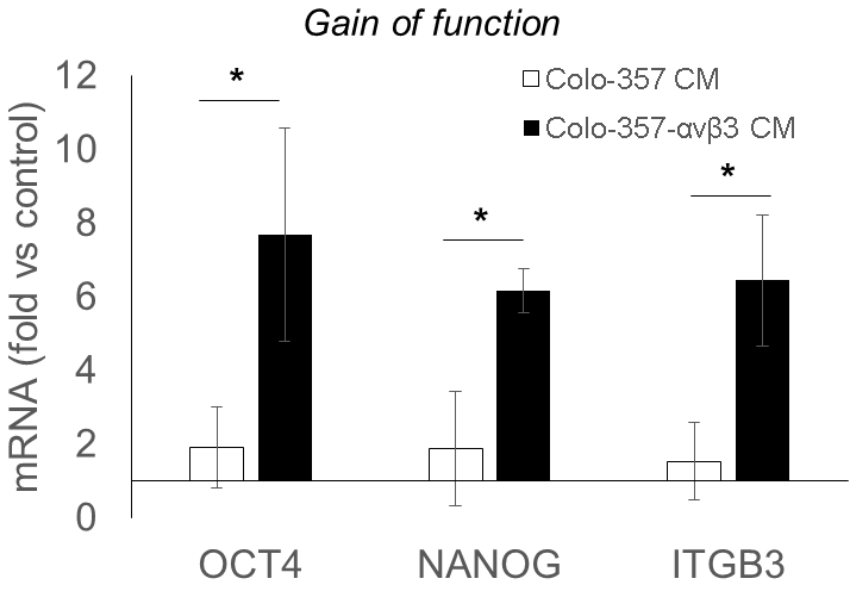


Figure 2.3. Conditioned media from αvβ3 expressing cells induce expression of stem genes. qPCR data showing mRNA induction of stem genes when incubated with conditioned media from αvβ3 expressing Colo-357 FG cells. Bar graphs represent mean±SD of n = 3 independent experiments. Statistical analysis was done using Student *t* test. *, $P < 0.05$

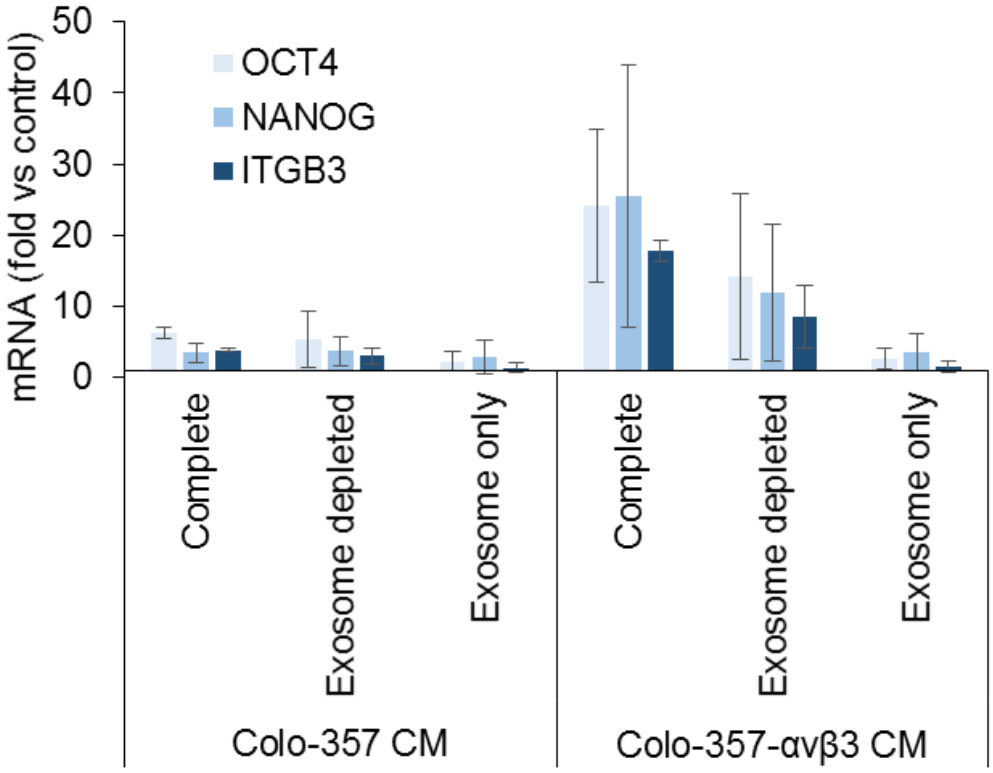


Figure 2.4. Exosome depletion on conditioned media from αvβ3 expressing cells does not reduce induction of stem genes. qPCR data illustrating the effects of exosome depletion of conditioned media from αvβ3 expressing Colo-357 FG cells on stem gene induction. Bar graphs represent n = 3 independent experiments.

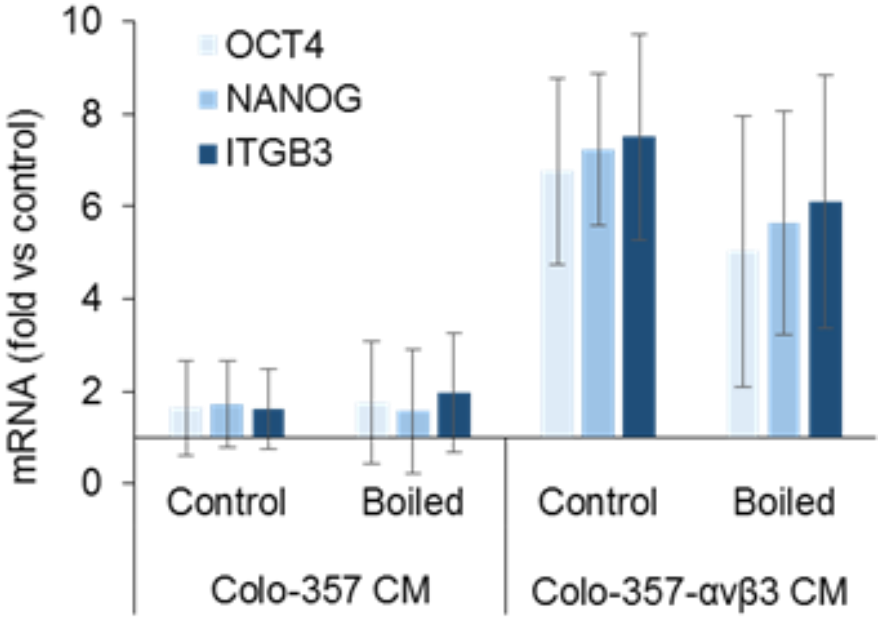


Figure 2.5. Boiling conditioned media from αvβ3 expressing cells does not have an effect on stem gene induction . qPCR data illustrating the effects of heat denaturation (boiling) of conditioned media from αvβ3 expressing Colo-357 FG cells on stem gene induction. Bar graphs represent n = 3 independent experiments

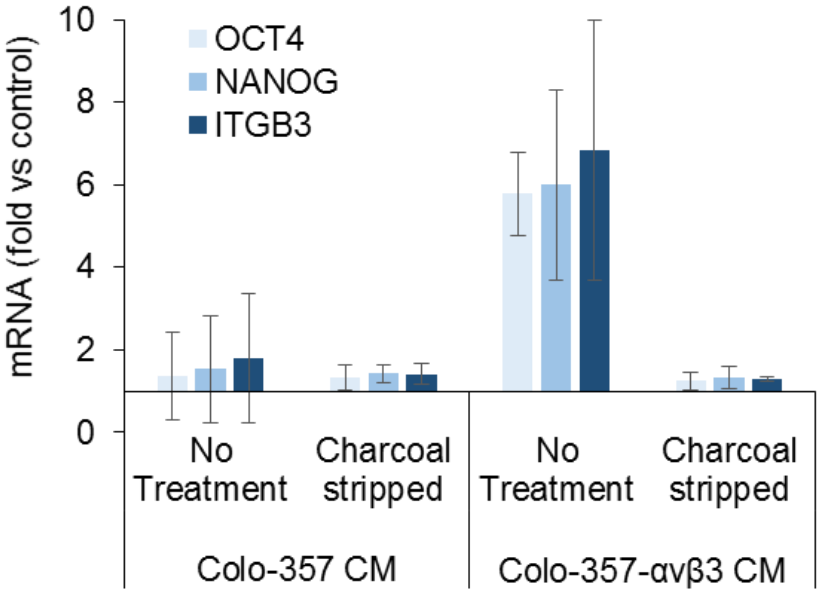


Figure 2.6. Charcoal stripping of conditioned media from αvβ3 expressing cells decreases induction of stem genes. qPCR data illustrating the effects of charcoal stripping (depletion of lipids) from conditioned media from αvβ3 expressing Colo-357 FG cells on stem gene induction. Bar graphs represent n = 3 independent experiments

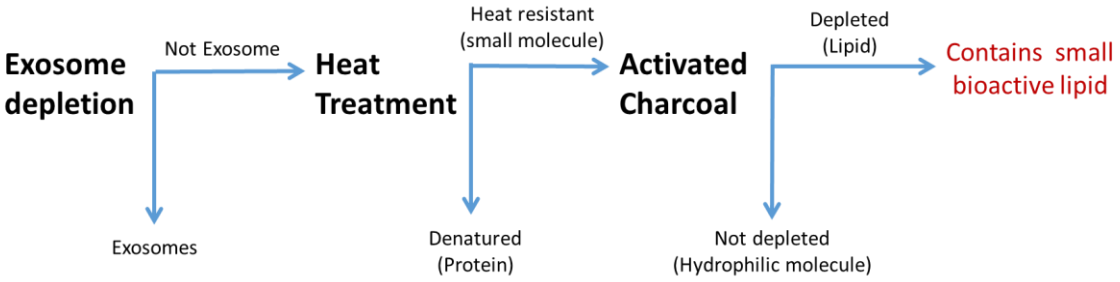


Figure 2.7. Schematic illustrating workflow of characterizing conditioned media. Workflow illustrating methods used to determine characteristic of secreted factor. First I depleted exosomes from conditioned media which determined the activity is not from exosomes. Then I boiled the conditioned media which determined the activity was heat resistant and, therefore, a small molecule. Finally, I depleted the conditioned media of lipids by charcoal stripping and discovered loss of activity. Therefore, I concluded the factor secreted by αvβ3 expressing cells is a small bioactive lipid.

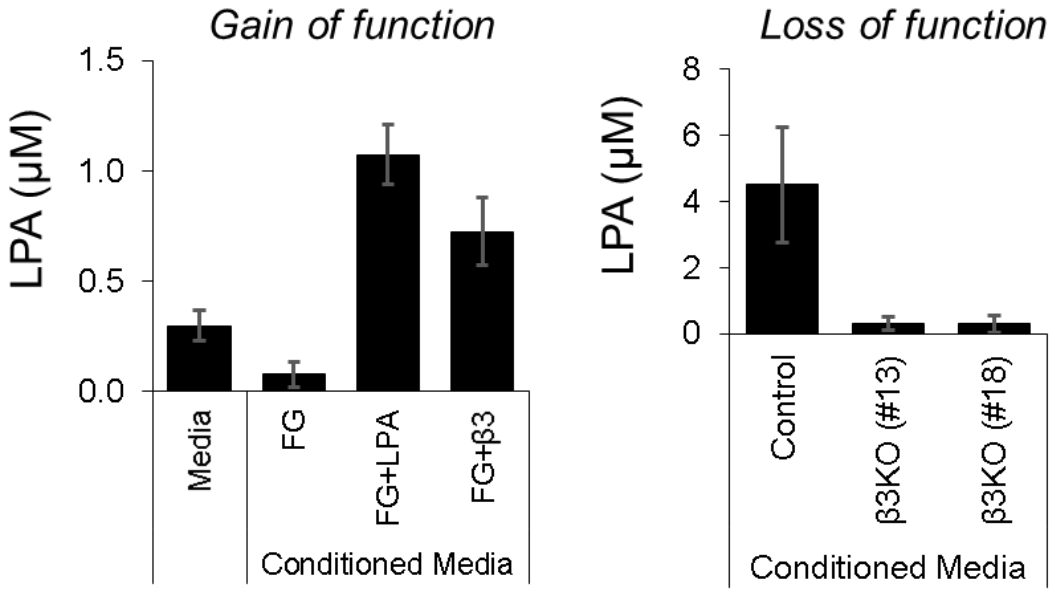


Figure 2.8. αvβ3 integrin is necessary and sufficient for LPA production and secretion. Competitive ELISA assay for detecting LPA in conditioned media from αvβ3 expressing cells using a gain-of-function (Colo-357 FG) and loss-of-function (PANC1) approach. Bar graphs represent n = 3 independent experiments.

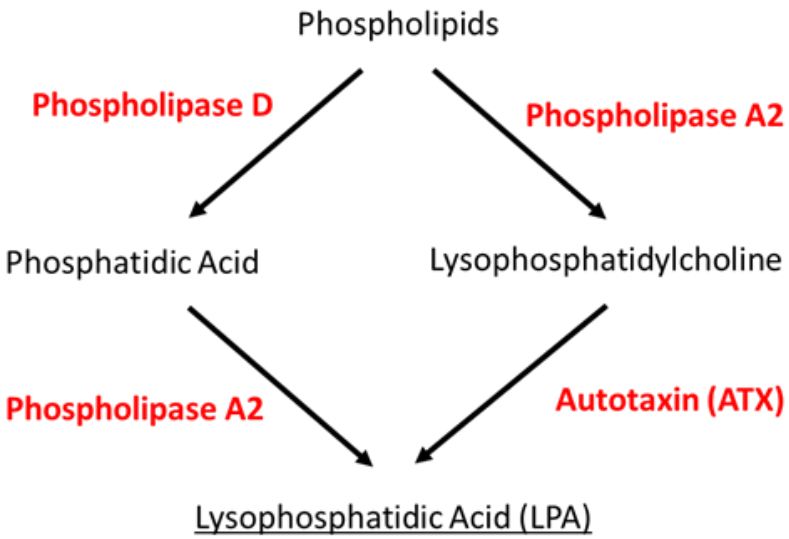


Figure 2.9. Schematic illustrating two metabolic pathways for producing LPA. Schematic illustrating the two pathways to producing LPA. One through Phospholipase D and Phospholipase A2 and another through Phospholipase A2 and Autotaxin.

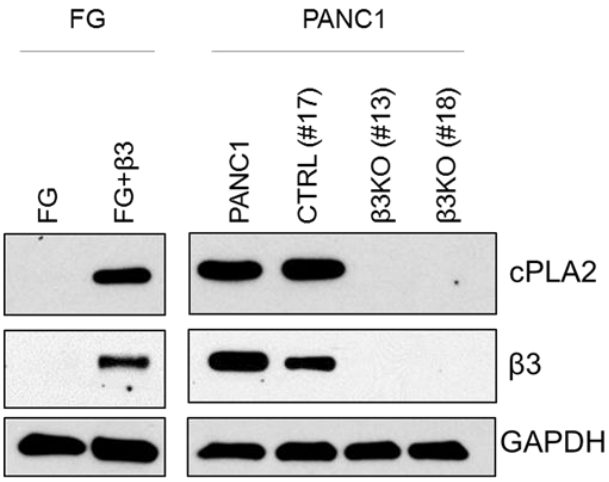


Figure 2.10. αvβ3 integrin is necessary and sufficient for the expression of cPLA2 Western blots showing cPLA2 expression in cells expressing αvβ3 integrin in a gain-of-function (Colo-357 FG) and loss-of-function (PANC1) approach. GAPDH was used as a loading control.

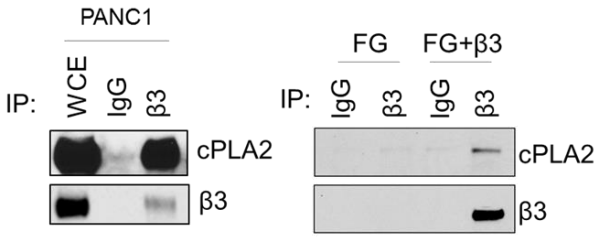


Figure 2.11. αvβ3 integrin complexes with cPLA2 Immunoprecipitation assay for detecting cPLA2 binding to αvβ3 integrin in cells expressing αvβ3 integrin in a gain-of-function (Colo-357 FG) and loss-of-function (PANC1)approach.

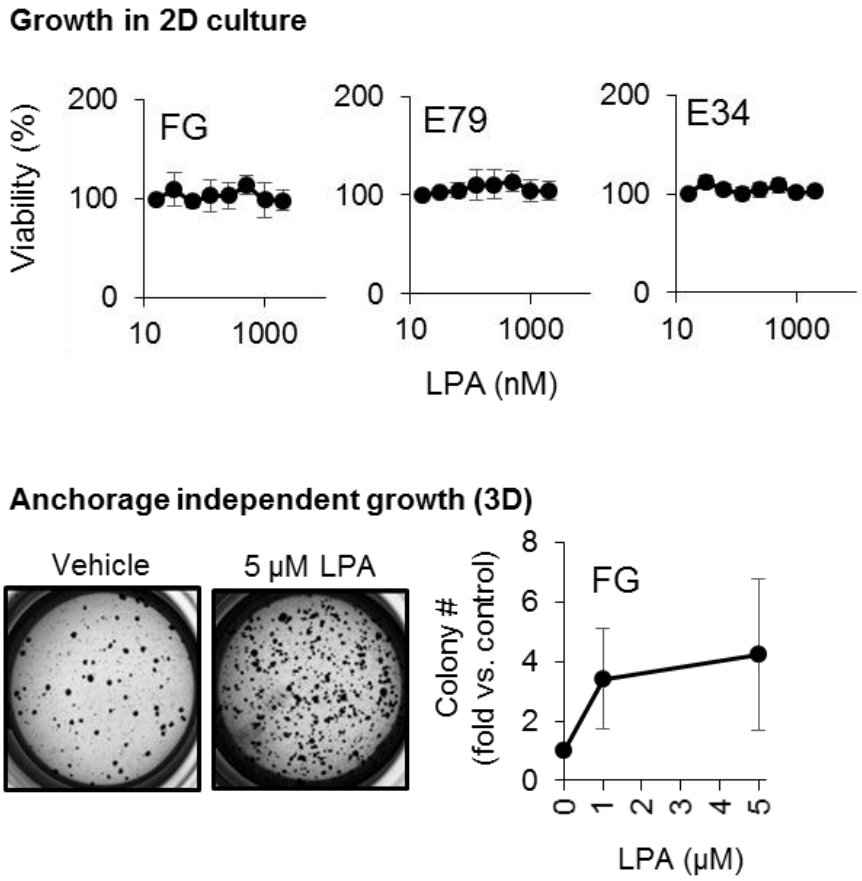


Figure 2.12. LPA increases anchorage independent growth. Upper panel: CellTiter Glo assay performed on adherent cells (2D) at different doses of LPA. Lower panel: Soft agar growth assay (3D) preformed on Colo-357 FG cells at different doses of LPA. Graphs represent mean±SD for n = 3 independent experiments

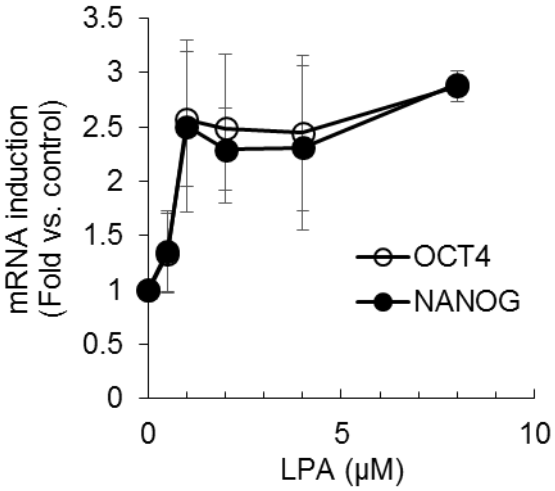


Figure 2.13. LPA induces the expression of stem genes *OCT4* and *NANOG*. qPCR data illustrating the effects of LPA on *OCT4* and *NANOG* expression in Colo-357 FG cells at different doses of LPA after 72 hours. Graph represent mean±SD for n = 2 independent experiments

Chapter 2.9 Supplemental Figures

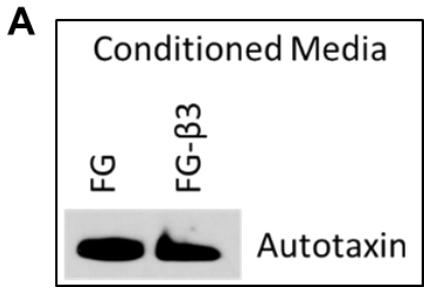


Figure S2.1. Tumor cell expression of αvβ3 does not affect expression of Autotaxin, a major enzyme in the production of LPA A. Western blot analysis of Autotaxin expression in cells ectopically expressing αvβ3

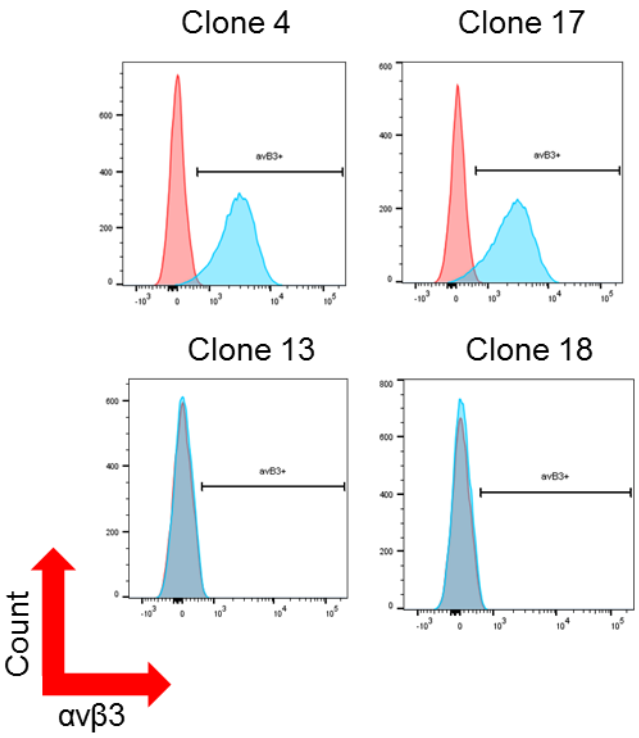


Figure S2.2. Validation of αvβ3 knockout in PANC1 cells A. Flow cytometry analysis quantifying αvβ3 expressing using LM609

	ITGB3	ITGA5	ITGB6	ITGB5	ITGB8	ITGA6	ITGB4	ITGA2	ITGA4	ITGB7	ITGB2
ENPP2	0.2	0.1	-0.3	-0.2	-0.1	-0.3	-0.5	-0.2	0.6	0.2	0.3
PLA2G10	-0.3	-0.3	0.2	0.1	-0.1	0.3	0.3	0.2	-0.4	0.0	-0.1
PLA2G12A	0.0	-0.3	-0.2	-0.3	0.0	-0.1	-0.4	-0.1	0.1	-0.3	-0.2
PLA2G1B	0.0	-0.2	0.1	-0.1	0.0	-0.1	-0.2	-0.1	0.1	0.0	0.1
PLA2G2A	0.1	0.1	-0.2	-0.1	0.0	-0.1	-0.3	-0.2	0.4	0.2	0.3
PLA2G2D	0.2	0.1	-0.1	-0.1	0.0	-0.2	-0.3	-0.1	0.6	0.6	0.5
PLA2G2F	-0.1	0.0	0.3	0.1	0.1	0.3	0.3	0.2	-0.2	0.0	0.0
PLA2G3	-0.1	-0.1	0.1	-0.1	0.0	0.1	0.1	0.1	-0.1	0.2	0.1
PLA2G4A	0.1	-0.1	0.2	0.1	0.2	0.3	0.1	0.3	0.0	-0.1	-0.1
PLA2G4C	0.1	0.1	-0.3	-0.2	0.0	-0.5	-0.4	-0.3	0.3	0.1	0.3
PLA2G5	0.3	0.6	-0.2	0.2	0.1	-0.3	-0.3	-0.2	0.5	0.2	0.4
PLA2G6	-0.3	-0.3	-0.3	-0.4	-0.3	-0.4	-0.1	-0.4	-0.2	-0.1	-0.3
PLA2G7	0.3	0.4	-0.1	0.2	0.1	-0.1	-0.1	0.0	0.5	0.4	0.7
PLA2R1	0.3	0.2	0.3	0.5	0.4	0.4	0.2	0.5	0.1	0.1	0.2
PLD1	0.1	-0.2	0.1	0.0	0.2	0.1	0.0	0.1	0.1	-0.1	0.1
PLD2	0.1	0.1	0.1	0.2	0.1	0.0	0.3	0.0	-0.1	0.1	0.1
PLD3	0.0	0.2	-0.3	0.1	-0.2	-0.4	-0.2	-0.3	0.1	0.0	0.3

Figure S2.3. Correlation analysis of phospholipases and integrins using TCGA dataset from PDAC patients A. Correlation coefficients between expression of all known integrins and all known phospholipases involved in production of LPA. Red, Positive correlation (≥ 0.3). Green, Negative Correlation (≤ -0.3).

Chapter 2.10 Materials and Methods

Cell lines:

Pancreatic ductal adenocarcinoma cell lines (PANC-1) were obtained from the American Type Culture Collection (ATCC) and grown in DMEM + 10% FBS. Cell line authentication was performed by the ATCC using short tandem repeat DNA profiles. The pancreatic carcinoma cell line COLO-357-FG was a gift from Dr. Shama Kajiji and Vito Quaranta (The Scripps Research Institute). Upon receipt, each cell line was expanded, cryopreserved as low-passage stocks, and tested routinely for mycoplasma. For ectopic expression, cells were transfected with a vector control and vector of interest using a lentiviral system as previously described [68] [44]. αvβ3 integrin expression was tested using qPCR analysis and flow cytometry as described below. CRISPR knockout efficiency was tested using flow cytometry.

Reagents, chemicals, and antibodies:

Lysophosphatidic Acid (18:1) was obtained from Avantii Polar Lipids, Inc. (Catalog #857130). LPA was diluted to a stock concentration of 1mM in dH2O. CellTiter Glo was obtained from Promega (Catalog #G7573). cPLA2 (Catalog #2832) and β3 integrin (Catalog #13166) antibody was obtained from Cell Signaling. GAPDH (Catalog #GTX627408) was obtained from GeneTex. LM609

antibody used to target αvβ3 integrin is produced in house and used at a 1:1000 concentration.

Quantitative RT-PCR:

Total RNA was collected from cancer cells using the RNeasy RNA Purification kit (Qiagen). cDNA was synthesized with SuperScript III reverse transcriptase using oligo(dT) primers (Invitrogen), and RT-PCR was performed on a LightCycler with SYBR Green (BioRad). RPL37A, Actin, and GAPDH expression was used as an internal reference to normalize input cDNA. Ratios of the expression level of each gene to that of the reference gene were then calculated. Primers used for this study are listed on **Supplementary Table 2.1**.

Gene expression analysis using public databases:

mRNA expression data in TCGA datasets was used to analyze the expression of integrins and phospholipases in Pancreatic Ductal Adenocarcinoma patients using cBioPortal for the TCGA RNA Seq V2 RSEM Pancreatic adenocarcinoma dataset (“PAAD”, TCGA, Provisional, 179 samples with progression data).

Anchorage-independent growth and cell viability assays:

Soft agar assays were performed as described [44] for 14-21 days with weekly media replacement. CellTiterGLO viability assays (Promega) were performed according to manufacturer instruction.

Flow cytometry:

Cell pellets were washed with HBSS, blocked with 1 % BSA in HBSS for 30 minutes at room temperature and stained with indicated primary antibodies or IgG isotype controls with or without fluorescently labeled secondary antibodies at room temperature for 1 hr respectively. After the staining, the cells were incubated with propidium iodide (PI) (Sigma, P4864), and flow cytometry was performed on BD LSRFortessa™ and analyzed using FlowJo (Treestar) software.

Statistical analysis:

All statistical analyses were performed using Prism software (GraphPad), Microsoft Excel (Microsoft), and SPSS (IBM). Two-tailed Mann Whitney U tests, Chi-squared tests, Fisher's exact tests, one-way ANOVA tests or t-tests were used to calculate statistical significance. $P<0.05$ was considered significant.

Table S2.1 List of qPCR primers used in this study

Target	Direction	Sequence
Actin	**Forward**	GCTGTGCTACGTCGCCCTG
	Reverse	GGAGGAGCTGGAAGCAGCC
GAPDH	**Forward**	AAGGTGAAGGTCGGAGTCAAC
	Reverse	GGGGTCATTGATGGCAACAATA
RPL37A	**Forward**	ATTGAAATCAGCCAGCACGC
	Reverse	GATGGCGGACTTTACCGTGA
POU5F1 (OCT4)	**Forward**	CTTGAATCCCGAATGGAAAGGG
	Reverse	GTGTATATCCCAGGGTGATCCTC
NANOG	**Forward**	TTTGTGGGCCTGAAGAAAACT
	Reverse	AGGGCTGTCCTGAATAAGCAG
ITGB3	**Forward**	GTGACCTGAAGGAGAATCTGC
	Reverse	TCACTCACTGGGAACTCGATG

Table S2.2 List of gRNAs used in this study

Target		Sequence
ITGB3	#1	ACCTCGCGTGGTACAGATGT
	#2	CCCAACATCTGTACCACGCG

Table S2.3 List of expression vectors used in this study

Target	Ectopic Expression
ITGB3	F12-β3
	F12-β3 759x
Control	F12-GFP

Chapter 2.11 Acknowledgements

Chapter 2 is coauthored with Tami Von Schalscha and David A. Cheresh. The dissertation author is the primary author of this chapter.

I'd like to acknowledge Tami Von Schalscha for her extraordinary technical help. I'd also like to thank Dr. Mohit Jain and his lab for their input in designing experiments to isolate and identify the secreted factor from αvβ3+ conditioned media.

Chapter 3 - Stress-inducible LPA Receptor 4 drives KRAS-mediated pancreatic cancer progression

Chapter 3.1 Abstract

Pancreatic cancer depends on both KRAS and a complex microenvironment, one component of which is lysophosphatidic acid (LPA), a bioactive lipid that promotes tumor progression. Here, I report that pancreatic cancer cells overcome the effects of cellular stress by exploiting a LPA/LPA Receptor 4 (LPAR4) signaling axis to further tumor progression by promoting cell invasion, macropinocytosis, and ROS elimination, adaptations to stress that have been linked to oncogenic KRAS. Stress or drug treatment specifically upregulates expression of LPAR4, that not only enhances stress mitigation and macropinocytosis, but promotes tumor initiation and metastasis. LPAR4 drives these processes via cAMP/PKA-mediated activation of Vasodilator Stimulated Phosphoprotein (VASP), which coordinates actin reorganization events supporting macropinocytosis and cell invasion. These findings demonstrate how KRAS mutant cells utilize LPAR4 as an inducible coping mechanism to overcome stresses imparted by the tumor microenvironment or induced by therapy, thereby promoting an aggressive pancreatic cancer phenotype.

Chapter 3.2 - Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer with dense stroma that impedes drug delivery and creates a nutrient-poor environment. The vast majority of PDAC tumors harbor activating KRAS mutations that provide cells with diverse survival advantages, allowing them to overcome the cellular stresses inherent in their environment in order to metastasize and spread throughout the peritoneal cavity.

While KRAS mutant tumors were once considered “undruggable”, identifying how such tumors are able to thrive is beginning to unlock new opportunities for therapeutic intervention [69]. For example, KRAS mutant tumors readily utilize macropinocytosis to engulf nutrients such as albumin [32], and this consumption provides a key source of amino acids to support malignant growth [70]. We recently reported that a Galectin-3 inhibitor can disable the macropinocytosis process and halt tumor growth [71], while Liu and colleagues exploited the enhanced macropinocytotic state to facilitate uptake of albumin-conjugated doxorubicin [72]. Hyperactive KRAS also controls reactive oxygen species (ROS) detoxification through increased expression of antioxidant genes, allowing cells to hold ROS at levels permissive for advanced cancer progression and metastasis but not trigger senescence or cell death [73]. Accordingly, blocking KRAS-controlled antioxidants such as Nrf2 [74] or HMOX-1 [75] can sensitize tumors to the effects of therapies that induce oxidative stress. Thus, understanding the basic biology of how pancreatic cancer cells adapt and survive

within their unique environment is a critical step in the design of new therapeutic strategies to control disease progression.

PDAC is known for a particularly dense stromal tumor microenvironment that impairs oxygen and nutrient perfusion, forcing tumor cells to adapt in order to survive. Another defining feature is malignant ascites that fosters an invasive phenotype and metastatic dissemination throughout the abdominal cavity. One component of ascites is lysophosphatidic acid (LPA), a bioactive lipid that supports a variety of cellular functions to boost cancer progression. Not only are levels of LPA significantly increased in serum and ascites from pancreatic cancer patients [76], but expression of Autotaxin, a primary enzyme responsible for LPA production, has recently been linked to pancreatic cancer progression, metastasis, and drug resistance [67].

Here, I report that KRAS mutant pancreatic cancer cells exposed to multiple forms of cellular stress become hypersensitive to the effects of lysophosphatidic acid (LPA). In the presence of stress or standard of care therapy, pancreatic cancer cells selectively upregulate LPA receptor 4, allowing them to utilize LPA to boost multiple hallmarks of KRAS mutant cancer, including macropinocytosis, ROS elimination, tumor initiation, cell invasion, and metastasis.

Chapter 3.3 - Stress sensitizes cells to LPA

Lysophosphatidic acid (LPA), a component of the tumor microenvironment and driver of pancreatic cancer progression [57] is known to promote stress tolerance [77]. Therefore, I asked whether LPA contributes to progression of pancreatic cancer in the context of cellular stress by investigating whether different forms of cellular stress can enhance pancreatic cancer sensitivity to LPA. While LPA is known to drive cell migration [78, 79], I show here that this response is amplified for pancreatic cancer cells that are challenged with sublethal levels of nutrient stress, oxidative stress, or standard of care chemotherapies, gemcitabine and paclitaxel (**Figure 3.1)**.

Hallmarks of KRAS-mediated stress tolerance include mitigation of nutrient stress through increased macropinocytotic uptake of extracellular nutrients [32, 80-82] and adaptation to oxidative stress by decreasing mitochondrial reactive oxygen species (ROS) levels [83-85]. Therefore, I asked whether these KRAS-mediated functions are enhanced during cellular stress in the presence or absence of LPA. When cells are challenged with nutrient stress, LPA not only induces macropinocytosis measured by uptake of TMR-dextran, but it increases cell viability only in the absence of stress (**Figure 3.2**). Strikingly, when cells are exposed to EIPA, a well-established inhibitor of macropinocytosis, the viability enhancement induced by LPA is completely diminished (**Figure 3.2**). Therefore, LPA provides protection against nutrient deprivation through increased macropinocytotic uptake of nutrients. In addition, when cells are exposed to

oxidative stress, LPA reduces mitochondrial ROS levels as measured using MitoSOX (**Figure 3.3**). Together, these findings demonstrate that cells exposed to LPA become resistant to multiple forms of stress, including nutrient deprivation, oxidative stress, or chemotherapy. When tumor cells encounter these stresses, LPA not only enhances cell migration/invasion but alleviates the effects of stress through increased macropinocytosis and mitochondrial ROS detoxification.

Chapter 3.4 – Pancreatic cancer cells selectively induce LPAR4 in response to cellular stress

LPA is a phospholipid derivative that can act as a signaling molecule by binding to 6 cognate receptors, LPAR1-LPAR6. In KRAS mutant pancreatic cancer cells, LPA can enhance migration, macropinocytosis, and ROS mitigation in the presence of cellular stress (**Figure 3.1-3.3**). Therefore, I examined a panel of pancreatic cancer cells lines for changes in LPA receptor levels following their exposure to cellular stress. Interestingly, the basal mRNA expression levels for each receptor show considerable heterogeneity in the absence of stress (**Figure 3.4A)**. However, nutrient stress, which sensitizes cells to LPA, selectively upregulates LPA receptor 4 (LPAR4) mRNA expression across all cell lines tested (**Figure 3.4B**). Similarly, oxidative stress induced by hydrogen peroxide treatment induces LPAR4 mRNA and protein expression (**Figure 3.5**). Although basal LPAR4 expression is generally low among pancreatic cancer cells, it is the only

LPA receptor that becomes significantly upregulated in response to stress, including the standard of care chemotherapies, paclitaxel and gemcitabine (**Figure 3.6**). To validate the clinical relevance of these findings, I examined the TCGA pancreatic adenocarcinoma (PAAD) dataset. Interestingly, among the six LPA receptors, LPAR4 as well as LPAR1 mRNA expression levels are significantly higher in tumor tissue from patients who progressed after primary therapy compared with patients who did not progress. (**Figure 3.7**). While LPAR1 and LPAR4 are linked to progression in these patients, only LPAR4 specifically responds to stress or therapeutic intervention.

Chapter 3.5 – LPAR4 induction is dependent on an open chromatin formation and the FOXO1 transcription factor

Since stress-induced chromatin remodeling contributes to cancer progression and drug resistance [86], I assessed whether the distal and proximal promoter regions of LPAR4 might undergo chromatin remodeling upon exposure to cellular stress. Indeed, markers of open chromatin (H3K27Ac and H3K4me3) were increased while H3K27me3 (a marker of closed chromatin) was decreased upon H_2O_2 treatment (**Figure S3.4**). To further dissect the mechanism of LPAR4 induction, I utilized the ENCODE database to identify a list of transcription factors predicted to bind to the LPAR4 promoter region (**Figure S3.5A**). Of these transcription factors, I conducted a TCGA analysis to identify transcription factor

whose expression positively correlated with LPAR4 expression in PDAC patients. Among these, I conducted siRNA knockdown for 2 transcription factors with established roles in stress response pathways (ZEB1 and FOXO1). Knockdown of FOXO1 prevented the induction of *LPAR4* mRNA expression in response to cellular stress (**Figure S3.5C**). Together, these findings indicate that the cellular stresses, including chemotherapies used in the clinic, may induce a unique dependence on LPAR4 through epigenetic and transcriptional changes.

Chapter 3.6 - LPAR4 drives LPA mediated stress-dependent pancreatic cancer cell invasion, macropinocytosis, tumor initiation and metastasis

Since LPAR4 is upregulated by cellular stress, I examined if LPAR4 expression in the presence of LPA was necessary and sufficient for pancreatic cancer cell response to stress and whether this might impact cancer progression. Indeed, while LPA promotes enhanced cell migration/invasion in response to stress, preventing LPAR4 expression reverses this effect. In fact, ectopic expression of LPAR4 is sufficient to promote LPA-induced migration in the absence of stress (**Figure 3.8A**). In order to validate the knockdown model, I conducted a rescue experiment by ectopically expressing LPAR4 in the shLPAR4 background. Indeed I found that LPAR4 expression in the knockdown model rescued the knockdown phenotype an increased migration capacity (**Figure S3.8**). These findings indicate that LPAR4 is necessary and sufficient for stress-induced

LPA-mediated pancreatic cancer cell migration/invasion. Given the role that macropinocytosis plays in overcoming nutrient stress of KRAS mutant tumor cells [32] I considered whether LPAR4 expression induced by nutrient stress might play a role in regulating the macropinocytotic response. I found that ectopic expression of LPAR4 increases macropinocytotic uptake while LPAR4 knockdown decreases macropinocytotic uptake when cells are exposed to nutrient stress (**Figure 2.8B**). Similarly, ectopic expression of LPAR4 decreases ROS levels while knockdown of LPAR4 increases ROS levels (**Figure 3.9**). Given that macropinocytosis and ROS mitigation are hallmarks of KRAS-addicted cells, our findings reveal that LPAR4 plays a key role in these KRAS-dependent properties of pancreatic cancer cells.

While LPA has been shown to promote tumor initiation and progression [57] little in known regarding which LPA receptor(s) are involved in this process. It is likely that tumor initiation required overcoming stress in the tumor microenvironment. I hypothesize this would result in LPAR4 expression enabling cells to adapt to the tissue microenvironment and form a productive tumor. This notion is supported by the finding that LPAR4 knockdown dramatically reduced the tumor initiation of pancreatic cancer cells implanted in mice. Accordingly, ectopic expression of LPAR4 on tumor cells was sufficient to increase tumor initiation (**Figure 3.10 and S3.7**). Moreover, for human tumors growing orthotopically in the pancreas of immunocompromised mice, LPAR4 expression significantly increased the incidence of metastasis to the hilar lymph node (**Figure 3.11A**), a metastatic site that is consistent with regional lymph nodes in patients [44]. Importantly, this

increase in metastasis is not due to a difference in primary tumor size (**Figure 3.11B**), indicating that LPAR4 enhances the invasive properties of tumor cells in these animals. Together, these experiments illustrate that LPAR4, the only stress inducible LPA receptor, contributes to the well-established role that KRAS plays in promoting stress tolerance, tumor initiation, and progression.

Chapter 3.7 - LPAR4-mediated PKA activity induces Vasodilator Stimulated Phosphoprotein (VASP) activation leading to cell migration/invasion and macropinocytosis

LPA can promote migration and proliferation by signaling through multiple LPA receptor and G protein-coupled receptor pairings to activate RAS and PI3K [87, 88]. However, LPAR4 is unique among the LPA receptors based on its specific ability to signal through $G_{\alpha s}$ leading to the activation of PKA [89]. To assess whether the PKA pathway is responsible for LPAR4/stress-mediated functions of LPA, I treated cells with the PKA activator Forskolin (FSK) [90], the PKA inhibitor H89 [91], and expression of Protein Kinase Inhibitor (PKI), an endogenous negative regulator of PKA [92]. I found that Forskolin can promote migration in the absence of stress or LPA, while H89 and PKI expression blocks the stress dependent LPA/LPAR4 induced migration/invasion (**Figure 3.12**).

At the biochemical level, I found that LPAR4 stimulates the phosphorylation and activation of Vasodilator Stimulated Phosphoprotein (VASP) (**Figure 3.13**), a well-established downstream target of PKA [93] that is involved in actin

polymerization [94]. In the absence of stress, ectopic LPAR4 was sufficient to drive LPA-mediated phosphorylation of VASP on Serine 157, a phosphorylation site indicating the active and membrane bound form of VASP [95]. Furthermore, in the presence of stress, LPAR4 knockdown prevented VASP pS157 in response to LPA. Mechanistically, VASP coordinates actin polymerization to facilitate formation of cell membrane protrusions on the leading edge of migrating cells or formation of the macropinosome [96]. Indeed, LPA enhances cell migration and macropinocytosis in response to stress (**Figure 3.1 and 3.2**) or ectopic LPAR4 (**Figure 3.8A-B**). VASP knockdown reverses this LPA/LPAR4 dependent migration and macropinocytotic response (**Figure 3.14**). These finding reveal stress-induced LPAR4 expression, once activated by LPA, can drive PKA and VASP dependent pancreatic cancer cell migration and macropinocytosis.

Together, these findings indicate that pancreatic cancer cells can respond to stress by selectively upregulating expression of LPAR4, a receptor that is otherwise minimally expressed. This pathway offers a unique opportunity for stress mitigation by utilizing LPA known to be present in the pancreatic cancer stroma or ascites to activate the actin polymerization protein, VASP, that coordinates cytoskeletal remodeling events to support multiple KRAS-mediated adaptations to stress leading to increased tumor progression and metastasis (**Figure 3.15**).

Chapter 3.8 - Discussion

Pancreatic cancers contain a notorious stromal component that not only impedes drug delivery, but also contributes to an aggressive tumor phenotype. LPA present within this tumor microenvironment has been linked to increase cancer progression. Accordingly, Sherman and colleagues recently reported the conversion of lysophosphatidylcholine derived from pancreatic stroma cells into LPA by tumor cell-derived Autotaxin [67]. This cooperation between PDAC cells and the cancer-associated stroma promotes tumor growth that can be reversed upon LPA depletion through Autotaxin inhibition. Furthermore, LPA is well-known for its involvement in wound healing [97-99], and its ability to exacerbate cancer progression relates to the postulation that tumors are “wounds that never heal” [100]. While LPA contributes to wound repair and cancer progression, little is known about how cells respond to LPA and which LPA receptors contributes to these processes. Our findings reveal that pancreatic cancer cells utilize LPA to potentiate several stress mitigating functions of mutant KRAS through expression of LPAR4 upon oxidative stress and nutrient deprivation, stresses that are commonly associated with both cancer and tissue injury.

While LPAR1-3 are known to promote tumor cell migration [101, 102] and tumor initiation [57], the functions of LPA receptors 4-6 are less clear. Here, I find that LPAR4 expression levels are undetectable on KRAS mutant pancreatic cancer cells in the absence of stress. In fact, LPAR4 expression is unlikely to be detected in cultured cells due to their growth in a serum containing nutrient rich environment

typically used to propagate cells *in vitro*. However, LPAR4 is expressed in tumor cells after exposure to various stresses including: nutrient deprivation, ROS, and cancer therapies. Our findings reveal that tumor cells exposed to these stresses selectively upregulate LPAR4 to unlock a series of specific LPA-induced biological properties that contribute to stress mitigation.

LPA is an upstream activator of KRAS and PI3K [103]. Interestingly, in pancreatic cancer, oncogenic KRAS has been linked to ROS elimination [104], migration [82] and macropinocytosis [32], which are critical for stress mitigation and tumor progression *in vivo*. We recently reported that αvβ3 integrin, a marker of mesenchymal tumor cells, promotes a state of KRAS addiction since it facilitates nutrient uptake by macropinocytosis and ROS mitigation [71]. As seen in KRAS addicted cells, I show that LPA/LPAR4 enhances migration, macropinocytosis, as well as ROS mitigation *in vitro*. Moreover, LPAR4 promotes enhanced tumor initiation and spontaneous metastasis *in vivo* suggesting that LPA/LPAR4 is able to potentiate KRAS-dependent functions leading to tumor progression. Indeed, LPAR4 specifically expressed by pancreatic cancer cells in response to microenvironmental stress and/or therapeutic intervention, suggests a previously undefined role for LPA in stress mitigation that is triggered when LPAR4 becomes upregulated.

Among the six known LPA receptors, LPAR4 is unique with regard to its capacity to initiate intracellular signaling through $G\alpha_s$ [89] to induce activation of PKA and phosphorylation of VASP on S157. Once phosphorylated and activated,

VASP can promote actin polymerization leading to lamellipodia formation [105] and migration. Interestingly, I found that VASP is necessary for LPA/LPAR4 dependent migration. Previously, Mills and colleagues have found that LPA can promote lamellipodia formation in prostate cancer cells via VASP activation [63] but it is not clear that this required LPAR4. In conjunction with VASP's role in actin polymerization, macropinocytosis, a type of cellular endocytosis, relies on actin polymerization resulting in membrane protrusions to engulf large quantities of extracellular fluid to overcome nutrient stress. Strikingly, I found that VASP is necessary for both LPA and LPAR4 dependent macropinocytosis which facilitated increased cell survival when cells were exposed to nutrient stress. These findings indicate an important role that the LPAR-LPAR4-PKA signaling axis plays in stress mitigation through enhanced migration and macropinocytosis via activation of VASP.

Additionally, our findings raise the question of whether the LPA-LPAR4-PKA-VASP signaling axis can be targeted for future potential therapies. Currently there are two commercially available PKA inhibitors, H-89 and KT 5720, although they have off-target effects [91]. LPA receptor inhibitors, such as Ki16425 that inhibits LPAR1-3, have unfavorable bioavailability *in vivo*. Interestingly, the Autotaxin inhibitor, ONO8430506, drastically diminished the production of LPA [106] and decreased pancreatic cancer tumor progression [67] with relatively favorable pharmacokinetic and pharmacodynamics properties [106]. Our findings suggest that by blocking LPA production or LPAR4 receptor activity it may be

possible to sensitize KRAS mutant pancreatic cancers to standard-of-care chemotherapeutics by targeting upstream of LPAR4.

Overall, our study highlights how pancreatic cancer cells change their repertoire of LPA receptors in order to better cope with a variety of stresses, including nutrient scarcity, oxidative stress, or cancer therapy. Our findings are consistent with the concept that the fibrotic component of pancreatic cancer creates a nutrient-poor, wound-like microenvironment [67] that can cause cancer cells to express LPAR4. By allowing them to utilize LPA produced from stromal cells, cancer cells can enhance their capacity for nutrient uptake through macropinocytosis and to mitigate ROS to boost survival in the face of limited nutrients or other forms of stress. In conclusion, our results demonstrate that stress induced expression of LPAR4 in the presence of external sources of LPA enables pancreatic cancer cells to counteract the effects of cellular stress or cancer therapy thereby enhancing their malignant properties.

Chapter 3.9 Figures

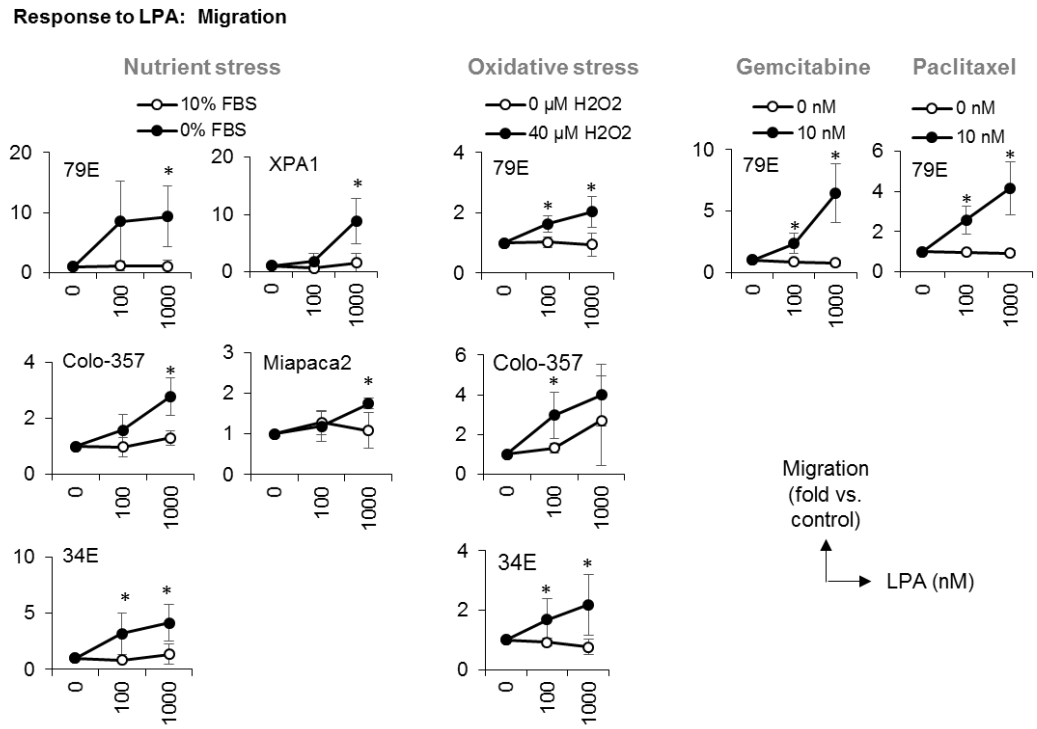


Figure 3.1. Stress sensitizes pancreatic cancer cells to lysophosphatidic acid (LPA)-mediated migration. Graphs show cell migration measured by Transwell assay to evaluate the effect of increasing doses of LPA (0, 100, 1000nM) between normal growth conditions vs. stress conditions, including nutrient stress (serum deprivation), oxidative stress (hydrogen peroxide), and cancer therapy (gemcitabine and paclitaxel). Data is normalized to vehicle control. Graph shows mean±SD for n=3 or more independent experiments. *, P<0.05 compared to control using Student *t* test.

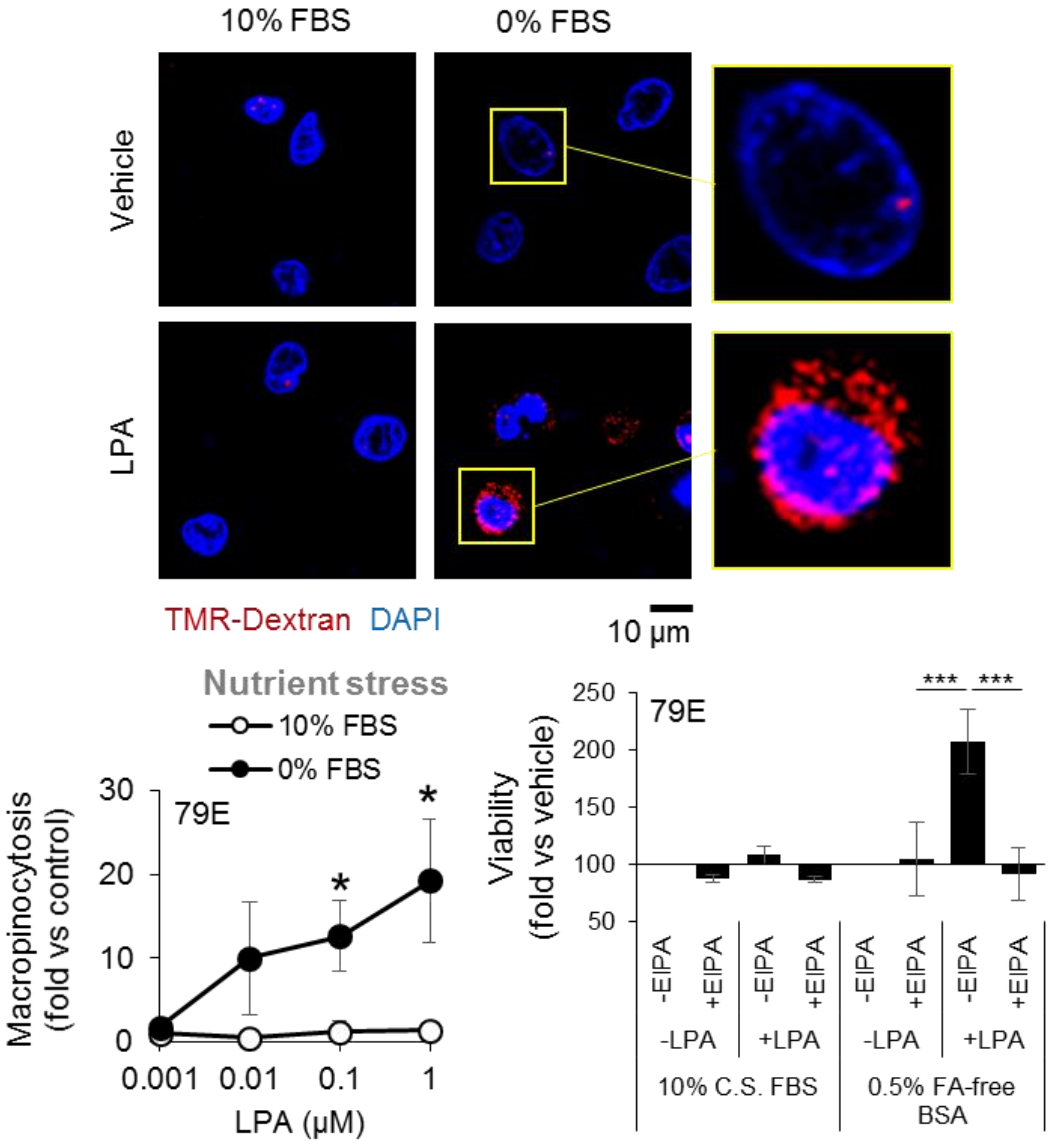


Figure 3.2. LPA increases macropinocytotic uptake in the presence of nutrient stress leading to increased viability Macropinocytosis uptake was evaluated using TMR-Dextran as a marker of macropinosomes (red), illustrating LPA dose-dependent effect on macropinocytosis between normal conditions and nutrient deprived conditions +/- treatment with LPA. CellTiter-Glo assay was preformed to assess viability. EIPA was used at 10 µM. LPA was used at 1 µM. Pictures are representative of 3 independent experiments. Scale bar = 10 µm. Graph shows mean±SD for n=3 independent experiments. Viability was assessed using CellTiter Glo. *, P<0.05. ***, P<0.0005 compared to control using Student *t* test

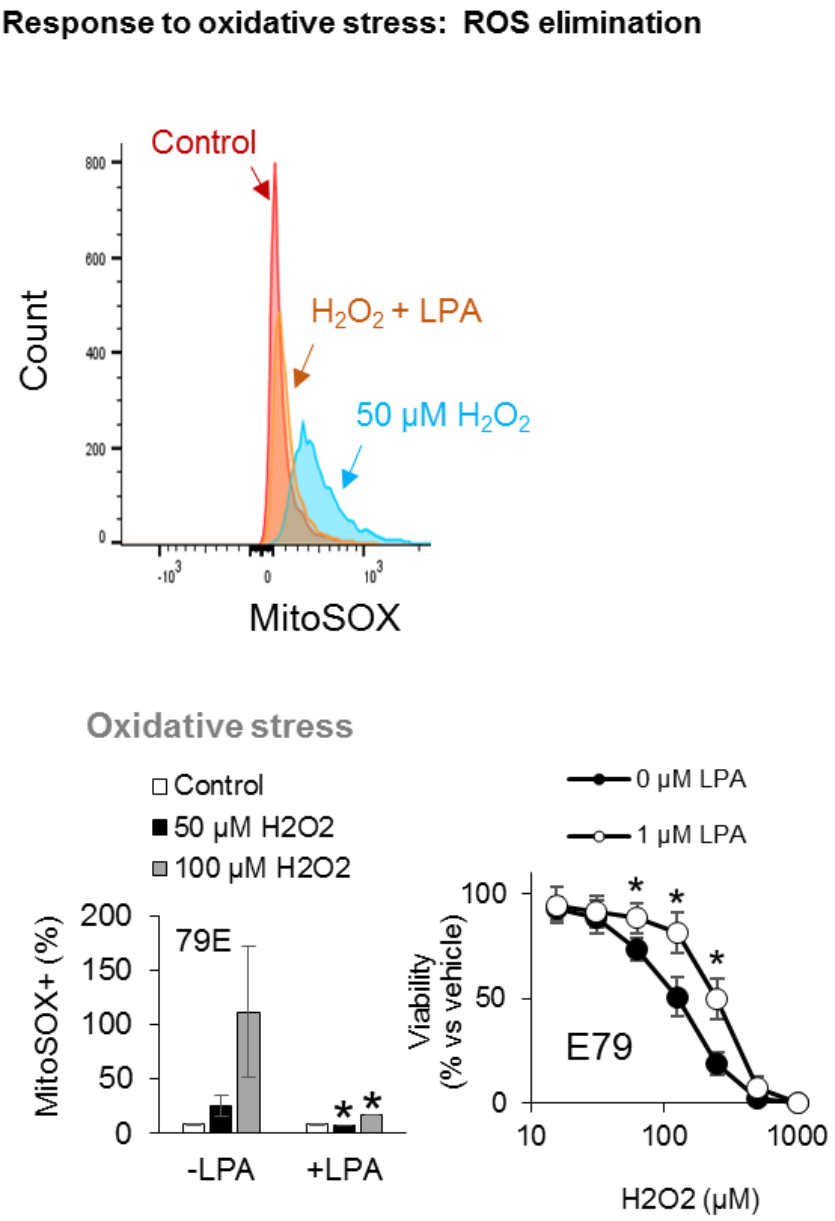


Figure 3.3. LPA reduces Mitochondrial ROS levels when exposed to hydrogen peroxide leading to increased viability. Flow cytometry analysis for MitoSOX, a marker of mitochondrial reactive oxygen species (ROS) content, upon treatment of LPA in the presence or absence of oxidative stress (hydrogen peroxide). Bars represent mean±SD for at least three independent experiments. Viability was assessed using CellTiter Glo. Statistical significance was measured using the two-tailed Student *t* test. *, P<0.05 compared to control.

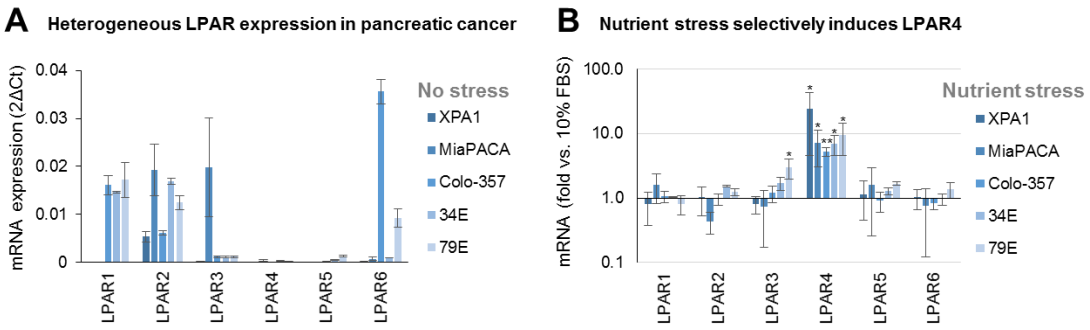


Figure 3.4. Cancer cells selectively induce LPAR4 in response to nutrient deprivation. **A**. Graph shows mean±SD for n=3 independent experiments for basal mRNA expression of each LPA receptor in each pancreatic cancer cell line incubated in complete media. Data is normalized to housekeeping genes *RPL37A*, *GAPDH*, and *ACTB*. **B.** Graphs show mean±SD for n=3 independent experiments for mRNA expression for LPA Receptors 1-6 for cells subjected to nutrient stress (serum deprivation) normalized to complete media. Statistical significance was measured using the two-tailed Student *t* test. *, P<0.05, **, P<0.005

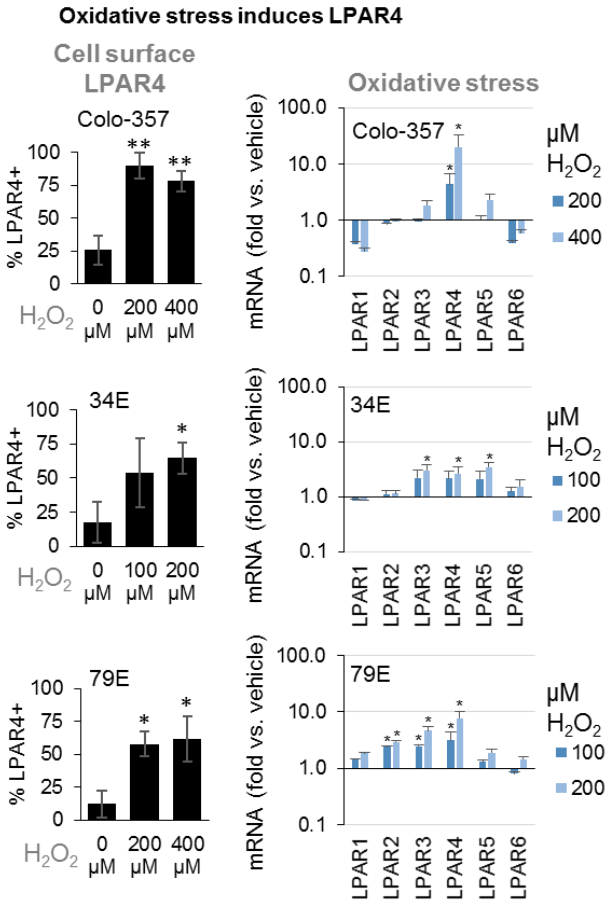


Figure 3.5. Cancer cells induce LPAR4 expression in response to oxidative stress. Flow cytometry and qPCR analysis of LPAR4 expression for cells treated with increasing doses of oxidative stress (hydrogen peroxide). Bar graphs show mean±SD for n=3 independent experiments. Flow cytometry is represented by percent of cells positive for LPAR4 cell surface expression. qPCR is represented by fold increase compared to control. Statistical significance was measured using the two-tailed Student *t* test. *, P<0.05, **, P<0.005

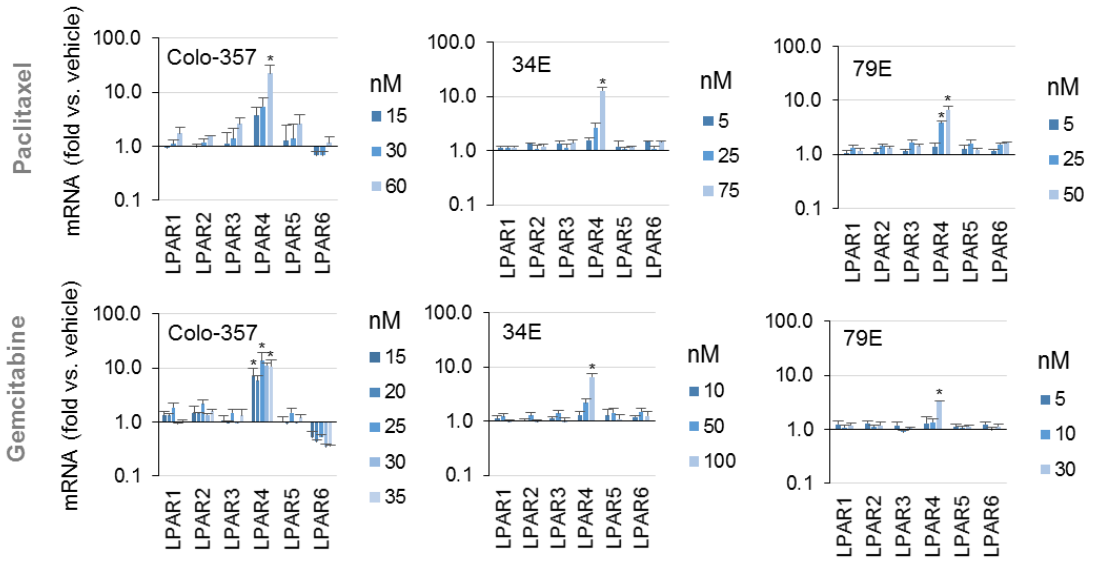


Figure 3.6. Cancer cells selectively induce LPAR4 in response to standard-of-care chemotherapy. Graphs show mean±SD for n=3 independent experiments for mRNA expression of LPA Receptors 1-6 in cells treated with cancer therapies paclitaxel or gemcitabine normalized to vehicle treatment. Statistical significance was measured using the two-tailed Student *t* test. *, P<0.05,

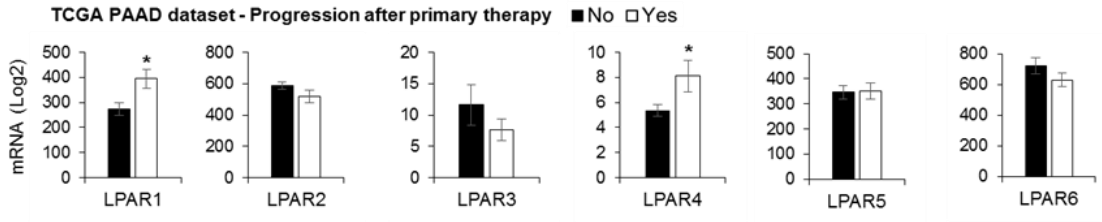


Figure 3.7. TCGA analysis shows that progression after primary therapy in PDAC patients correlate with increased LPAR4 expression. Graphs show mean±SD mRNA expression levels for each LPA receptor for tumors that did not progress after primary therapy (No, black bars) vs. tumors that did progress (Yes, open bars). Statistical significance was measured using the two-tailed Student *t* test. *, P<0.05

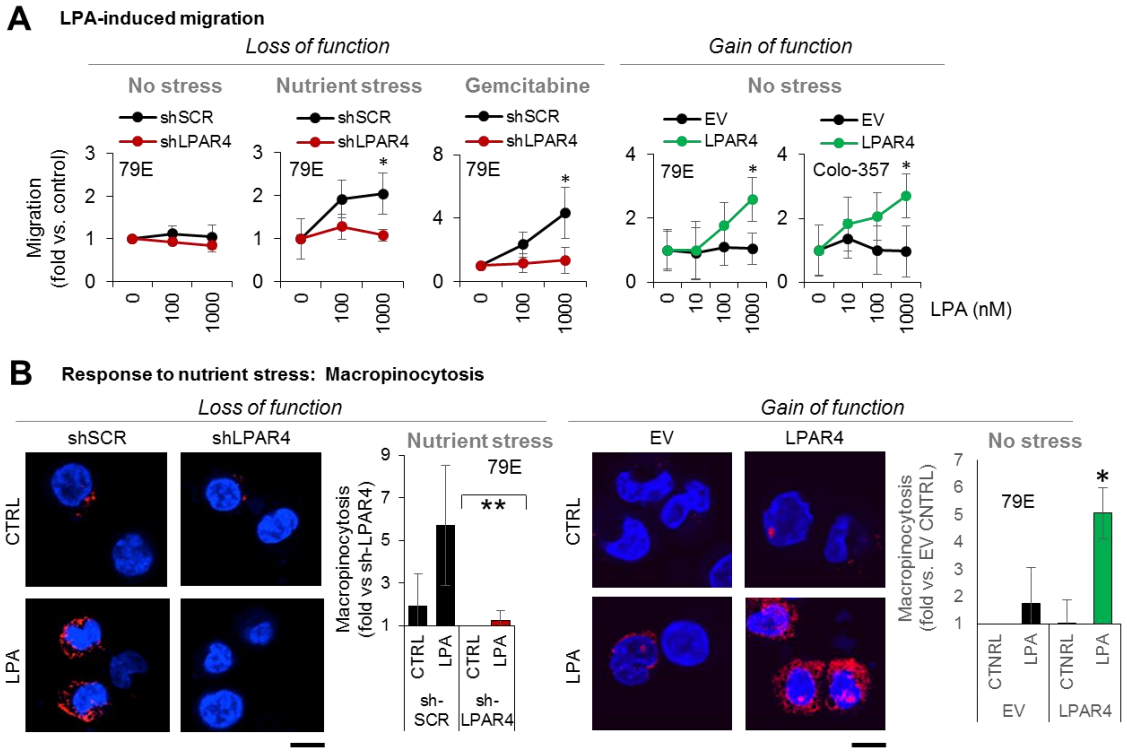


Figure 3.8. LPAR4 drives LPA mediated stress-dependent pancreatic cancer cell migration and macropinocytosis. A. Graphs show cell migration measured by Transwell assay to evaluate the effect of knockdown of LPAR4 with increasing doses of LPA (0, 100, 1000nM) for cells exposed to nutrient stress (0% FBS) and cancer therapy (10nM gemcitabine). Cell migration was also assessed with ectopic expression of LPAR4 in the absence of stress. Data is normalized to vehicle control. Graph shows mean±SD for n=3 independent experiments. *, P<0.05 compared to control using Student *t* test. **B.** Macropinocytosis was quantified by TMR-Dextran uptake (red), illustrating effects of knockdown of LPAR4 upon stress and ectopic expression of LPAR4 in the absence of stress on macropinocytosis. Blue, DAPI. Scale bar = 10 µm. Graph shows mean±SD for n=5 independent experiments. *, P<0.05 compared to control using One-way ANOVA.

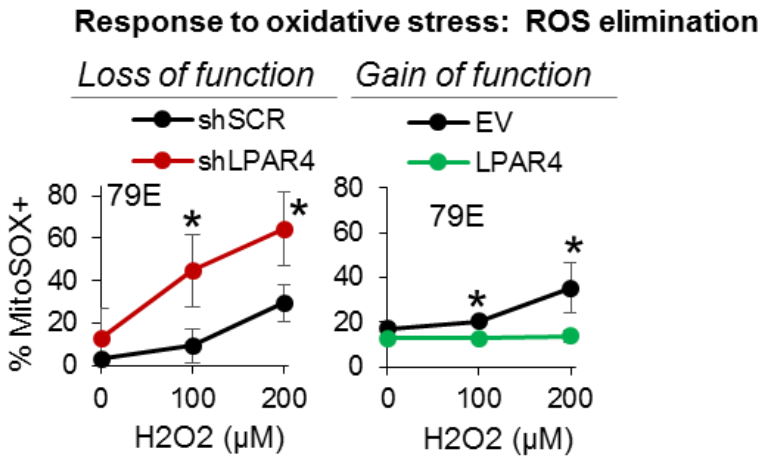


Figure 3.9. LPAR4 drives ROS mitigation. Flow cytometry analysis for MitoSOX, a marker of mitochondrial reactive oxygen species (ROS) content, upon knockdown of LPAR4 and ectopic expression of LPAR4 in the presence or absence of oxidative stress (hydrogen peroxide). Bars represent mean±SD for at least three independent experiments. Statistical significance was measured using the two-tailed Student *t* test. *, P<0.05 compared to control

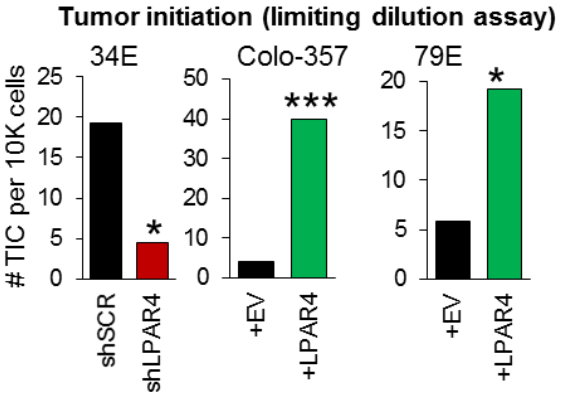


Figure 3.10. LPAR4 drives tumor initiation. Tumor initiating capacity was determined using limiting dilution experiments. Graphs show the estimated number of tumor initiating cells (TIC) calculated using ELDA software. *P <= 0.05; ***P <= 0.0005 compared to control.

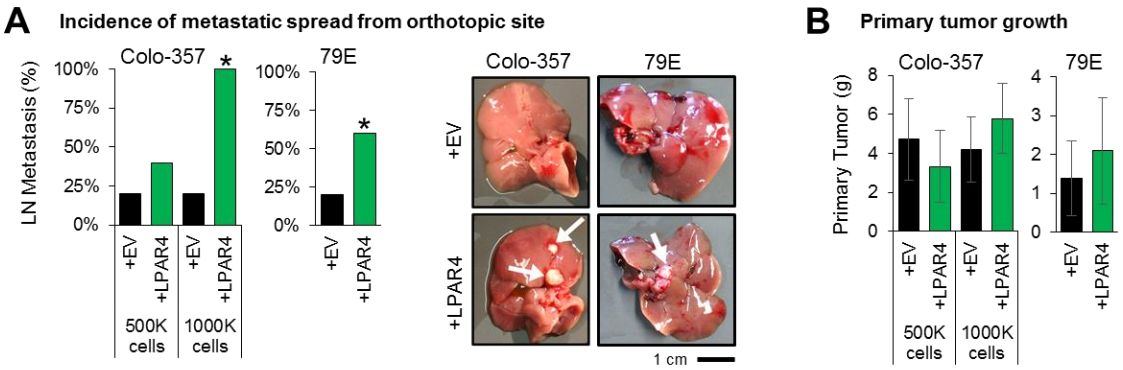


Figure 3.11. LPAR4 drives metastasis A. Representative images show large metastatic nodules (white arrows) 8 weeks after tumor cells were implanted orthotopically to the pancreas. Graphs show the percent of mice with detectable spontaneous metastasis to the hepatic hilar lymph nodes. N = 10 mice per group. *P <= 0.05 compared to control using Fisher's exact test. **B.** Primary tumor weight, shown as mean±SD. n=10 mice per group. *P <= 0.05 compared to control using Student *t* test.

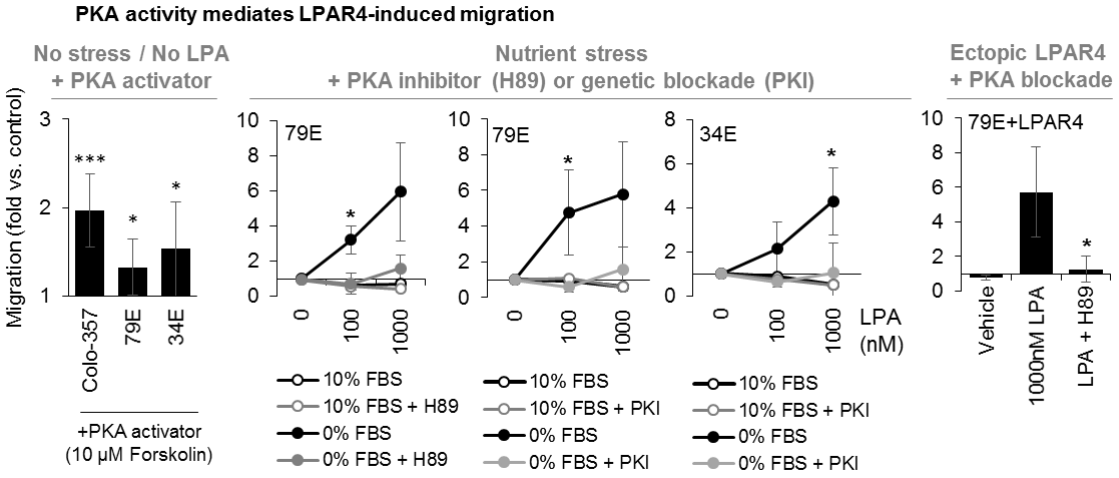


Figure 3.12. PKA activity is necessary and sufficient for LPAR4 mediated migration. Graphs show the effect of Forskolin (PKA activator, 10 µM), PKI (Protein Kinase Inhibitor), and H89 (pharmacological PKA inhibitor, 5 µM) on cell migration. Graph shows mean±SD for n=5 independent experiments. * P<0.05, *** P<0.0005 compared to control using Student *t* test.

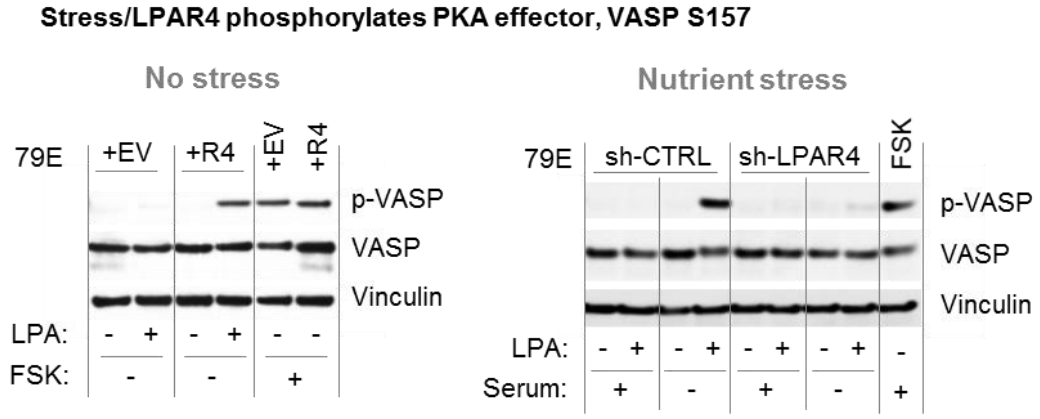


Figure 3.13. LPAR4-mediated PKA activity phosphorylates Vasodilator Stimulated Phosphoprotein (VASP) activation. Western blot analysis of activation and phosphorylation of VASP S157, a downstream target of the PKA pathway, between control, knockdown, and ectopic expression of LPAR4. Representative western blot of n = 2 independent experiments.

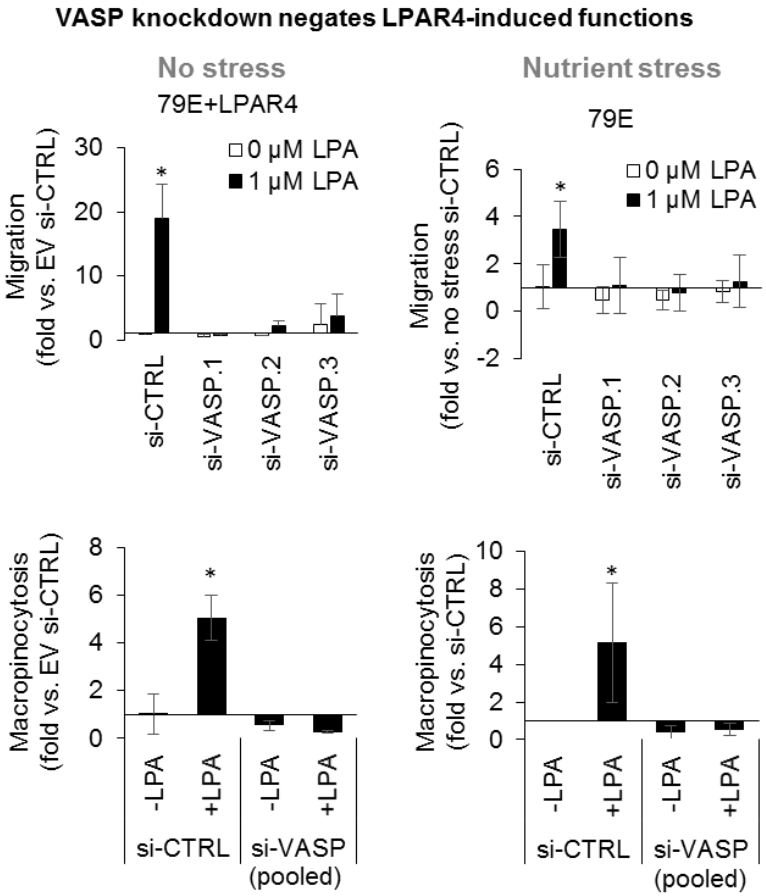


Figure 3.14. VASP is necessary for LPAR4 mediated migration and macropinocytosis. Upper panel: Cell migration analysis of VASP knockdown in an ectopic expression of LPAR4 and a stress-induced model. Lower panel: Macropinocytosis uptake analysis performed on cells with and without VASP knockdown in an ectopic expression of LPAR4 and a stress-induced model treated with LPA at 1 µM. Graphs show mean±SD for n=3-5 independent experiments. * P<0.05 compared to control using One-Way ANOVA.

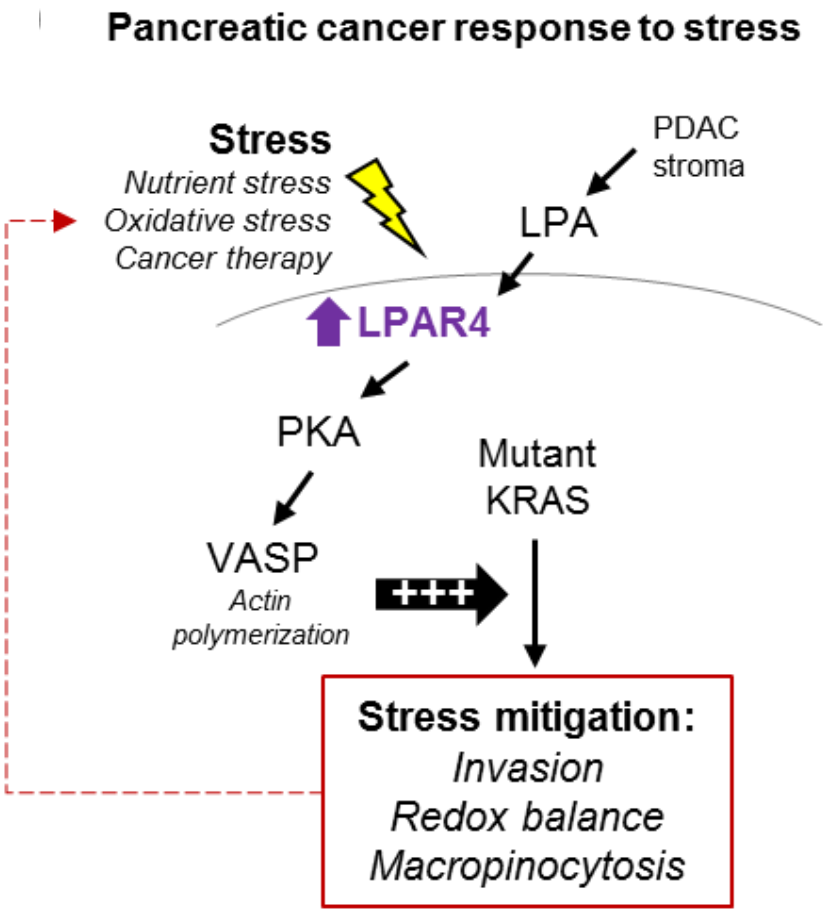


Figure 3.15. LPAR4-mediated PKA activity induces Vasodilator Stimulated Phosphoprotein (VASP) activation leading to cell migration/invasion and macropinocytosis, Continued. D. Schematic illustrating how multiple stresses can induce the expression of LPAR4. LPA produced from the pancreatic cancer stroma can lead to the activation of PKA and VASP. VASP can then promote macropinocytosis and migration, which are KRAS-mediated functions. Pancreatic Ductal Adenocarcinoma, PDAC; Lysophosphatidic Acid, LPA; LPA Receptor 4, LPAR4; Protein Kinase A, PKA; Vasodilator Stimulated Protein, VASP.

Chapter 3.10 Supplemental Figures

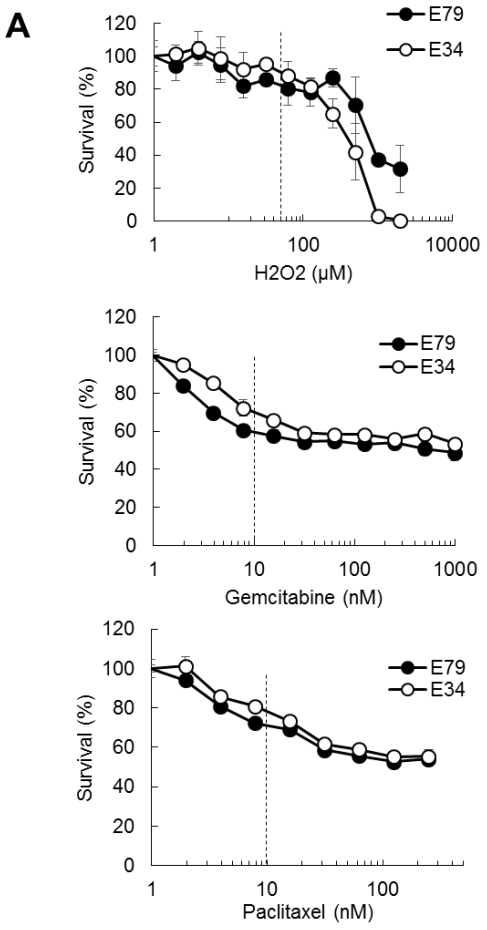
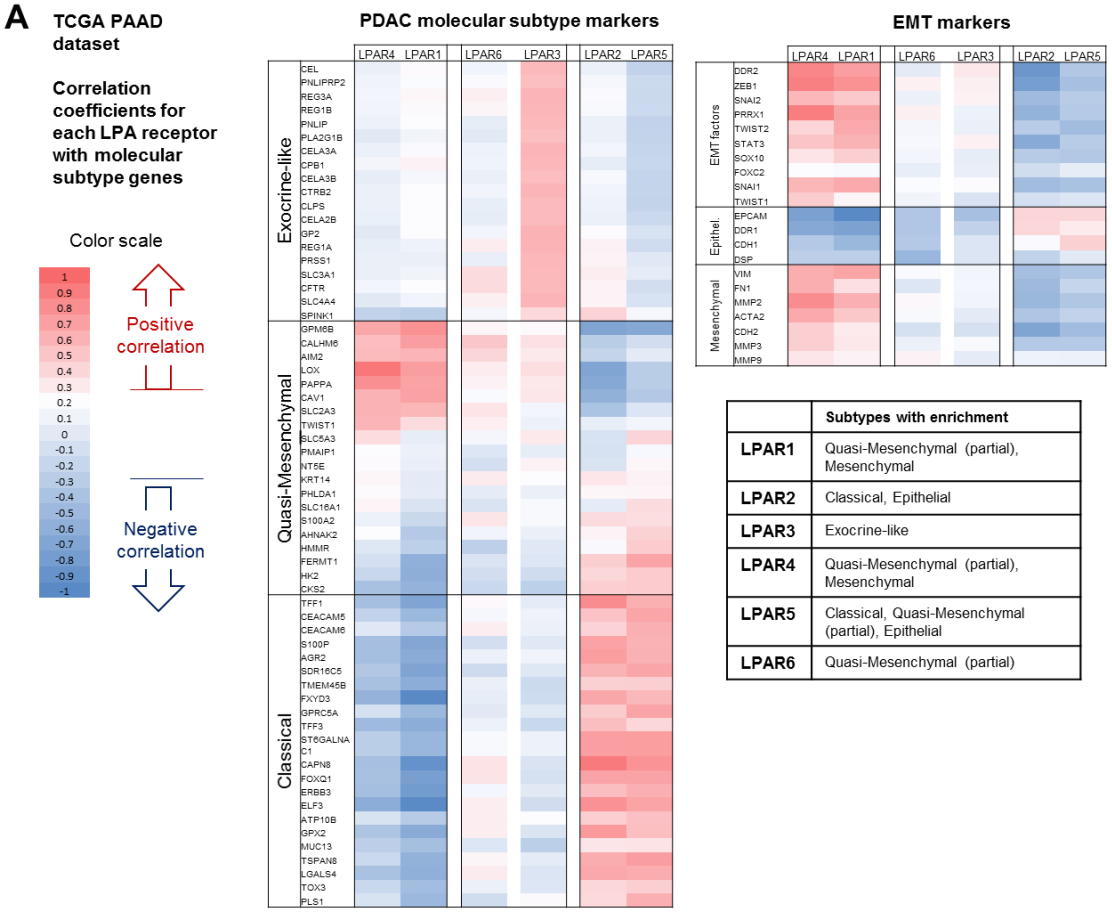
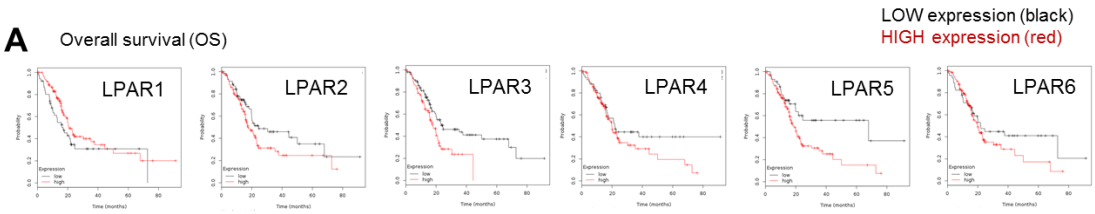


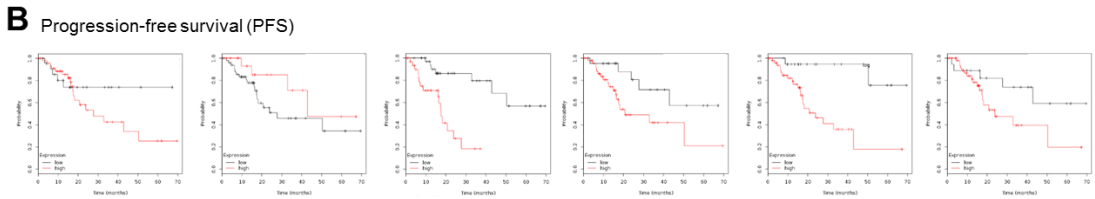
Figure S3.1. Confirmation of sublethal doses of stress. A. CellTiter Glo was used to assess viability at different doses of hydrogen peroxide, gemcitabine, and paclitaxel. Dashed line represents sub-lethal dose used to pre-stress cells. Data is normalized to vehicle control. Graph shows mean±SD for n=3 independent experiments.



	Subtypes with enrichment
LPAR1	Quasi-Mesenchymal (partial), Mesenchymal
LPAR2	Classical, Epithelial
LPAR3	Exocrine-like
LPAR4	Quasi-Mesenchymal (partial), Mesenchymal
LPAR5	Classical, Quasi-Mesenchymal (partial), Epithelial
LPAR6	Quasi-Mesenchymal (partial)

Figure S3.2. TCGA analysis from PDAC patients showing correlation of LPA Receptors to EMT genes. A. Heatmaps show Pearson's correlation coefficients for each LPA receptor with markers of pancreatic cancer molecular subtypes (Exocrine-like, Quasi-Mesenchymal, Classical) and EMT genes (EMT factors, Epithelial markers, Mesenchymal markers). Color scale indicates levels of positive correlation (red) vs. negative correlation (blue).





C Summarizing statistics

		LPAR1	LPAR2	LPAR3	LPAR4	LPAR5	LPAR6
Log rank P	OS	0.10	0.08	0.01	0.20	0.01	0.26
	PFS	0.25	0.16	0.00	0.02	0.00	0.06
Hazard ratio	OS	0.7	1.5	1.7	1.4	2.0	1.3
	PFS	1.8	0.5	6.4	3.1	7.9	2.6

Figure S3.3 Kaplein-Meier Curves associating expression of each LPA receptor to survival. A. Kaplan-Meier curves comparing overall survival of PDAC patients between high (red) and low (black) expression of each LPA receptor. **B.** Kaplan-Meier curves comparing Progression-free survival of PDAC patients between high (red) and low (black) expression of each LPA receptor. **C.** Summarized statistics showing Hazard Ratio and Log rank P.

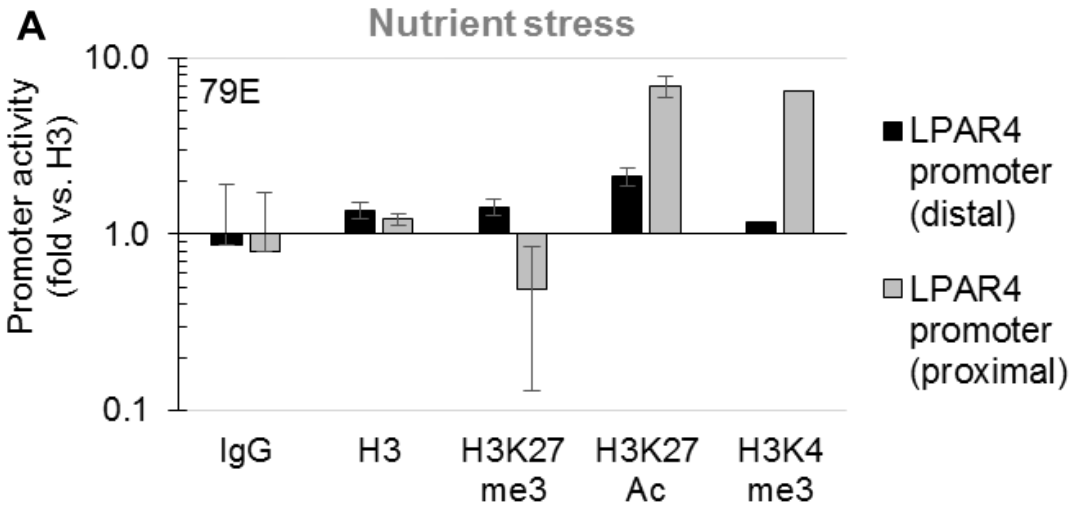


Figure S3.4. ChIP-qPCR analysis of LPAR4 promoter region upon nutrient stress. Fold increase of presence of histone markers at the LPAR4 promoter region (distal and proximal) upon nutrient deprivation. H3, control. H3K27me3, Marker of heterochromatin (closed chromatin). H3K27Ac & H3K4me3, Marker of euchromatin (open chromatin)

A

1 – Transcription factors predicted to bind LPAR4 promoter from ENCODE database

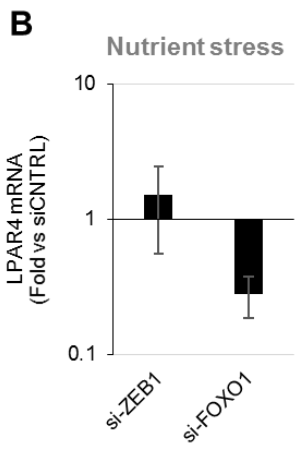
CTCF
ESR1
FOXM1
FOXO1
HSF1
IRF4
MEF2C
NFIC
RAD21
RUNX3
SOX9
SPI1
TAF1
TBP
ZEB1

2 – Positive correlation with LPAR4 for the TCGA Pancreatic Adenocarcinoma (PAAD) dataset

	Rho	*P-value*	*Q-value*
ZEB1	0.76	2E-34	2E-31
MEF2C	0.69	6E-27	1E-24
ESR1	0.55	2E-15	6E-14
FOXO1	0.51	3E-13	5E-12
IRF4	0.47	5E-11	6E-10
RUNX3	0.41	2E-08	1E-07
SPI1	0.40	2E-08	2E-07

3 – Roles in stress response (literature)

ZEB1
FOXO1



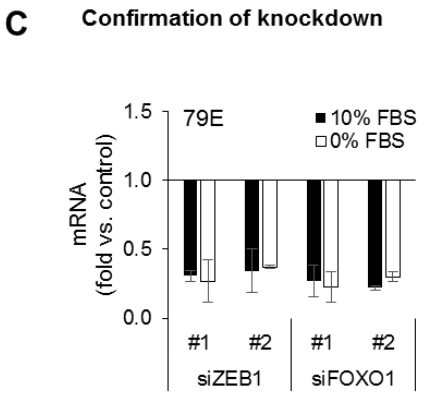


Figure S3.5 FOXO1 is a transcription factor required for LPAR4 induction upon nutrient deprivation. A. Schematic shows list of transcription factors identified using the ENCODE dataset as putative binding events at the LPAR4 promoter region. Candidates were selected based on their correlation with LPAR4 for the TCGA PAAD dataset and established roles in stress pathways. **B.** LPAR4 mRNA induction upon siRNA knockdown of ZEB1 and FOXO1 upon nutrient stress. **C.** Confirmation of siRNA-mediated knockdown for transcription factors evaluated in Figure S2.4B.

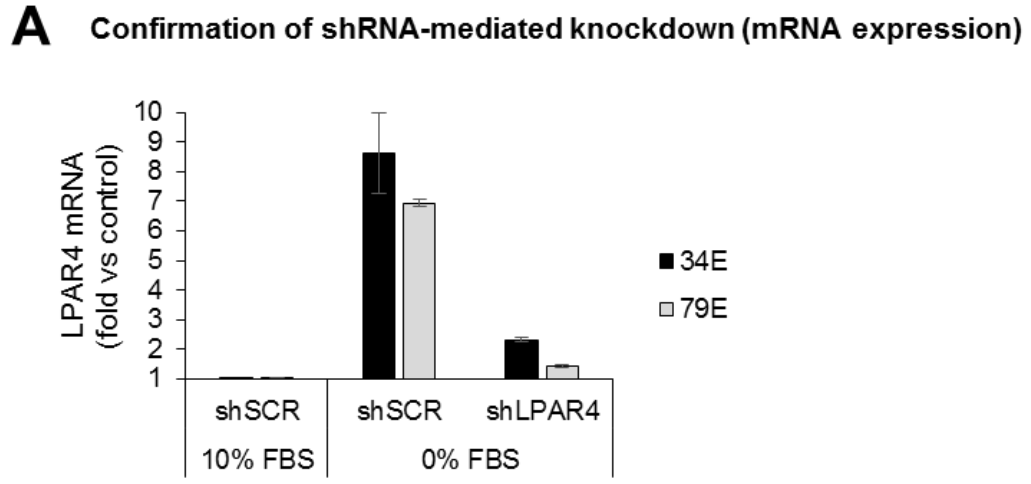


Figure S3.6. Confirmation of shRNA-mediated knockdown of stress induced LPAR4 (mRNA expression) A. qPCR analysis to assess the induction of *LPAR4* mRNA expression in response to nutrient stress (0% FBS) for cells with stable expression of shRNA for *LPAR4* (shLPAR4) or scramble control (shSCR). Bar graphs show mean±SD for n=2 independent experiments.

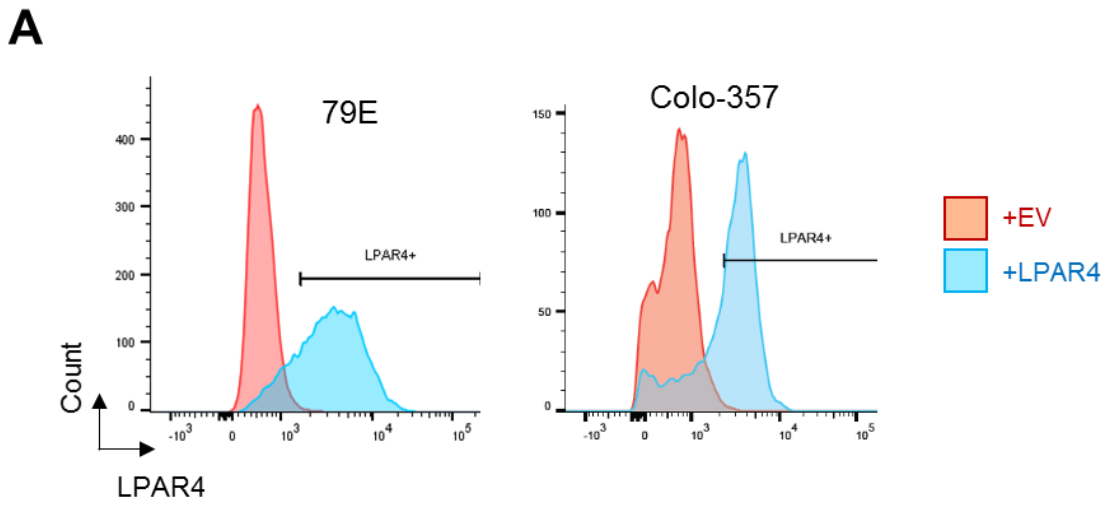


Figure S3.7. Confirmation of ectopic expression (cell surface protein expression) **A**. Flow cytometry analysis to assess LPAR4 cell surface protein expression for cells with stable expression of empty vector (+EV) or LPAR4 (+LPAR4).

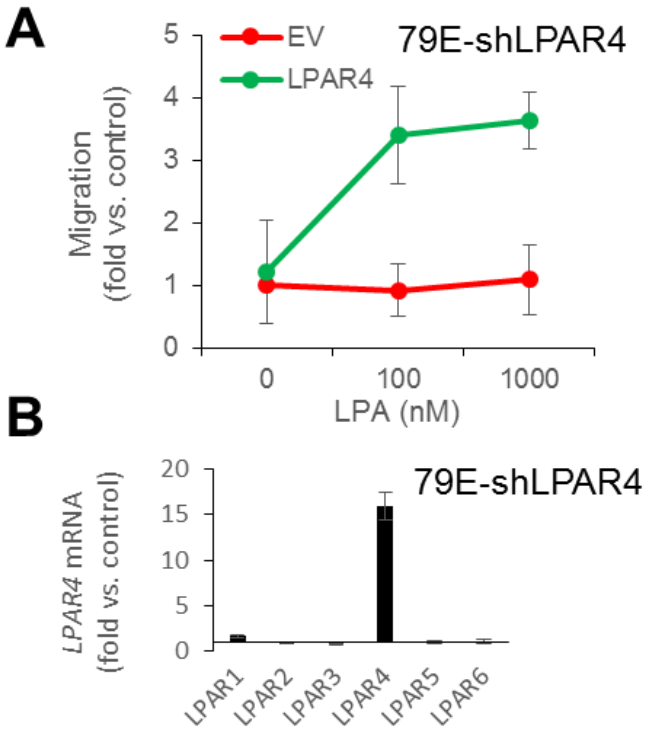


Figure S3.8. LPAR4 ectopic expression in shLPAR4 background rescues knockdown migration phenotype. A. Migration assay ectopically expressing LPAR4 in a 79E-shLPAR4 knockdown model at different doses of LPA. **B.** qPCR analysis validating overexpression of LPAR4 in the 79E-shLPAR4 knockdown model. Bar graphs show mean±SD for n=3 independent experiments.

A

34E *Loss of function*

# cells	shSCR	shLPAR4
100K	4/4	4/4
10K	8/8	8/8
1K	7/8	3/8
100	2/12	0/12
10	0/12	0/12

Colo-357 *Gain of function*

# cells	EV	LPAR4
1M	4/4	4/4
100K	4/4	4/4
10K	7/8	8/8
1K	5/8	8/8
100	1/12	3/12
10	0/12	1/12

79E *Gain of function*

# cells	EV	LPAR4
100K	4/4	4/4
10K	8/8	8/8
1K	4/8	6/8
100	0/12	4/12
10	0/12	0/12

Figure S3.9. Tumor initiation in nu/nu mice (limiting dilution assay). A. Table shows the incidence of palpable tumors formed by the indicated number of cells injected with loss of function (cells stably expressing sh-SCR (scramble) vs. sh-LPAR4) and gain of function (cells stably expressing ectopic empty vector (EV) vs. LPAR4).

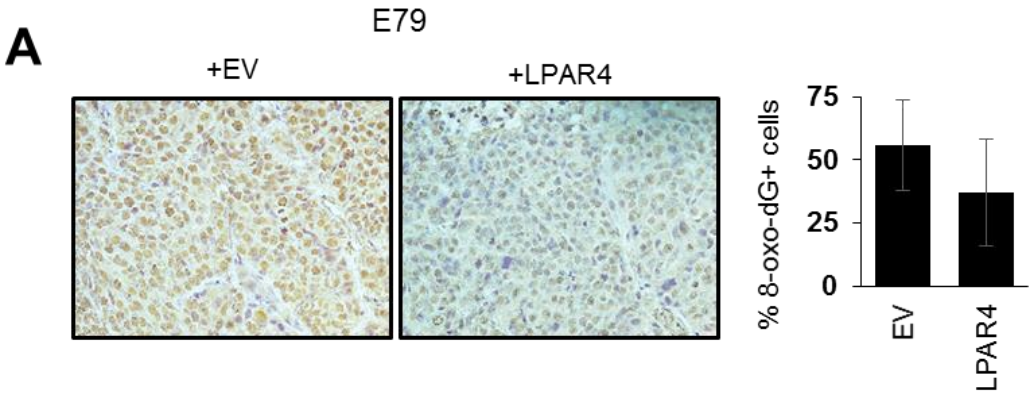


Figure S3.10. IHC staining for ROS marker in orthotopic xenograph model A. IHC staining of 8-oxo-dG, a reactive oxygen species (ROS) marker, between control vs. ectopically expressing LPAR4 cells in a immunocompromised mouse orthotopic xenograph model. Bar graphs represent mean±SD of n = 15 samples of independent sections

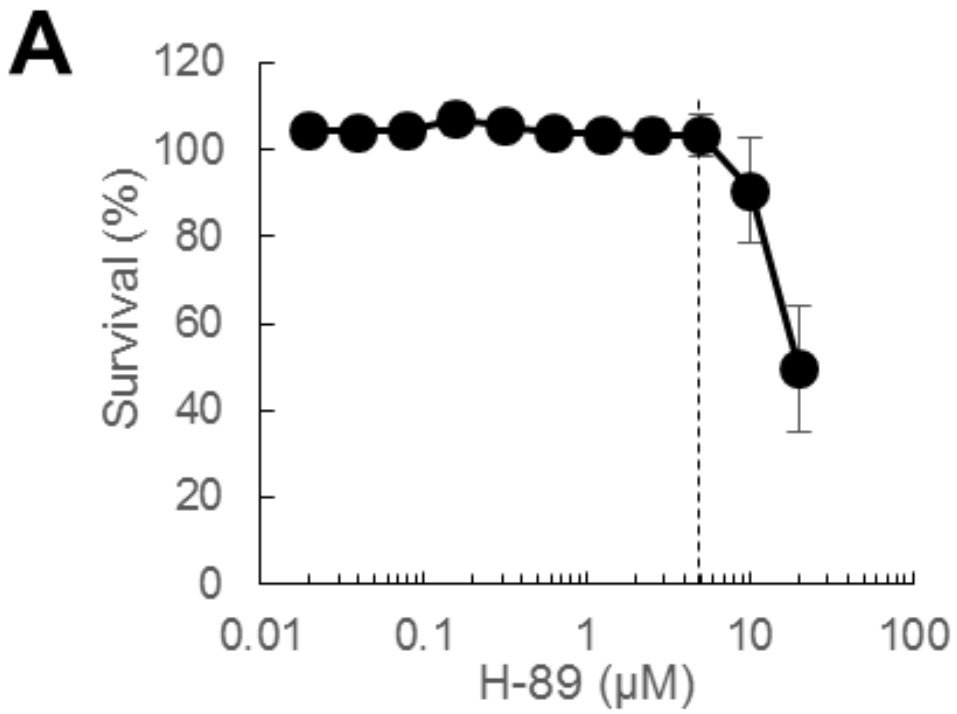


Figure S3.11. H-89 Dose response curve. A. CellTiter Glo was used to assess viability at different doses of H-89. Dashed line represents dose used to inhibit PKA. Data is normalized to vehicle control. Graph shows mean±SD for n=2 independent experiments.

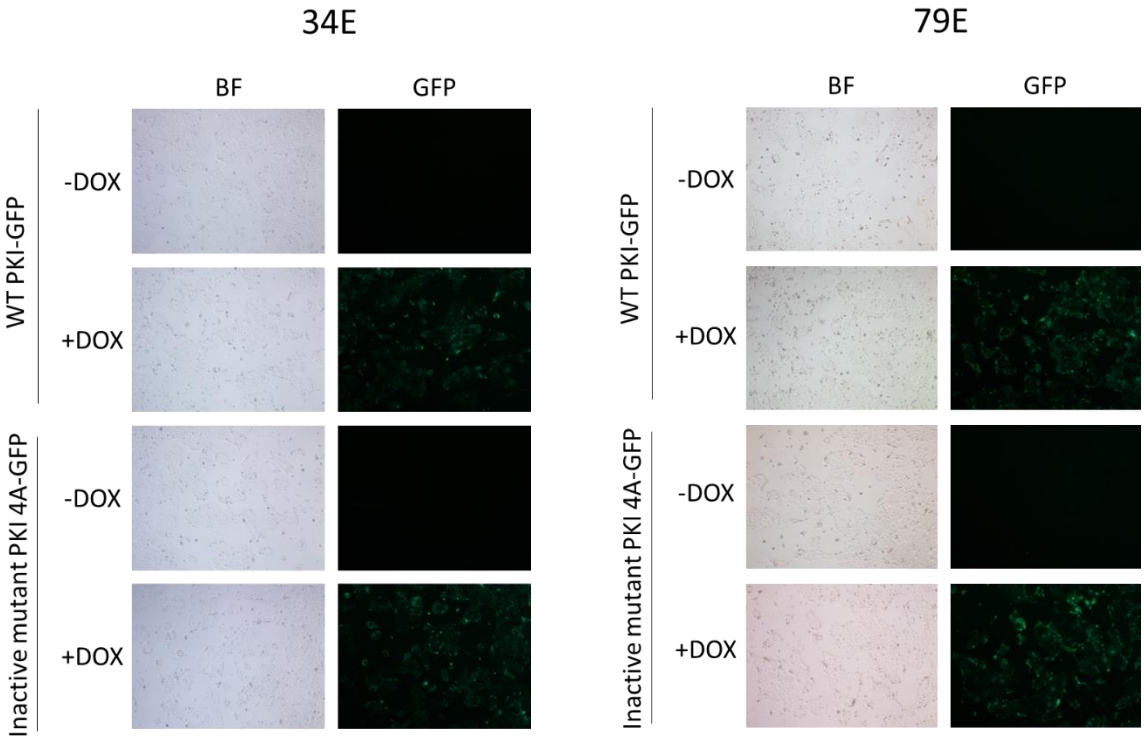


Figure S3.12. Validation of PKI expression upon doxycycline treatment. 34E and 37E cells expressing a Tet-inducible PKI-GFP construct were plated and treated with doxycycline. Pictures were taken after 24 hours of incubation using an inverted fluorescent microscope.

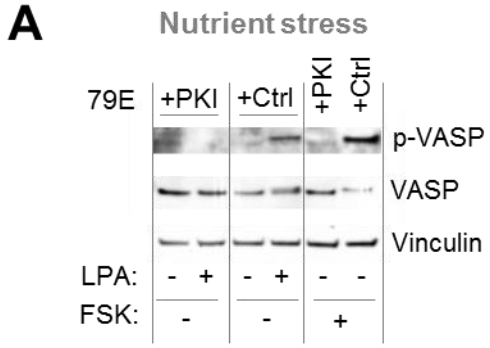


Figure S3.13. Validation of PKA/VASP inhibition by PKI. A. Western blot analysis of inhibition of VASP S157 upon expression of Protein Kinase Inhibitor (PKI).

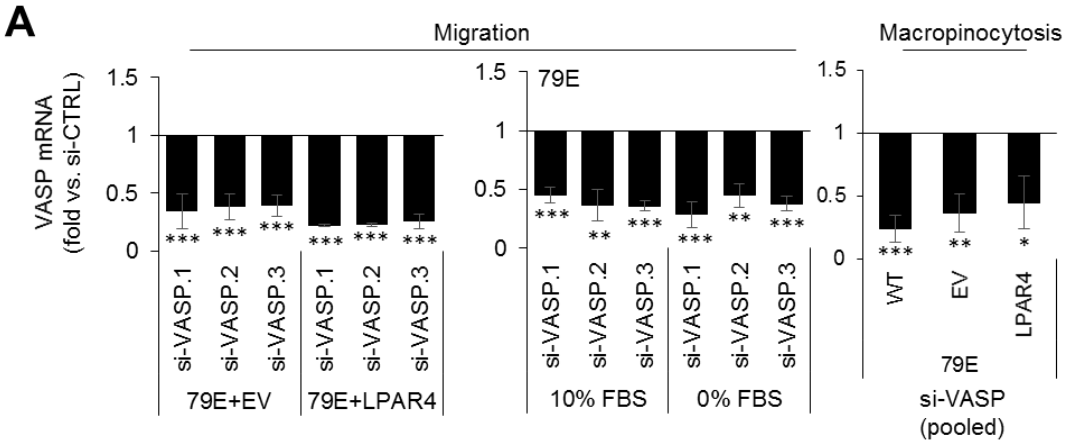


Figure S3.14. VASP knockdown validation. A. qPCR analysis of VASP mRNA expression for three separate siRNA molecules and pooled siRNA. Bar graphs show mean±SD for n=4 independent experiments. Graph shows mean±SD for n=3 or more

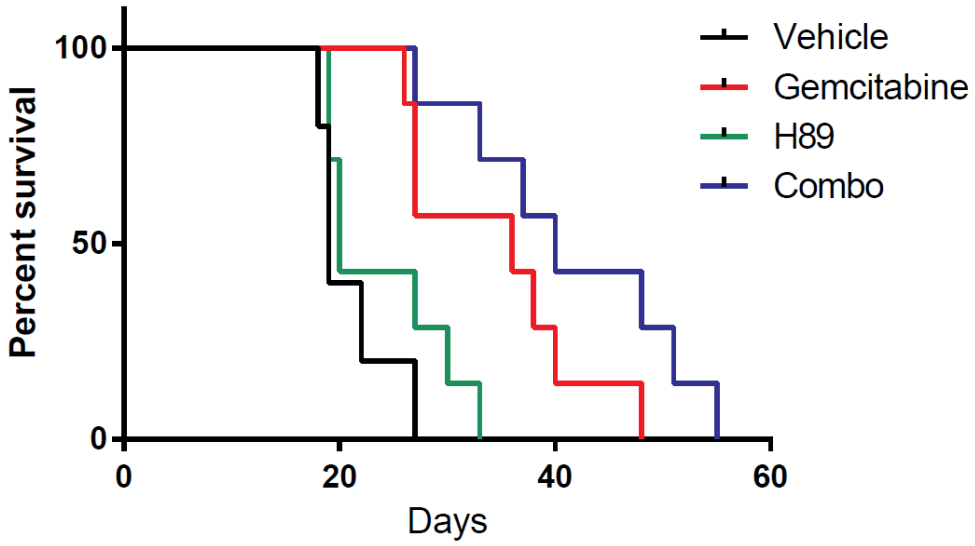


Figure S3.15. Survival experiment with PKA inhibitor H-89 in combination with Gemcitabine. Kaplan-Meier curve showing survival of F1 hybrid mice orthotopically injected with KPC484 mouse PDAC organoids treated with vehicle, H-89 along (10 mg/kg), Gemcitabine alone (2.5 mg/kg), or combination. Each treatment group is n=7 mice. Statistics was conducted using the Log Rank (Mantel-Cox) test. Only Gemcitabine and Vehicle were statistically significant.

Chapter 3.11 Materials and Methods

Cell lines:

Pancreatic ductal adenocarcinoma cell lines (XPA1, MiaPACA-2, PANC-1) were obtained from the American Type Culture Collection (ATCC) and grown in DMEM + 10% FBS. Cell line authentication was performed by the ATCC using short tandem repeat DNA profiles. 79E and 34E cells were derived from patient-derived xenograft (PDX) models established by Dr. Andrew Lowy (University of California, San Diego). The pancreatic carcinoma cell line COLO-357-FG was a gift from Dr. Shama Kajiji and Vito Quaranta (The Scripps Research Institute). Upon receipt, each cell line was expanded, cryopreserved as low-passage stocks, and tested routinely for mycoplasma. For ectopic expression or shRNA knockdown vectors, cells were transfected with a vector control and vector of interest using a lentiviral system as previously described [68] [44]. LPAR4 expression was tested using qPCR analysis and flow cytometry as described below. shRNA knockdown efficiency was quantified using qPCR analysis.

Reagents, chemicals, and antibodies:

Lysophosphatidic Acid (18:1) was obtained from Avantii Polar Lipids, Inc. (Catalog #857130). LPA was diluted to a stock concentration of 5mM in dH2O. CellTiter Glo was obtained from Promega (Catalog #G7573). MitoSOX was

obtained from ThermoFisher Scientific (Catalog #M36008) and was used in conjunction with LIVE/DEAD Fixable Far Red dead cell stain from ThermoFisher Scientific (Catalog #L10120). Forskolin (Catalog #S2449), H-89 (Catalog #S1582), Gemcitabine (Catalog #S1149), and Paclitaxel (Catalog #S1150) were obtained from SelleckChem and suspended with the manufacturer suggested solvent and stock concentration. TMR-Dextran was obtained from ThermoFisher Scientific (Catalog #D1868). VASP (Catalog #3132T) and phospho-VASP (Ser157) (Catalog #3111T) antibodies were obtained from Cell Signaling. Vinculin (Catalog #MA1103) was obtained from Boster. LPAR4 antibody was obtained from Protein Tech (Catalog #22165-1-AP) and used at a final concentration of 1:500 for flow cytometry.

Gene expression analysis using public databases:

mRNA expression data in TCGA datasets was used to analyze the expression of *LPAR4* between patients who did and did not progress after standard-of-care therapy using cBioPortal for the TCGA RNA Seq V2 RSEM Pancreatic adenocarcinoma dataset (“PAAD”, TCGA, Provisional, 89 samples with progression data).

Quantitative RT-PCR:

Total RNA was collected from cancer cells using the RNeasy RNA Purification kit (Qiagen). cDNA was synthesized with SuperScript III reverse transcriptase using oligo(dT) primers (Invitrogen), and RT-PCR was performed on a LightCycler with SYBR Green (BioRad). RPL37A, ACTB, and GAPDH expression was used as an internal reference to normalize input cDNA. Ratios of the expression level of each gene to that of the reference gene were then calculated. Primers used for this study are listed on **Supplementary Table 3.1**.

Flow cytometry:

Cell pellets were washed with HBSS, blocked with 1 % BSA in HBSS for 30 minutes at room temperature and stained with indicated primary antibodies or IgG isotype controls with or without fluorescently labeled secondary antibodies at room temperature for 1 hr respectively. After the staining, the cells were incubated with propidium iodide (PI) (Sigma, P4864), and flow cytometry was performed on BD LSRFortessa™ and analyzed using FlowJo (Treestar) software.

Evaluation of reactive oxygen species (ROS):

MitoSOX™ Red (Thermo Fisher) was used according to manufacturer instructions to assess mitochondrial oxidation by superoxide in live cells using flow cytometry.

Macropinosome visualization and quantification:

Macropinocytosis was quantified as described previously [107]. Briefly, cells were incubated with TMR-dextran (Thermo Fisher) at the final concentration of 1 mg/mL for 1 hour at 37°C, fixed in 4 % formalin, and stained with DAPI. Images were captured using a Nikon Eclipse C1 confocal microscope. Particle area per cell was determined using ImageJ (NIH).

Cell viability Assay:

Cell TiterGlo (Promega) was used according to manufacturer instructions to assess viability using an luciferase-based luminescence system.

Transwell Migration Assay:

Cell migration was measured using Transwell inserts (Corning Life Sciences, Tewksbury, MA) according to manufacturer's instructions. Cells were grown in normal vs stress conditions for 72 hours. Next, cells were counted and

placed in Transwell inserts in DMEM 0.5% Fatty Acid-free BSA. The lower chamber was incubated with DMEM 10% Charcoal-stripped FBS. After 24 hours of incubation, cells were fixed with 20% methanol and 0.1% Crystal Violet. Images were taken of inserts which were then analyzed using ImageJ (NIH). Cell counts were then used in statistical analysis, and all experiments were repeated in triplicate.

Mouse tumor experiments

Study approval: All experiments involving mice were conducted under protocol S05018 and approved by the UC San Diego Institutional Animal Care and Use Committee. All studies are in accordance with the guidelines set forth in the NIH Guide for the Care and Use of Laboratory Animals.

Tumor initiation (limiting dilution analysis): 10, 10^2,10^3, 10^4, 10^5 and 10^6 cells were suspended in a mixture of Basement Membrane Matrix Phenol Red-free (BD Biosciences) and HBSS 1:1 and injected in the flanks of 6-to-8-week-old female immune compromised nu/nu mice. Mice were examined for the presence of palpable tumors, and the frequency of tumor-initiating cells was calculated using ELDA software as described [108].

Orthotopic pancreatic cancer and metastasis model: Pancreatic carcinoma cells (1 x 10^6 or 0.5 x 10^6 tumor cells in 50 µl of PBS) were injected into the pancreas of 6-to-8-week-old female nude mice as previously described [44].

Tumors were established for 4-8 weeks (tumor sizes were monitored by ultrasound). Mice were sacrificed after 8 weeks, tumors and organs harvested, and organs scanned for GFP+ metastatic sites using the IVIS Spectrum In Vivo Imager (PerkinElmer).

Survival Experiment: F1 Hybrid KPC mouse model was used to produce Pancreatic Ductal Adenocarcinoma organoids (KPC484). These organoids were grown *in vitro* using established protocols [109]. 10,000 cells were injected orthotopically into the tail of the pancreas. Treatment began 7 days post-surgery. Mice were in injected with Vehicle, H-89 (10 mg/kg, daily, formulation: 30% PEG, 1% Tween, 1% DMSO), Gemcitabine (2.5mg/kg, biweekly, formulation: Saline), or Combo intraperitoneally. Mice were sacrificed when moribund, have ascites buildup, or in great physical distress.

Statistical analysis:

All statistical analyses were performed using Prism software (GraphPad), Microsoft Excel (Microsoft), and SPSS (IBM). Fisher's exact tests, One-Way ANOVA tests or Student *t* tests were used to calculate statistical significance. $P<0.05$ was considered significant.

Table S3.1. List of qPCR primers used in this study

Target	Direction	Sequence
Actin	Forward	GCTGTGCTACGTCGCCCTG
	Reverse	GGAGGAGCTGGAAGCAGCC
GAPDH	Forward	AAGGTGAAGGTCGGAGTCAAC
	Reverse	GGGGTCATTGATGGCAACAATA
RPL37A	Forward	ATTGAAATCAGCCAGCACGC
	Reverse	GATGGCGGACTTTACCGTGA
LPAR1	Forward	GCTGCCATCTCTACTTCCATC
	Reverse	AAGCGGCGGTTGACATAGATT
LPAR2	Forward	ACAGCCCGACTTTCACTTGAG
	Reverse	GCCCACAATGAGCATGACCA
LPAR3	Forward	GCTGCCGATTTCTTCGCTG
	Reverse	AGCAGTCAAGCTACTGTCCAG
LPAR4	Forward	TCCTTACCAACATCTATGGGAGC
	Reverse	ACGTTTGGAGAAGCCTTCAAAG
LPAR5	Forward	GTGTCCTGACTACCGACCTA
	Reverse	TGCGTAGTAGGAGAGACGA
LPAR6	Forward	TTGTATGGGTGCATGTTCAGC
	Reverse	GCCAATTCCGTGTTGTGAAGT
LPAR4 promoter (proximal)	Forward	GACTGGTGACTAAGGGAACG
	Reverse	AGGACACAAAAAGGCCTGAA
LPAR4 promoter (distal)	Forward	GAAACATGCCCTACTTGGGA
	Reverse	TTTCTCTTGGCAGAGCAGAG
VASP	Forward	ATGGCAACAAGCGATGGCT
	Reverse	CGATGGCACAGTTGATGACCA
ZEB1	Forward	GATGATGAATGCGAGTCAGATGC
	Reverse	ACAGCAGTGTCTTGTTGTTGT
FOXO1	Forward	TGATAACTGGAGTACATTTCGCC
	Reverse	CGGTCATAATGGGTGAGAGTCT
SOX9	Forward	AGCGAACGCACATCAAGAC
	Reverse	CTGTAGGCGATCTGTTGGGG
HSF1	Forward	CCATGAAGCATGAGAATGAGGC
	Reverse	CTTGTTGACGACTTTCTGTTGC

Table S3.2. List of the siRNA and shRNA used in this study

Target	siRNA (Ambion)	shRNA (Sigma Aldrich)
LPAR4		TRCN0000357142
		TRCN0000357143
		TRCN0000357144
		TRCN0000008854
		TRCN0000008858
ZEB1	S229970	
	N269440	
HSF1	S6952	
	S6950	
FOXO1	S5259	
	S5258	
SOX9	S532659	
	S532658	
VASP	S14747	
	S14748	
	S14749	
Control	Silencer Select Negative Control #2	MFCD07785395

Table S3.3. List of the expression vectors used in this study

Target	Ectopic Expression (Genecopoeia)	Ectopic Expression (Silvio Gutkind)
LPAR4	EX-M0914-Lv122	
PKI WT		pLenti-Treg3G PKI WT GFP
PKI 4A (Negative mutant)		pLenti-Treg3G PKI 4A GFP
TetO		pLenti CMV rtTA3G Blast
Control	EX-NEG-Lv122	

Chapter 3.12 Acknowledgments

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TR, SMW, and DAC designed research studies and wrote the manuscript. TR, HIW, TVS, MM, MA, and JT conducted experiments and acquired data. BC and AML contributed to experimental designs. All authors contributed to analyzing data and reviewing the manuscript.

Chapter 4 – Discussion

Despite extensive efforts invested in the clinical development of cancer therapies, current treatments can control tumor growth initially but have produced only modest long term efficacy, because most of the patients ultimately relapse with the tumor becoming resistant against established therapies. Certain studies implicate tumor-initiating cells (TICs), also known as cancer stem cells or tumor-propagating cells, as contributors to tumor dormancy, metastasis and relapse [110, 111]. TICs represent a subpopulation of highly tumorigenic cancer cells that are capable of anchorage independence, self-renewal and asymmetric division, properties that render these cells particularly resistant to therapy [43, 112].

Integrin αvβ3 is a cell surface adhesion molecule that has been well established as a driver of tumor progression [25, 113]. Not only has the expression of αvβ3 integrin been correlated with poor outcome and higher incidence of metastasis for epithelial cancers [25], but its expression has also been reported on a subpopulation of breast [114, 115] and leukemia cancer stem cells [116]. Although the canonical function of integrins is the coordination of cell–matrix communication and adhesion[25], αvβ3 integrin is capable of triggering anchorage-independent cell survival and tumor metastasis in a ligand-independent manner [20]. Furthermore, αvβ3 integrin, through a KRAS mediated signaling pathway, increases tumor initiating capacity, stemness, and resistance against RTK inhibition [15]. Considering that we have established αvβ3 integrin as a

marker of stemness and drug resistance, I hypothesized that αvβ3 expressing cells can secrete factors that promote properties associated with high tumorigenic stem-like cells.

Here I report that, indeed, αvβ3 expressing cells, in both a gain-of-function and loss-of-function model, secrete factors that promote anchorage-independent growth in a soft agar assay and induce the expression of *OCT4* and *NANOG*, markers of pluripotent stem cells. Using several methods to interrogate the conditioned media from αvβ3 expressing cells, including exosome depletion, heat denaturation, and charcoal-stripping, I discovered that conditioned media from αvβ3 expressing cells contained a small bioactive lipid responsible for induction of stem genes. Previous studies indicated that the bioactive lipid Lysophosphatidic acid (LPA) can promote tumor initiation and progression [61]. Therefore, I assessed the LPA content of conditioned media from αvβ3 expressing cells using a competitive ELISA-based assay and found that αvβ3 expressing cells secrete LPA. Furthermore, LPA can induce stem genes and increase anchorage independent growth, the same phenotypes observed when incubating cells with conditioned media.

Given that LPA is known to promote stress tolerance [77], I asked whether LPA contributes to progression of pancreatic cancer in the context of cellular stress. I began by investigating whether different forms of cellular stress can enhance pancreatic cancer sensitivity to LPA. I found that multiple forms cellular

stress, including nutrient deprivation, oxidative stress, and chemotherapy treatment, can enhance LPA-mediated migration. Furthermore, LPA can mitigate nutrient deprivation and oxidative stress through enhanced macropinocytotic uptake of macromolecules, such as albumin, and decreased levels of mitochondrial ROS, respectively. To determine the mechanism of this phenotype, I assessed how cellular stress effects the repertoire of LPA receptors. Strikingly, I found that cellular stress selectively induces the expression of LPA Receptor 4 (LPAR4). I found this receptor necessary and sufficient for not only migration, macropinocytosis, and mitochondrial ROS mitigation but also tumor initiation and metastasis. Furthermore, I discovered that LPAR4 can activate the cAMP-PKA-VASP signaling pathway and that this pathway is critical for LPAR4-mediated migration and macropinocytosis through actin polymerization. In conclusion, my doctoral studies demonstrate two newly discovered phenomenon in pancreatic cancer: αvβ3 integrin is responsible for the production of LPA and that LPA, in the presence of stress, can promote tumor progression through selective expression of LPAR4.

Pancreatic ductal adenocarcinoma (PDAC) is still considered one of the deadliest aggressive solid tumors [117]. This outcome is owed to the aggressive behavior of the pancreatic cancer cells as well as the unique tumor microenvironment. PDAC is characterized by desmoplasia resulting in a thick, condensed stromal compartment. This microenvironment consists of abundant extracellular matrix and stromal cells, including cancer-associated fibroblasts

(CAF), immune, and endothelial cells [118]. This tumor microenvironment has been shown to impair drug delivery [4] and promote tumor progression through secretion of factors, such as LIF [36]. A recent study by Sherman and colleagues illustrated pancreatic stromal cells can secrete lysophosphatidylcholine (LPC) while PDAC cells secrete Autotaxin, an enzyme that uses LPC to produces LPA, leading to tumor progression that is sensitive to Autotaxin inhibition [38]. Although my work illustrates a dissimilar mechanism for the production of LPA in PDAC compared to Sherman and colleagues, my discoveries complement their work in that I demonstrate mechanistically how LPA can promote tumor progression.

Given my work demonstrates a new found mechanism for stress mitigation and tumor progression, an important future step is to develop targeted therapies to inhibit LPAR4 mediated signaling. As mentioned before, there are PKA [119] and LPA Receptor inhibitors [103], however they are not selective and/or have low bioavailability. Therefore, an effective approach would be to deplete the PDAC tumor of LPA using ONO-8430506, an Autotaxin inhibitor with good pharmacokinetic and pharmacodynamics properties [120]. My findings suggest that a combination therapy with Gemcitabine, a standard-of-care chemotherapy for PDAC, and ONO-8430506 can have favorable survival benefits because ONO-8430506 will deplete the tumor of LPA such that LPAR4 induced by Gemcitabine will have no effect on tumor progression.

Furthermore, patient overall survival data demonstrates that approximately ~40% of patients that express low levels of LPAR4 survive past 80 months (6 years) after diagnosis (**Figure S3.3A**). This is unprecedented considering the 5 year survival rate for pancreatic ductal adenocarcinoma is ~7% [1]. This leads to question why that portion of patients expressing low levels of LPAR4 survive much longer compared to the other ~60% of patients that express low levels of LPAR4 who do not survive past 20 months (~1.5 years). One hypothesis is that LPAR4 expression cooperates with other signaling mechanism and only upon inactivation or limited expression of both these signaling mechanisms do patients survive longer. In order to test this hypothesis, we can compare the TCGA RNAseq data of patients expressing low levels of LPAR4 between those that survive before and after 20 months. Candidates that are differentially expressed between these two groups can be assessed for their cooperative effects with LPAR4 activation. This can lead to further classification of stress response pathways and lead to better targeted therapeutics.

Additionally, my work raises a few questions that can provide some future directions. I discovered that LPAR4 is selectively induced upon stress and can activate the PKA signaling pathway. However, five other LPA receptors exist at different, yet relevant expression levels. How do each of these receptors play a role in PDAC progression? LPA receptor 1 (LPAR1) expression is significantly increased upon failure after therapy, similar to LPAR4. LAPR1 is known to activate the KRAS, PI3K, and Rho signaling pathway [103] which are also involved in

macropinocytosis [121] and actin polymerization [122]. Therefore, a study assessing the role each receptor plays in pancreatic cancer progression is necessary to understanding the full scope of how LPA effects the tumor. Moreover, further mechanistic studies need to be conducted to determine the mechanism of ROS mitigation by LPA. Blocking oxidative stress response elements such as HMOX1 [123] and NRF2 [74] can sensitize tumors to the effects of therapies that induce oxidative stress. Additionally, LPA has exhibited an ability to stabilize and increase nuclear localization of NRF2 in a panel of epithelial cancers [124]. Therefore, a study assessing the role NRF2 plays in LPA/LPAR4 mediated ROS mitigation can provide further insight on developing combination therapies. Finally, further studies need to be conducted to understand the role αvβ3 integrin plays in LPA mediated PDAC progression. My work illustrates how αvβ3 integrin can produce LPA *in vitro*. However, the exact mechanism of how the LPA produced from αvβ3 expressing PDAC cells can promote tumor progression, whether dependent or independent of LPAR4, is not well-understood. Sherman and colleagues have outlined a mechanism for LPA production involving Autotaxin, which, interestingly, strongly interacts with and is activated by αvβ3 integrin [58]. My work, however, suggests that αvβ3 integrin expressing cells directly produce LPA through cPLA2. Experiments delineating the effect of each of these mechanism on PDAC progression is essential. I hypothesize that only upon chemotherapy treatment or at the metastatic nodules, two events that are known to induce αvβ3 expression, will αvβ3-dependent LPA production be an important

factor. Therefore, co-culture and co-injection studies of αvβ3 expressing and non-expressing cells need to be conducted in order to determine the role αvβ3 integrin plays in LPA-mediated PDAC progression.

In conclusion, I discovered two new pathways important in understanding the role of LPA in PDAC. I found that αvβ3 integrin expression leads to secretion of LPA through cPLA2 and that LPA can promote tumor progression and stress mitigation through induction of LPAR4. These two new pathways can provide new targets to treat PDAC patients as well as further understanding how cancer reacts, mitigates, and overcomes different classes of cellular stresses.

Chapter 5 - References

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