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Role of a Fibronectin-Enriched Extracellular Matrix Niche in Promoting Isolation Stress-Tolerance in Pancreatic Cancer

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Bioengineering

by

Chinmayi Kashyap

Committee in charge:

Professor David A. Cheresh, Chair
Professor Adam Engler, Co-Chair
Professor Stephanie Fraley

2023

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The Thesis of Chinmayi Kashyap is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2023

DEDICATION

To

My mother, Rashmi,

For encouraging me to pursue my passion for research and make a significant impact in the world

My father, Venkatesh,

For his unparalleled love and sacrifices that have enabled me to pursue my dreams

My sister, Shashwati,

For her constant love and support

My friends,

For giving me a home away from home throughout my challenging yet empowering journey as a graduate student.

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LIST OF ABBREVIATIONS

CAF	Cancer-Associated Fibroblast
CREB	cAMP Response Element-Binding protein
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSC	Cancer Stem Cell
CTGF	Connective Tissue Growth Factor
CYR61	Cysteine-Rich angiogenic inducer 61
ECM	Extracellular Matrix
EMT	Epithelial to Mesenchymal Transition
EV	Empty Vector
FACS	Fluorescence-Activated Cell Sorting
FAK	Focal Adhesion Kinase
FN or FN1	Fibronectin
FN-KO	Fibronectin Knockout
gRNA	Guide Ribonucleic Acid
IF	Immunofluorescence
LPA	Lysophosphatidic Acid
LRAR4 or R4	Lysophosphatidic Acid Receptor-4
NTgRNA	Non-Targeting Guide Ribonucleic Acid
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
ROS	Reactive Oxygen Species
shRNA	short hairpin Ribonucleic Acid
TIC	Tumor-Initiating Cell
YAP	Yes-Associated Protein

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Chapter 2 contains unpublished data which was acquired with the help of Dr. Chengsheng Wu from the Cheresh lab. The thesis author was the primary author of this chapter.

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ABSTRACT OF THE THESIS

Role of a Fibronectin-Enriched Extracellular Matrix Niche in Promoting Isolation Stress-Tolerance in Pancreatic Cancer

by

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Pancreatic cancer is notoriously known for being aggressive and resistant to chemotherapy. It has one of the highest rates of recurrence among all cancers and a poor survival rate even after treatment. The tumor microenvironment in pancreatic cancer, also known as a “niche”, is characterized by the deposition of a dense extracellular matrix (ECM). To establish a tumor at a primary or metastatic site, tumor-initiating cells (TICs) must adapt and survive in a highly hostile environment

characterized by hypoxia, nutrient deprivation, and oxidative stress, together known as isolation stress. TICs accomplish this by gaining stem-like features which ultimately lead to a stress-tolerant phenotype. Pancreatic cancer cells upregulate a receptor called lysophosphatidic acid receptor-4 (LPAR4) in response to isolation stress. LPAR4 expression is necessary and sufficient to establish a tumor-initiating niche, which plays an important role in cancer progression. A characteristic of this niche is the enrichment of the ECM component fibronectin (FN), which interacts with cells through cell-surface integrin receptors. These interactions activate various signaling molecules such as the transcriptional regulator Yes-associated protein (YAP), consequently driving cancer stemness. In this study, I investigate the adaptive gain of a stress-tolerant phenotype in LPAR4- cells mediated by a FN-enriched ECM deposited by LPAR4+ cells. I report that the ectopic expression of LPAR4 in pancreatic cancer cells is sufficient to induce a stress-tolerant phenotype and YAP activation, through the elevated expression of FN. Knockout of FN in LPAR4+ cells can hamper YAP activation, emphasizing the crucial role of FN in YAP signaling. Furthermore, the ECM deposited by LPAR4+ cells is sufficient to confer the stress-tolerant phenotype to neighboring LPAR4- cells through paracrine signaling by a fibronectin-dependent pathway, leading to integrin-mediated activation of YAP and expression of YAP targets CTGF and CYR61. A pure FN-matrix is sufficient to induce YAP activation in LPAR4- cells, indicating that FN is the key ECM component that interacts with cell surface integrin receptors to drive cancer stemness in response to isolation stress. Together, all these factors help tumor-initiating cells to survive isolation stress, resist chemotherapy, and form aggressive tumors, leading to pancreatic cancer progression.

CHAPTER 1: INTRODUCTION

Pancreatic cancer is an aggressive form of cancer which is projected to become the second leading cause of cancer-associated deaths in the United States by the year 2040 [1]. The rising incidence rate along with a low 5-year survival rate of ~10% [2], results in a very high economic burden, especially to patients with metastatic pancreatic cancer [3]. The prognostic outcomes of pancreatic cancer are poor as symptoms are often non-specific until later stages [4], limiting timely diagnosis and treatment. The recurrence rate remains high at ~84% even after treatment [5], and half of the new diagnoses are cases of metastatic cancer [4]. A characteristic of pancreatic cancer is desmoplasia, which is the deposition of a dense extracellular matrix (ECM) in the tumor microenvironment [6]. The rigid ECM blocks the delivery of anti-cancer drugs to the target cancer cells by compressing the tumor vasculature [7], making pancreatic tumors resistant to chemotherapy. This compression of blood vessels also results in hypoxia and reduced nutrient bioavailability in the tumor microenvironment [6]. It is important to understand the cellular mechanisms which contribute to the aggressiveness of pancreatic cancer to develop effective diagnostic methods and therapeutic interventions.

Tumor-initiating cells (TICs) face a unique challenge of non-optimal growth conditions including nutrient deprivation, hypoxia, loss of cell-cell contact and adhesion to the ECM, surveillance by immune cells, and chemotherapy-induced oxidative stress. “Isolation stress” is an umbrella term coined by the Cheresh laboratory to describe these growth-limiting conditions that the solitary TICs must adapt to and overcome to successfully establish a tumor at a primary or metastatic site [8]. Since isolation stress is a very new concept that is being explored in our lab, it is important to elucidate the

mechanisms by which tumor-initiating cells gain isolation stress-tolerance and drive cancer progression. The cancer stem cell (CSC) theory states that tumor initiation is enabled by a stress-tolerant subpopulation of tumor cells with stem-like properties i.e., self-renewal and the ability to differentiate into tumor cell types [9]. While CSCs have been studied in various types of cancer, they were first identified in pancreatic cancer by Li *et. al.* by using a xenograft model showing a subpopulation of cells that exhibited enhanced tumorigenic potential upon orthotopic tail vein injections into mice [10]. The CSC theory has evolved over the years, and recent advances indicate that in addition to being a pre-existing subpopulation, CSCs can also arise from tumor cells that undergo "adaptive reprogramming" when challenged with stress to gain cancer stemness [11].

Previous studies in our lab by Wu *et. al.* established that pancreatic cancer cells selectively upregulate Lysophosphatidic Acid Receptor-4 (LPAR4) in response to isolation stress [8]. The ligand for LPAR4 is the bioactive lipid, lysophosphatidic acid (LPA). Earlier studies revealed that pancreatic cancer cells survive in hypoxic conditions by scavenging fatty acids from lysophospholipids, including LPA [12]. Recently, Wu *et. al.* described a new role for LPAR4 that appears to be independent of its function as an LPA receptor. In response to stress, downregulation of the microRNA miR-139-5p releases a brake on *LPAR4* mRNA, allowing pancreatic cancer cells to gain expression of LPAR4. One of the well-established readouts of stress-tolerance in cells is their ability to mitigate the accumulation of mitochondrial reactive oxygen species (ROS) such as superoxide (MitoSOX) [13]. Wu *et. al.* showed that in 79E pancreatic cancer cells subjected to nutrient stress, the knockdown of LPAR4 using short hairpin RNA (shRNA) resulted in increased accumulation of MitoSOX, indicating that the ability to upregulate LPAR4 is necessary for

stress-tolerance in pancreatic cancer cells [8]. On the other hand, cells with ectopic expression of LPAR4 mitigated the accumulation of MitoSOX when challenged with nutrient stress as compared to LPAR4- cells, indicating that LPAR4+ is sufficient for stress-tolerance in pancreatic cancer cells [8]. These observed effects are independent of the presence of exogenous LPA, since the serum is the major source of LPA and the cells in these studies were cultured in serum-free media.

To identify which genes are linked to *LPAR4* expression in response to isolation stress, Wu *et. al.* performed gene signature analysis using publicly available datasets. Overlap analysis revealed that the ECM protein fibronectin (FN) encoded by the *FN1* gene is one of 13 upregulated genes in common between cells that ectopically express LPAR4 and in LPAR4-rich pancreatic tumors [8]. Our lab demonstrated that stress-induced LPAR4 results in a cascade of signaling pathways, involving the activation of the kinase AKT and cAMP response element-binding protein (CREB), ultimately leading to cell-autonomous production of fibronectin [8]. In human cancer, high FN expression correlates with poor prognostic outcomes of pancreatic cancer, both in terms of overall survival and relapse-free survival [8].

Fibronectin is a key component of the ECM and its role in cancer progression has been extensively studied [14]. FN has been identified as an “extracellular driver of malignancy” [15], and an activator of inflammatory responses [16] in various cancers. FN is enriched in the stroma of pancreatic tumors but not in normal tissue [17], further emphasizing its involvement in cancer progression. FN plays a crucial role in the overall organization of the ECM by acting as a scaffold for the assembly of various other ECM proteins [18], including collagen-I [19]. Fibronectin is a glycoprotein that exists in two

forms: globular plasma FN and fibrillar cellular FN [20]. FN assembly into its fibrillar form is a cell-mediated process known as fibrillogenesis, which involves FN interaction with cell-surface integrins [20]. Integrins are transmembrane receptors which play a key role in adhesive interactions of cells with ECM proteins [21]. The role of integrins as regulators of cancer stemness is well studied, particularly in response to the tumor microenvironment cues including stress [22]. Many members of the integrin superfamily bind to various ECM proteins by recognizing the tripeptide motif Arg-Gly-Asp (RGD), and these RGD-recognizing integrins have been linked to cancer progression and metastasis [23]. While several integrins are capable of interacting with FN, integrin α5β1 is a FN-specific receptor [14] due to the additional recognition requirement for the Pro-His-Ser-Arg-Asn (PHSRN) synergy domain, located near the RGD domain. Another key integrin, αvβ3, is known to bind non-specifically to several RGD-containing ECM proteins including fibronectin [24] and it plays a role in the initiation of FN fibrillogenesis [25]. Our lab has identified a ligand-independent role of integrin αvβ3 in supporting anchorage-independent growth of cancer stem cells in the absence of appropriate ECM, establishing integrin αvβ3 as a marker of cancer stemness [26].

The ability of LPAR4-expressing pancreatic cancer cells to autonomously produce FN is notable, since stromal cells are the typical sources of ECM deposition within a tumor [7]. Cancer-associated fibroblasts (CAFs) [27] are the primary cellular component of the tumor microenvironment which secrete various cytokines and growth factors that promote pancreatic cancer progression through paracrine signaling [28]. In established tumors, CAFs are predominantly responsible for depositing and remodeling the ECM [18], which the tumor cells benefit from during cancer progression. While tumor cells also produce

their own ECM, this is often overlooked as the contribution of CAFs in ECM deposition is considered more significant. The ECM deposited by CAFs in enriched with fibronectin, and CAF contractility induces FN fibrillogenesis through integrin signaling [18]. However, the microenvironment that tumor-initiating cells encounter at a primary or metastatic site is growth-limiting due to the absence of an appropriate CAF-deposited ECM. Thus, the findings from our lab by Wu *et. al.* on the ability of LPAR4+ pancreatic cancer cells to autonomously produce a fibronectin-enriched ECM niche when challenged with isolation stress [8] are significant in explaining the role of LPAR4 in tumor initiation and cancer stemness.

One of the key cellular processes which drives cancer stemness in response to stress is the epithelial-to-mesenchymal transition (EMT) [29]. Cells undergoing EMT exhibit a distinct stress-tolerant “mesenchymal” phenotype, characterized by migratory projections and colony formation [30]. A key molecule which has been linked to tumor-initiation, EMT, and chemoresistance in cancer cells is the transcriptional regulator Yes-associated protein (YAP) [31], [32]. YAP is a downstream effector of the Hippo pathway, which plays a significant role in various developmental processes including organ size control and renewal of stem cells [33]. YAP is involved in maintaining a balance between cell proliferation and apoptosis [34], and regulating the cellular responses to mechanical cues through mechanotransduction [35]. YAP activity is regulated by the Hippo pathway, which sequesters the phosphorylated form of YAP within the cytoplasm to keep it in an inactive state by preventing its nuclear translocation [36]. The inactivation of YAP by the Hippo pathway acts as a checkpoint to control cell proliferation in response to cell density through contact inhibition [37].

Upon dephosphorylation at serine 127, YAP translocates to the nucleus and drives the transcription of various target genes [36], including two members of the CCN family: *CCN2* [38] which encodes the connective tissue growth factor (CTGF), and *CCN1* [39] which encodes the cysteine-rich angiogenic inducer 61 (CYR61). CTGF and CYR61 are matricellular proteins which act as an interface between cells and the ECM [40], and regulate various processes including cell survival, adhesion, and angiogenesis [41]. These proteins also interact with integrin αvβ3 [41], which has been established as a marker of cancer stemness by previous studies in our lab [26].

In the context of cancer, dysregulation of the Hippo pathway leads to a sustained activation of YAP, resulting in uncontrolled cell proliferation, stress-tolerance, and tumor progression [42]. The YAP targets CTGF and CYR61 have also been linked to cancer stemness and chemoresistance in tumor cells [43]. Fibronectin is one of the several mesenchymal markers expressed during EMT [44], and several studies have linked fibronectin-integrin interactions to YAP activation. Adhesion to fibronectin negatively regulates the Hippo pathway through a focal adhesion kinase (FAK)-Src signaling axis, which ultimately leads to YAP nuclear localization and hence its activation [45].

My thesis project builds on the previous findings in our lab by Wu *et. al.* that pancreatic cancer cells upregulate LPAR4 in response to isolation stress, which is necessary and sufficient to induce stress-tolerance [8]. The ectopic expression of LPAR4 results in the cell-autonomous production of the ECM component fibronectin through an AKT/CREB signaling axis, which establishes a tumor-initiating niche and drives cancer stemness **(Figure 1.1: Steps 1-7)** [8]. The current study aims to elucidate how pancreatic cancer cells respond to and utilize this FN-enriched niche to adapt to isolation stress. I

hypothesize that the tumor-initiating niche established by ectopic LPAR4 expression drives the activation of the transcriptional regulator Yes-associated protein (YAP), mediated by fibronectin-integrin signaling. YAP activation results in the expression of YAP targets CTGF and CYR61 **(Figure 1.1: Steps 8-10)**, which induce an adaptive gain of a stress-tolerant "mesenchymal" phenotype in pancreatic cancer cells. Furthermore, LPAR4+ cells can confer this characteristic colony-forming and migratory stress-tolerant phenotype to neighboring LPAR4- cells through ECM-mediated paracrine signaling, which drives cancer stemness and pancreatic tumor progression.

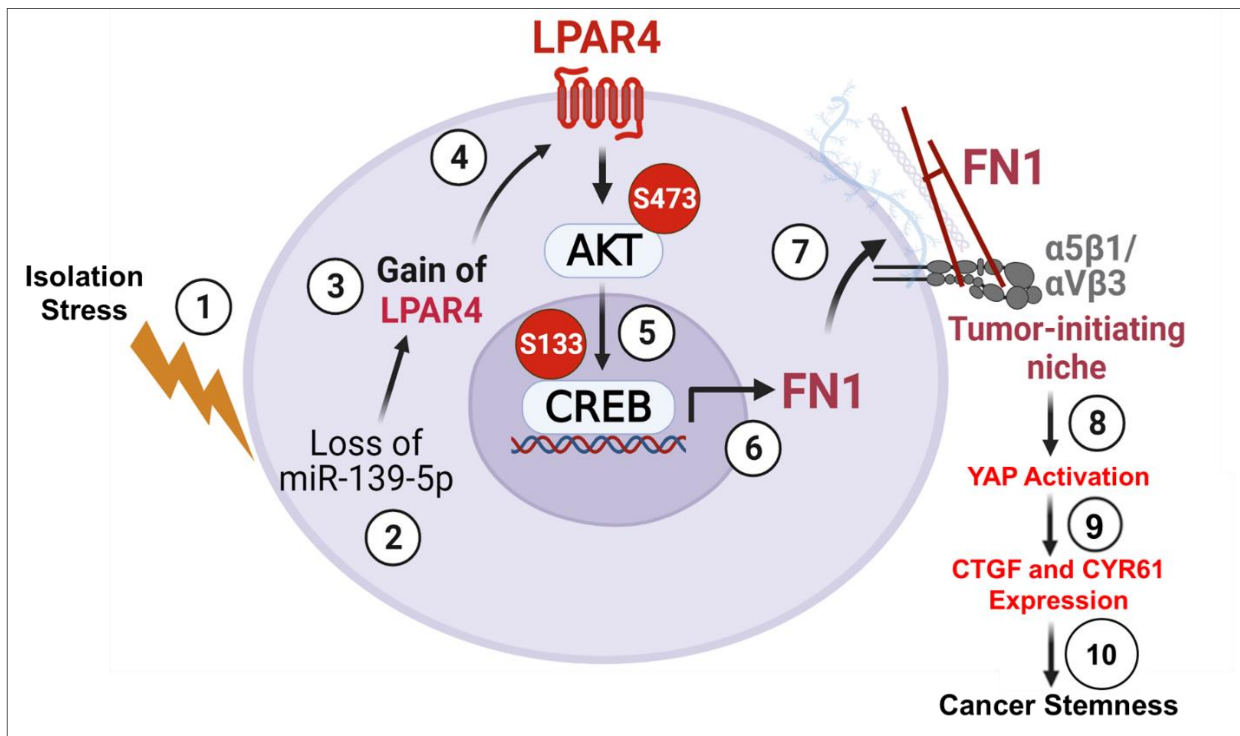

Figure 1.1: A graphical summary of the establishment and utilization of a tumor-initiating niche in pancreatic cancer cells. The upregulation of LPAR4 in response to isolation stress results in an enhanced production of fibronectin (FN) through an AKT/CREB signaling axis. Fibronectin-integrin interactions drive cancer stemness through the activation of YAP, and the expression of YAP targets CTGF and CYR61. (Modified from Wu *et. al.*, Nature Cell Biology, 2023 [8].)

CHAPTER 2: ECTOPIC EXPRESSION OF LYSOPHOSPHATIDIC ACID RECEPTOR-4 PROMOTES ISOLATION STRESS-TOLERANCE IN PANCREATIC CANCER CELLS

2.1 LPAR4+ cells exhibit a stress-tolerant phenotype and enhanced proliferation under isolation stress.

Previous studies in our lab by Wu *et. al.* have established that pancreatic cancer cells upregulate LPAR4 in response to isolation stress [8]. While these studies demonstrated that ectopic LPAR4 expression in pancreatic cancer is necessary and sufficient for stress-tolerance indicated by the mitigation of mitochondrial reactive oxygen species (ROS) [8], it is unclear how the LPAR4+ cells undergo adaptive reprogramming to gain stem-like properties. I hypothesized that ectopic LPAR4 expression drives cancer stemness and stress-tolerance in pancreatic cancer cells through adaptive gain of a stress-tolerant “mesenchymal” phenotype which enhances their proliferation when challenged with isolation stress. Paired cell lines were generated using 34E pancreatic cancer cells: the control Empty Vector (34E+EV) cells and the ectopic LPAR4 expression (34E+R4) cells. The paired cell lines were separately cultured at low cell density in serum-free media to mimic the effects of isolation stress that a solitary tumor cell might experience during tumor initiation, invasion, or metastatic dissemination in the body. Since the serum in cell culture media is the major source of Lysophosphatidic Acid (LPA), which is the ligand for LPAR4, serum-free conditions also ensured that the observed effects would be independent of the presence of exogenous LPA.

Live cell imaging using the IncuCyte S3 instrument at various time points (0h, 12h, 24h, 48h, 72h) after overnight cell adhesion to the tissue culture plate revealed that

34E+R4 cells exhibited a stress-tolerant "mesenchymal" phenotype **(Figure 2.1)**, characterized by the formation of migratory projections and coalescence into dense colonies. These characteristics resemble the phenotype of tumor-initiating cells which overcome isolation stress to proliferate and form tumors at primary or metastatic sites. In contrast, the 34E+EV control group cells remained relatively isolated and stationary when subjected to isolation stress **(Figure 2.1)**, indicating poor stress-tolerance. Cell growth characteristics were analyzed using the phase object count measurement in the IncuCyte software. It was observed that 34E+R4 cells attain a higher confluence over time as compared to the 34E+EV control group cells **(Figure 2.2)**, indicating enhanced proliferation of LPAR4+ cells under isolation stress conditions. Thus, ectopic LPAR4+ expression results in an isolation stress-tolerant phenotype in 34E cells.

2.2 YAP is activated in LPAR4+ cells in the absence of exogenous LPA.

Yes-associated protein (YAP) is a transcriptional regulator and an effector of the Hippo pathway. Various studies have linked YAP activation to epithelial-to-mesenchymal transition (EMT) [31] and resistance to chemotherapy in cancer [47]. YAP, in its inactive form, is phosphorylated and sequestered in the cytoplasm [46]. Dephosphorylation of YAP at serine 127 leads to its activation and nuclear translocation, where it drives the transcription of various target genes. Two YAP target genes *CCN2* [38] which encodes the connective tissue growth factor (CTGF) protein, and *CCN1* [39] which encodes the cysteine-rich angiogenic inducer 61 (CYR61) protein, are involved in driving cancer stemness in pancreatic cancer [43]. These proteins belong to the CCN family of matricellular signaling modulators [48], and play a significant role in facilitating cell-ECM

interactions [40]. CTGF and CYR61 are also known ligands of integrin αvβ3 [41], which has been established as a marker of cancer stemness by previous studies in our lab [26].

I hypothesized that the ectopic expression of LPAR4+ in pancreatic cancer cells drives cancer stemness through the activation of YAP and expression of YAP targets CTGF and CYR61. Gene expression data obtained using qRT-PCR showed that the YAP target genes, *CCN2* (CTGF) and *CCN1* (CYR61), are significantly elevated in 34E+R4 cells, indicating that YAP is activated in LPAR4+ cells **(Figure 2.3)** in the absence of exogenous LPA.

2.3 Fibronectin is necessary to drive YAP activation in LPAR4+ cells.

Our lab has previously established that stress-induced upregulation of LPAR4+ results in cell-autonomous production of the ECM component fibronectin through the AKT/CREB signaling axis [8]. It is important to elucidate how LPAR4+ cells utilize this fibronectin to establish a tumor-initiating niche and gain isolation stress-tolerance. Based on the evidence from studies that linked YAP activation in tumor cells to FN adhesion [45], I hypothesized that ectopic LPAR4+ expression leads to YAP activation which is mediated by elevated secretion of fibronectin. Western blot data comparing two paired (+EV and +R4) cell lines, 34E and 79E, showed that LPAR4+ cells have an elevated expression of fibronectin as well as lower levels of phosphorylated YAP at serine 127 **(Figure 2.4)**, which indicate that YAP is activated.

To establish that fibronectin is necessary for YAP activation in LPAR4+ cells, fibronectin knockout (FN-KO) cells were generated using CRISPR gene editing in 34E+R4 cells, and single-cell knockout clones were sorted using fluorescence-activated cell sorting (FACS). The knockout of FN in the FACS sorted single-cell clones was

validated using Western Blot **(Figure 2.5)**, using 34E cells transfected with a non-targeting guide RNA (NTgRNA) as a control. The validated FN-KO clones showed a reduced expression of the YAP targets CTGF and CYR61 as compared to the NTgRNA control **(Figure 2.5)**, indicating that the loss of FN in LPAR4+ cells hampered YAP activation. These results establish that LPAR4-linked YAP activation is mediated by fibronectin.

2.4 Figures

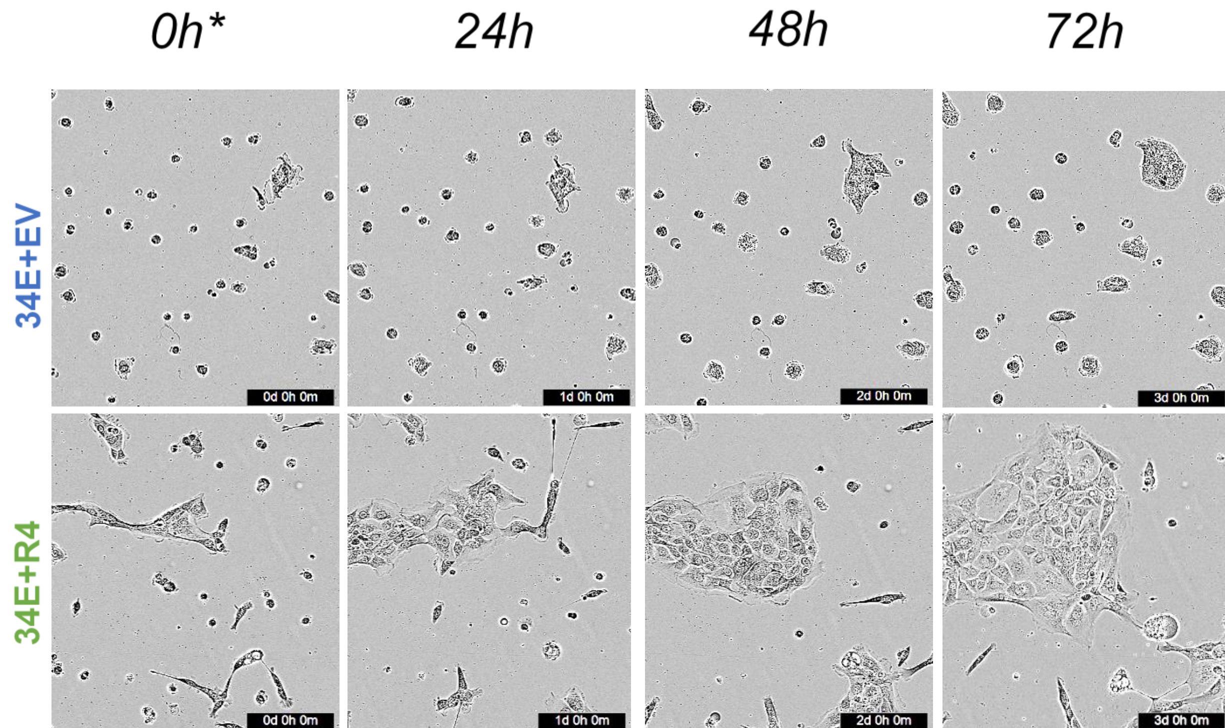

Figure 2.1: Ectopic LPAR4 expression induces an isolation stress-tolerant phenotype in pancreatic cancer cells. 34E paired cell lines (+EV or +R4) were cultured at low cell densities in serum-free conditions to produce a growth-limiting effect and mimic isolation stress. 0h* represents the first image captured post overnight cell adhesion to the tissue culture plate.

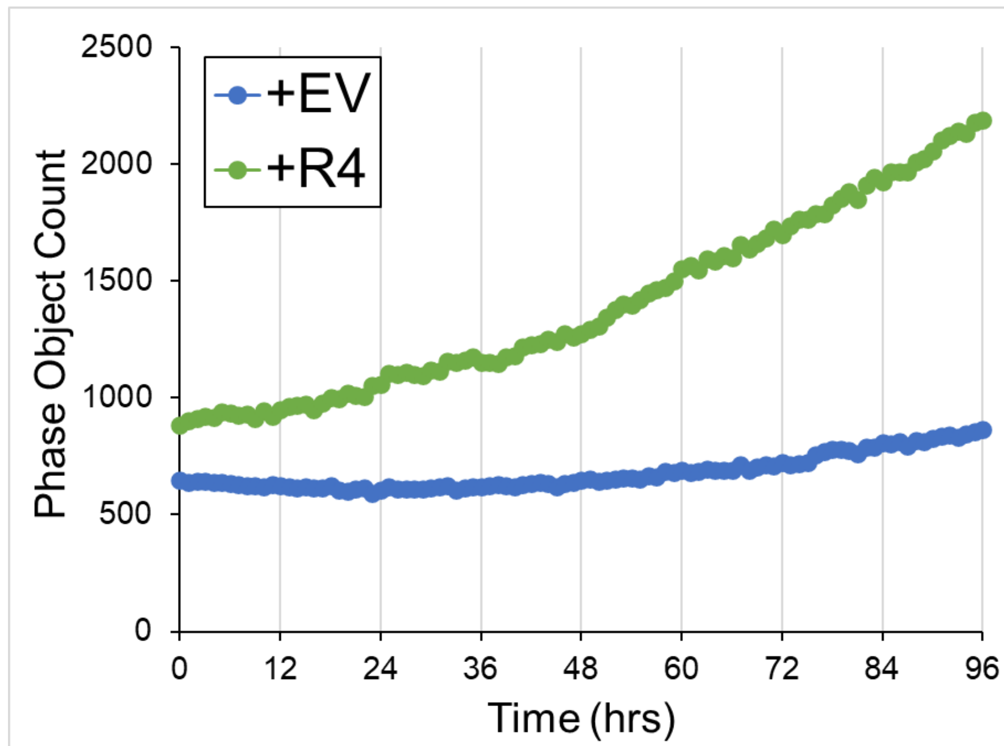

Figure 2.2: Ectopic LPAR4 expression enhances pancreatic cancer cell proliferation when challenged with isolation stress. 34E paired cell lines (+EV or +R4) were cultured at low cell densities in serum-free conditions to produce a growth-limiting effect and mimic isolation stress. 0h* represents the first image captured post overnight cell adhesion to the tissue culture plate.

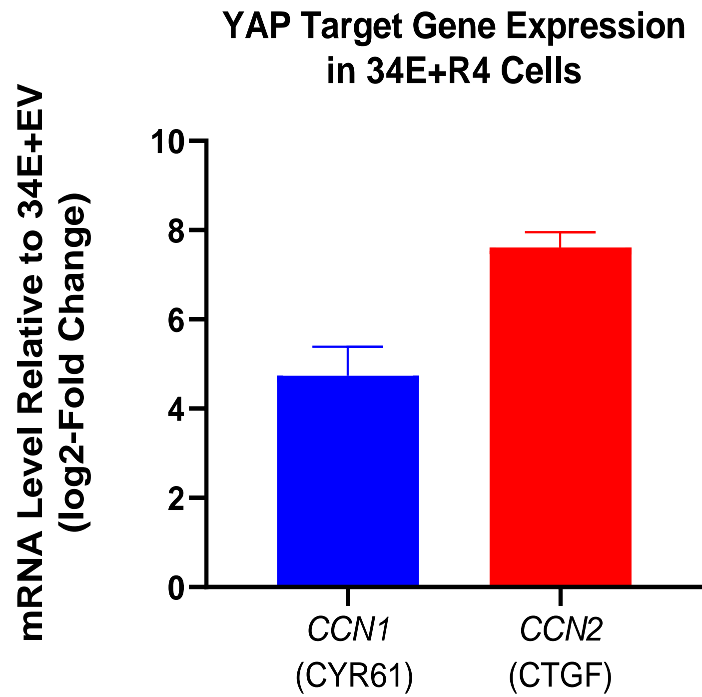

Figure 2.3: YAP target genes *CCN2* (CTGF) and *CCN1* (CYR61) are elevated in pancreatic cancer cells with ectopic LPAR4 expression. 34E paired cell lines (+EV and +R4) were cultured in the absence of exogenous LPA. Cells were harvested for qRT-PCR after 48 hours. Bar graphs represent n=3 independent experiments.

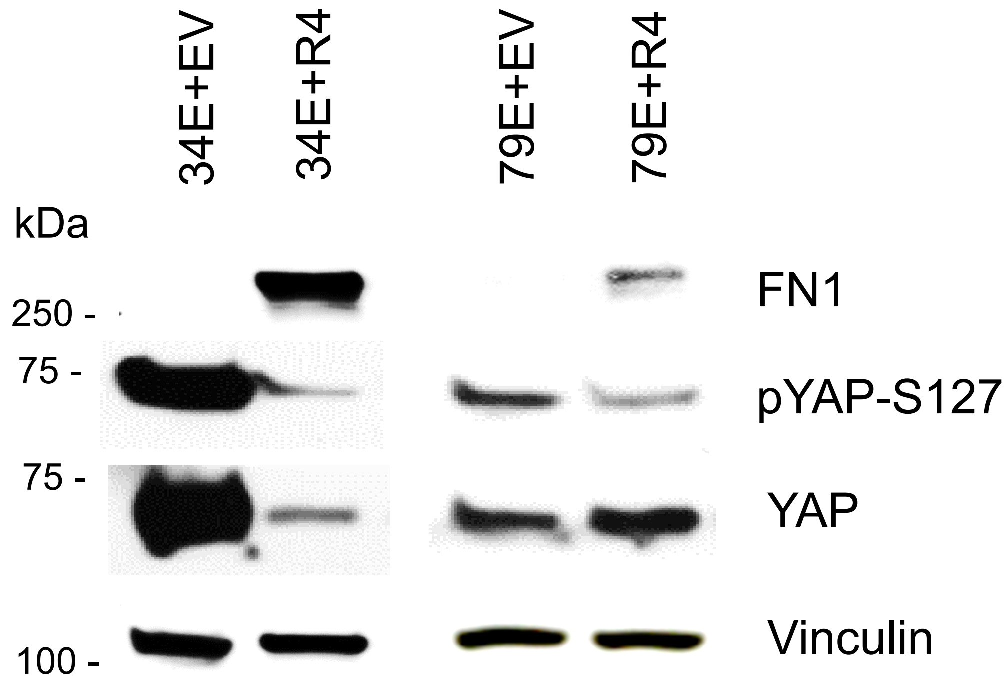

Figure 2.4: Ectopic LPAR4 expression enhances fibronectin production and YAP target (CTGF and CYR61) expression in pancreatic cancer cells. 34E and 79E paired cell lines (+EV and +R4) were cultured in the absence of exogenous LPA. Cells were harvested for western blot after 48 hours. Vinculin was used as the loading control.

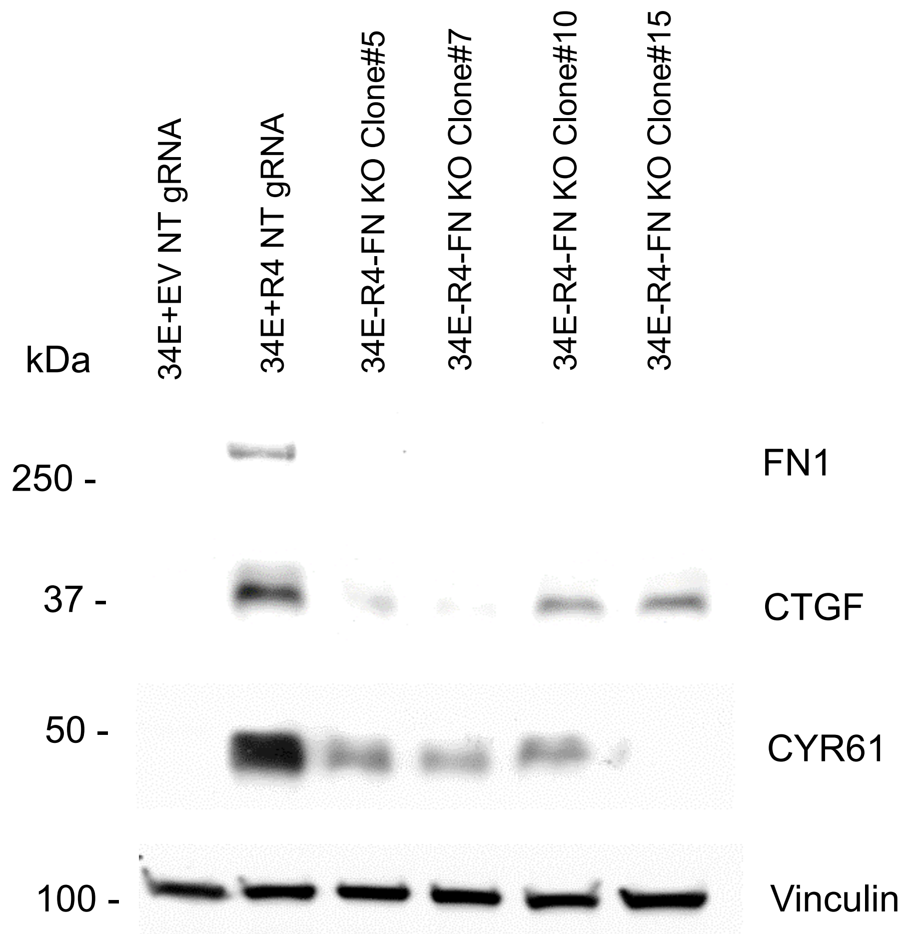

Figure 2.5: Fibronectin is necessary for YAP activation in pancreatic cancer cells with ectopic LPAR4 expression. Control and FN-knockout 34E+R4 cells were cultured in the absence of exogenous LPA. Cells were harvested for western blot after 48 hours. Vinculin was used as the loading control.

2.5 Materials and Methods

Cell Culture

34E and 79E parental cells were derived from patient-derived xenograft models established by Dr Andrew Lowy (University of California, San Diego). Paired cell lines were generated by transfecting the 34E and 79E cells with empty vector (EV) control or ectopic LPAR4 expression vector (R4) through a lentiviral system produced in 293T cells using Lipofectamine 3000 (Thermo Fisher Scientific). The expression vectors used in this study are listed in **Table 2.1.** Ectopic LPAR4 expression was validated using qRT-PCR and western blot. Cells were cultured in Dulbecco's modified Eagle medium containing 10% FBS (Gibco) and 1% penicillin–streptomycin (Thermo Fisher Scientific). For studies that required the absence of exogenous LPA, Dulbecco's modified Eagle medium (Thermo Fisher Scientific) containing charcoal-stripped fetal bovine serum (Corning) was used to culture cells.

Live Cell Imaging

34E paired cell lines (+EV and +R4) were seeded onto 6-well tissue culture treated plates at a low density of 100,000 cells per well and cultured in serum-free Dulbecco's modified Eagle medium to mimic isolation stress conditions. After allowing the cells to adhere overnight, the plates were moved into the incubation chamber of the IncuCyte S3 Live-cell imaging system at the Human Embryonic Stem Cell Core at the Sanford Consortium for Regenerative Medicine. Live cell imaging was performed to capture cell morphology at various time points (0h, 12h, 24h, 48h, 72h). The IncuCyte software was used to measure phase object count and quantify cell confluence over time.

Quantitative Reverse Transcriptase PCR

Total RNA was extracted from cells using the RNeasy RNA Purification kit (Qiagen). cDNA synthesis was done using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), and qRT–PCR was performed on a LightCycler using SYBR Green (Bio-Rad). For the calculation of fold changes in mRNA levels, *TBP, RPL37* and *RPL19* housekeeping genes were used to normalize the Ct values for target genes, qRT-PCR primer sequences used in this study are listed in **Table 2.2.**

Generation of Fibronectin Knockout Cell Clones

FN-knockout in 34E+R4 cells was generated using CRISPR gene editing using a commercially available *FN1*-targeting gRNA (Sigma #HSPD0000014750). The gRNA sequences used in this study are listed in **Table 2.3.** 34E+R4 cells were transiently transfected with control and *FN1*-targeting gRNAs through the p02-U6-gRNA: CMV-Cas9-2A-tRFP vector system (Millipore Sigma) using Lipofectamine 3000 (Thermo Fisher Scientific). Single cell clones were sorted using the Fluorescence-Activated Cell Sorting (FACS) instrument at the Stem Cell Core facility at Sanford Consortium for Regenerative Medicine, La Jolla, CA and cultured in Dulbecco's modified Eagle medium containing 10% FBS (Gibco) and 1% penicillin–streptomycin (Thermo Fisher Scientific). FN-knockout in the FACS sorted single cell clones was validated using western blot.

Western Blots

Cell lysates for western blots were prepared using 1X RIPA buffer containing protease and phosphatase inhibitors. Protein concentrations were estimated by BCA assay (Thermo Fisher Scientific). After denaturing the lysates with 4X Laemmli Sample buffer (BioRad) containing a the NuPAGE reducing agent (Invitrogen), 30ug of protein

per sample was loaded onto SDS-PAGE gels, followed by transfer onto polyvinylidene difluoride membranes. 5% fat-free milk in TBS-T buffer was used for blocking and probing. The following primary antibodies were used: Fibronectin (Novus #NBP-1-51723, 1:1000), Vinculin (Boster #MA1103, 1:3000), YAP (CST #4912S, 1:2000), pYAP-S127 (CST #4911S, 1:2000), CTGF (CST #86641S, 1:1000), CYR61 (Proteintech #26689-1-AP, 1:2000). The secondary antibodies used were goat anti-rabbit HRP (BioRad #170-6515) and goat anti-mouse HRP (BioRad #170-6516). ECL reagent (Thermo Fisher Scientific) was used to develop the blots. Vinculin was used as the loading control.

Table 2.1: List of expression vectors used in this study.

Target	Ectopic Expression (GeneCopoeia)
Empty vector (EV) control	EX-M0914-Lv122
LPAR4 (R4)	EX-NEG-Lv122

Table 2.2: List of qRT-PCR primers used in this study.

Target	Direction	Sequence
CCN2 (CTGF)	Forward	CAGCATGGACGTTCGTCTG
	Reverse	AACCACGGTTTGGTCCTTGG
CCN1 (CYR61)	Forward	CTCGCCTTAGTCGTCACCC
	Reverse	CGCCGAAGTTGCATTCCAG
TBP	Forward	CCACTCACAGACTCTCACAAC
	Reverse	CTGCGGTACAATCCCAGAACT
RPL37	Forward	TCGCCTCTAGTGTCCTCCG
	Reverse	GCGGGCCAAGGTGTTTTTC
RPL19	Forward	ATTGAAATCAGCCAGCACGC
	Reverse	GATGGCGGACTTTACCGTGA

Table 2.3: List of gRNAs used in this study.

Target	Sequence (5’ to 3’)
Non-targeting (NT) control	CGCGATAGCGCGAATATATT
Fibronectin (*FN1*)	ACTCTCGCAGTTAAAACCT

2.6 Acknowledgements

Chapter 2 contains unpublished data which was acquired with the help of Chengsheng Wu from the Cheresh lab. The thesis author was the primary author of this chapter.

CHAPTER 3: LPAR4+ CELLS CONFER EXTRACELLULAR MATRIX-MEDIATED ISOLATION STRESS-TOLERANCE TO LPAR4- CELLS

3.1 ECM deposited by LPAR4+ cells is sufficient to confer a stress-tolerant phenotype to LPAR4- cells.

The presence of an appropriate extracellular matrix (ECM) is critical for tumor-initiation and cancer progression [49]. Stromal cells such as cancer-associated fibroblasts (CAFs) are primarily responsible for ECM deposition [18], as well as the secretion of various factors that promote cancer progression [28]. While tumor cells also produce their own ECM, they mostly benefit from the CAF-deposited ECM to drive cancer progression. However, tumor-initiating cells face a unique challenge of isolation stress which includes the absence of a CAF-deposited ECM at the primary or metastatic site. Our lab has established that pancreatic cancer cells adapt to isolation stress by upregulating LPAR4, which consequently results in the autonomous production of various ECM components, particularly fibronectin [8]. Thus, the ability of LPAR4+ cells to deposit their own FN-enriched ECM could explain how they can survive under isolation stress and drive tumor-initiation.

Based on these findings and studies that have elucidated that paracrine signaling pathways play a crucial role in mediating cell-ECM communication in cancer [50], I hypothesized that the ECM deposited by the LPAR4+ cells is sufficient to promote stress-tolerance in LPAR4- cells, even in the absence of the ECM depositing LPAR4+ cells, establishing that isolation stress-tolerance is mediated by the ECM. To study the effect of the ECM secreted by LPAR4+ 34E cells (R4-ECM) on LPAR4- 34E cells, a decellularized ECM assay was performed as outlined in **Figure 3.1**. Live cell imaging using the IncuCyte

S3 instrument showed that LPAR4- cells gained the stress-tolerant “mesenchymal” phenotype when seeded on the decellularized ECM secreted by LPAR4+ cells under isolation stress conditions **(Figure 3.2)**. This phenotype resembles that of LPAR4+ cells under isolation stress, characterized by migratory projections and colony-formation. LPAR4- cells grown on the R4-ECM become confluent significantly faster than those grown on EV-ECM **(Figure 3.3)**, indicating enhanced proliferation under isolation stress. These results support the concept of a tumor-initiating niche which is formed because of LPAR4-driven FN expression in tumor-initiating cells, and further demonstrate that these LPAR4+ cells can confer the advantages of stress-tolerance to neighboring LPAR4- cells through paracrine signaling.

3.2 YAP is activated in LPAR4- cells grown on the ECM deposited by LPAR4+ cells.

The role of YAP [31] and its target genes, CTGF and CYR61, in driving cancer stemness has been well studied [43]. Based on my previous findings that ectopic expression of LPAR4 drives YAP activation in pancreatic cancer cells and on the ability of LPAR4+ cells to confer stem-like properties to LPAR4- cells, I hypothesized that the ECM deposited by LPAR4+ cells can confer stress-tolerance to neighboring LPAR4- cells by activating YAP through paracrine signaling. To test this hypothesis, the decellularized ECM assay **(Figure 3.1)** was performed. Western blot data revealed that LPAR4- cells grown on the ECM deposited by LPAR4+ cells (R4-ECM) have elevated expression of YAP targets CTGF and CYR61 as compared to cells grown on the ECM deposited by LPAR4- cells (EV-ECM) **(Figure 3.4)**, indicating that ECM produced by LPAR4+ cells is sufficient to activate YAP in neighboring LPAR4- cells.

3.3 Fibronectin in the ECM is sufficient to drive YAP activation in LPAR4- cells.

From the previous data generated in our lab, we learned that LPAR4+ pancreatic cancer cells exhibit an enhanced expression of fibronectin. However, the exact mechanism by which tumor-initiating cells utilize this FN to respond to isolation stress is unclear. I hypothesized that the tumor-initiating ECM-niche established by LPAR4+ cells (R4-ECM) is enriched with FN, and that this could account for the induction of a stress-tolerant phenotype in LPAR4- cells through paracrine signaling. To test this hypothesis, immunofluorescence (IF) staining for FN was performed on ECM with and without cells. The ECM deposited by cancer-associated fibroblast cells (CAF1299-ECM) was used as a control, since CAFs are known to deposit a FN-enriched ECM and they play an important role in cell-mediated FN assembly in the tumor microenvironment [18]. The IF staining revealed that FN is a major component of the R4-ECM, as compared to the EV-ECM which has significantly lower levels of FN **(Figure 3.5)**. While the CAF1299-ECM exhibits abundant and well-organized FN fibrils surrounding the cells **(Figure 3.5)**, the R4-ECM mainly comprises of globular fibronectin, indicating that FN is not optimally assembled into fibers. In the context of a tumor-initiating niche, it is likely that while the tumor-initiating LPAR4+ cells autonomously deposit the initial FN-enriched ECM, CAFs are the primary cellular component of the tumor stroma which continue to provide a FN-enriched ECM niche through paracrine signaling [28] while the tumor becomes established at a primary or metastatic site.

The results so far indicate that fibronectin is the key ECM component which drives YAP activation in response to isolation stress. However, the ECM is extremely complex in terms of composition, interactions, and signaling pathways [51]. Based on previous

studies which link YAP activation to FN adhesion through a negative regulation of the Hippo pathway [45], I hypothesized that fibronectin alone would be sufficient to drive YAP activation in LPAR4- cells. To study the effect of FN on YAP activation without the influence of other ECM components, LPAR4- KP4 cells were grown on a pure human plasma fibronectin matrix.

Western blot analysis showed an increased expression of the YAP targets CTGF and CYR61 in LPAR4- cells grown on a pure FN matrix **(Figure 3.6)**. These results establish that FN in the ECM is sufficient to drive YAP activation, and consequently cancer stemness, in LPAR4- cells.

3.4 Figures

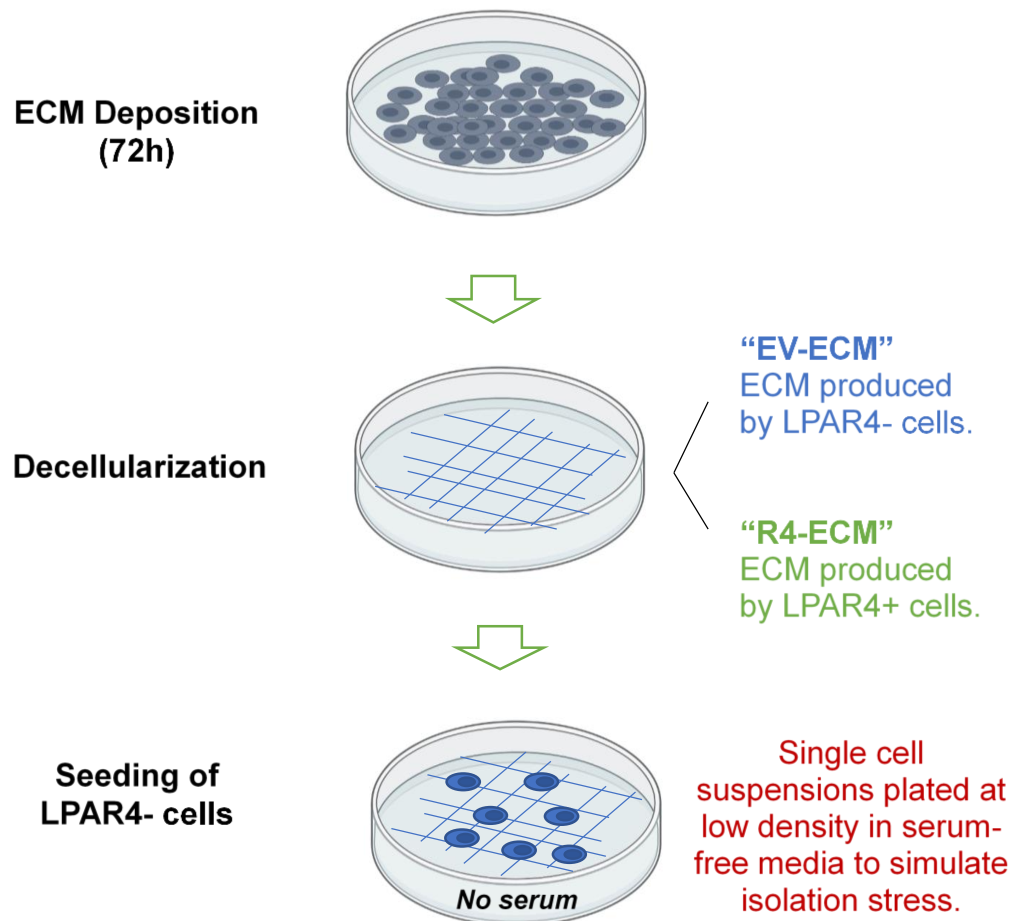

Figure 3.1: An overview of the decellularized ECM assay. The ECM depositing 34E+EV or 34E+R4 cells were cultured in the absence of exogenous LPA for 72 hours. Decellularized ECM was prepared using ammonium hydroxide treatment as described by Hellewell *et. al.* [52]. LPAR4- cells were seeded on the decellularized ECM at low cell densities and cultured in serum-free conditions to produce a growth-limiting effect and mimic isolation stress.

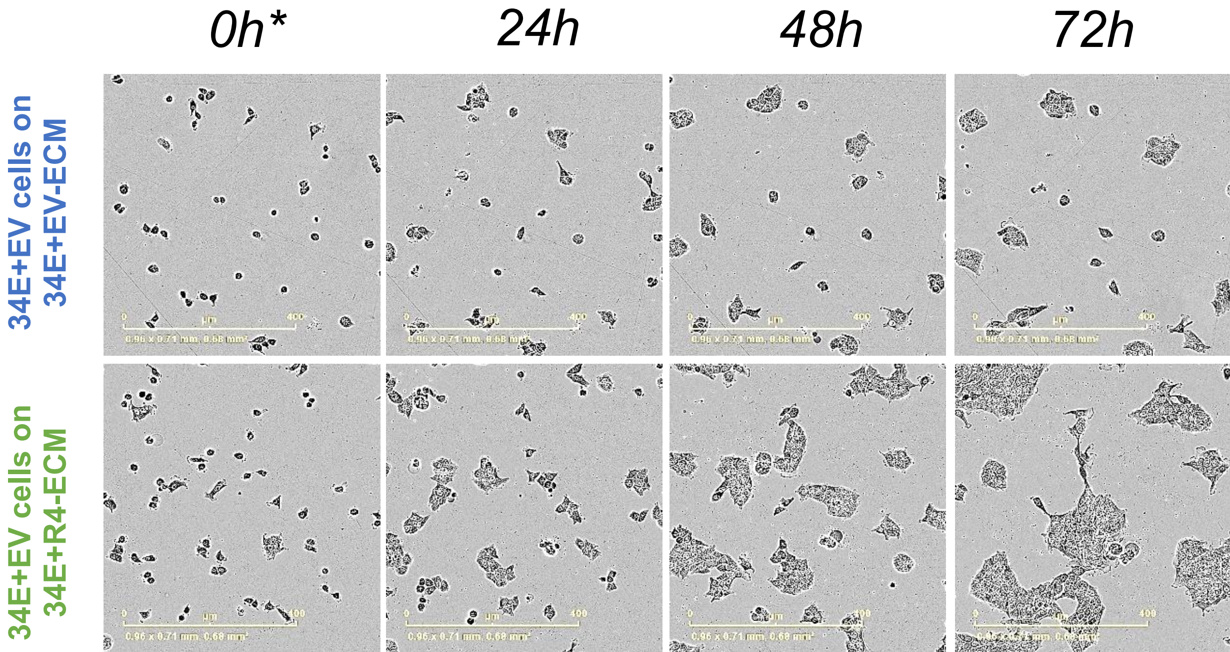

Figure 3.2: The ECM deposited by LPAR4+ cells can confer the isolation stress-tolerant phenotype to LPAR4- cells. The ECM depositing cells (+EV and +R4) were cultured in the absence of exogenous LPA for 72 hours. LPAR4- cells were seeded on the decellularized ECM at low cell densities and cultured in serum-free conditions to produce a growth-limiting effect and mimic isolation stress. 0h* represents the first image captured post overnight cell adhesion to the tissue culture plate.

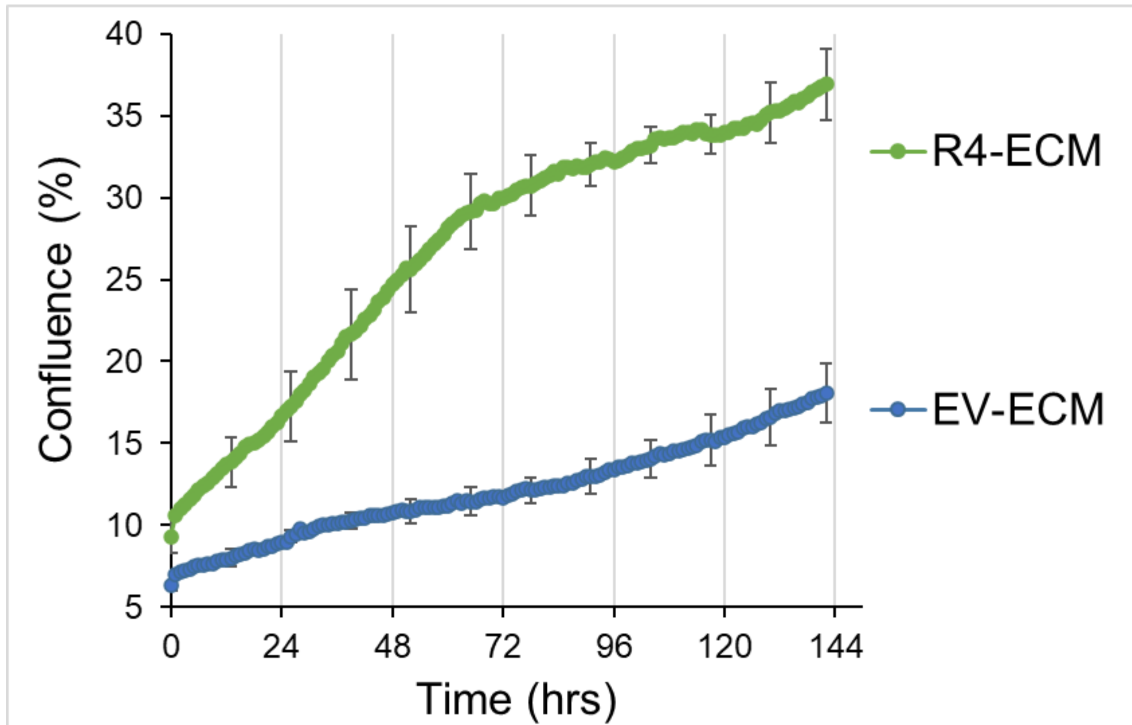

Figure 3.3: LPAR4- cells cultured on the ECM deposited by LPAR4+ cells exhibit enhanced cell proliferation when challenged with isolation stress. The ECM depositing cells (+EV and +R4) were cultured in the absence of exogenous LPA for 72 hours. LPAR4- cells were seeded on the decellularized ECM at low cell densities and cultured in serum-free conditions to produce a growth-limiting effect and mimic isolation stress. 0 hr represents the first image captured post overnight cell adhesion to the tissue culture plate.

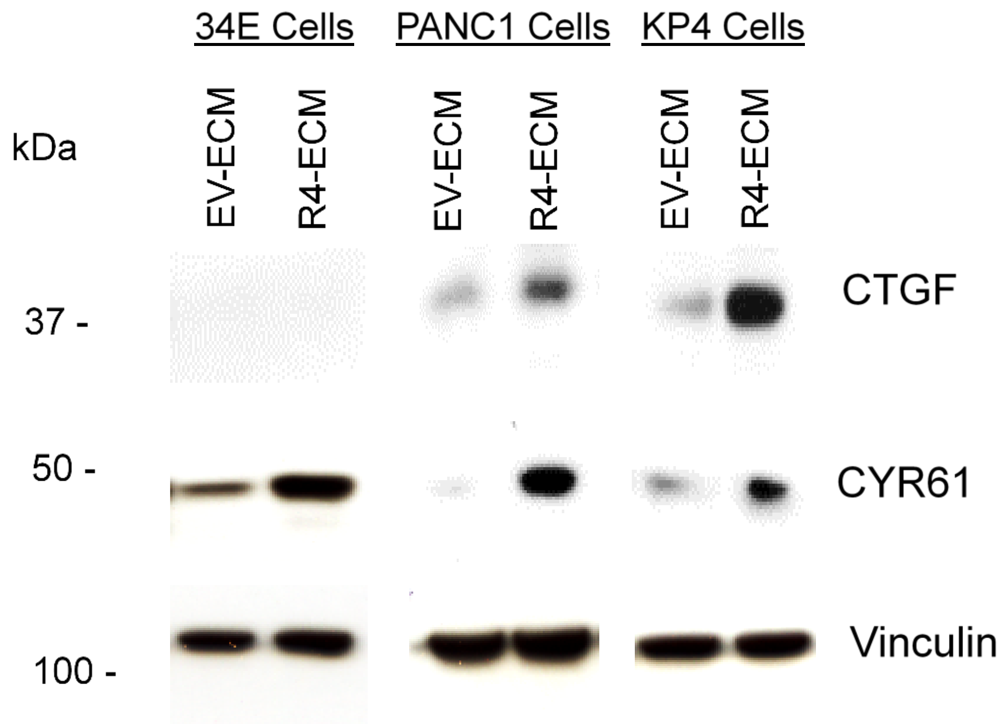

Figure 3.4: YAP targets CTGF and CYR61 are elevated in LPAR4- cells cultured on the ECM deposited by LPAR4+ cells. The ECM depositing cells (+EV and +R4) were cultured in the absence of exogenous LPA for 72 hours. LPAR4- cells were seeded on the decellularized ECM at low cell densities and cultured in serum-free conditions to produce a growth-limiting effect and mimic isolation stress. Cells were harvested for western blot after 48 hours. Vinculin was used as the loading control.

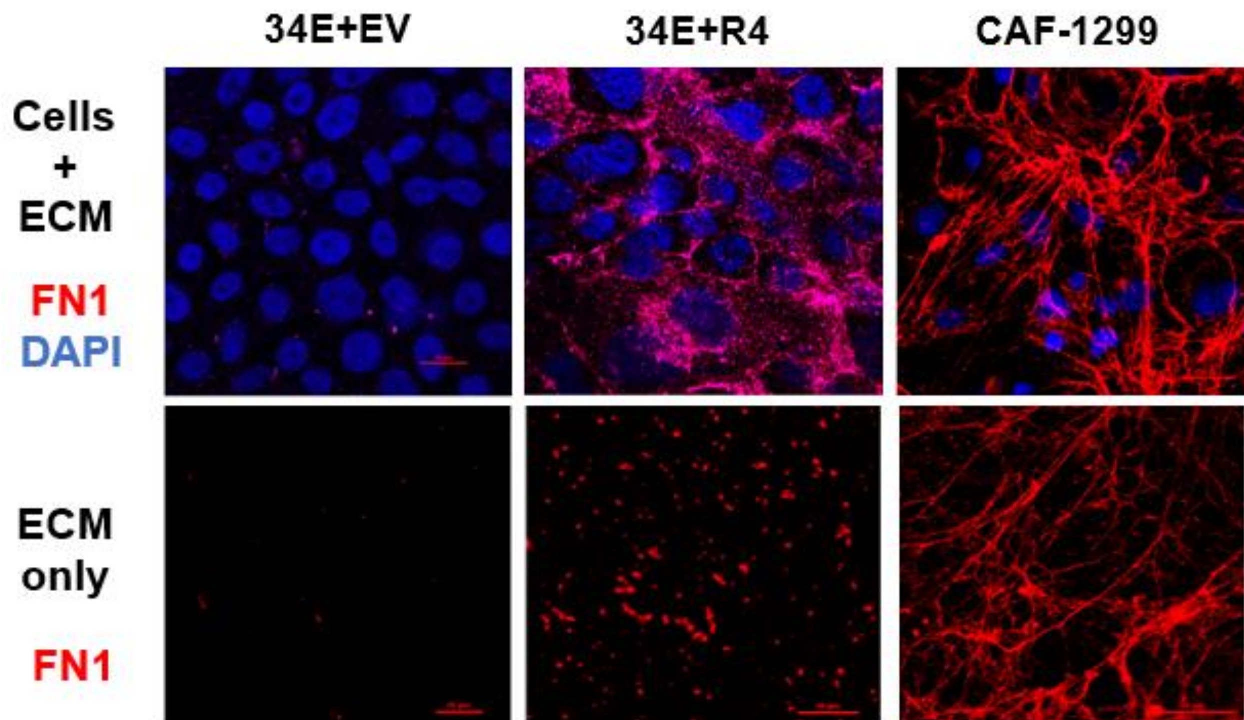

Figure 3.5: The ECM deposited by LPAR4+ cells is enriched with fibronectin. The ECM depositing cells (+EV and +R4) were cultured in the absence of exogenous LPA for 72 hours and immunofluorescence staining for FN was performed. CAF-1299 cells were used as a control. The nuclear stain DAPI was used to visualize the cells.

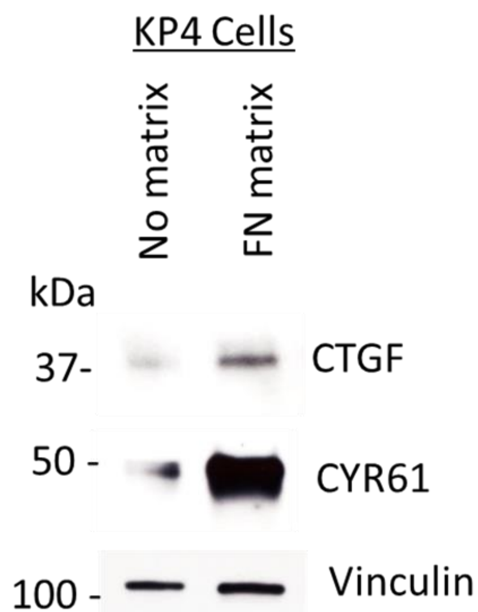

Figure 3.6 LPAR4- cells on a pure fibronectin matrix show an increased activation of YAP. The LPAR4- cells were seeded on the different matrices at low cell densities and cultured in serum-free conditions to produce a growth-limiting effect and mimic isolation stress. Cells were harvested for western blot after 24 hours. Vinculin was used as the loading control. All the blots were cut from the same SDS-PAGE gel.

3.5 Materials and Methods

Cell Culture

34E parental cells were derived from patient-derived xenograft models established by Dr. Andrew Lowy (University of California, San Diego). Paired cell lines were generated by transfecting the 34E cells with empty vector (EV) control or ectopic *LPAR4* expression vector (R4) using a lentiviral system produced in 293T cells using Lipofectamine 3000 (Thermo Fisher Scientific). The expression vectors used in this study are listed in **Table 3.1**. Ectopic LPAR4 expression was validated using qRT-PCR and western blot. 34E cells were cultured in Dulbecco's modified Eagle medium (Thermo Fisher Scientific) containing 10% FBS (Gibco) and 1% penicillin–streptomycin (Thermo Fisher Scientific). Pancreatic cancer cell lines PANC1 and KP4 were obtained from American Type Culture Collection (ATCC). Cancer associated fibroblasts (CAF1299) were obtained from the laboratory of Dr. Andrew Lowy (University of California, San Diego). PANC1, KP4 and CAF-1299 cells were cultured in Roswell Park Memorial Institute 1640 Medium (Thermo Fisher Scientific) containing 10% FBS (Gibco) and 1% penicillin–streptomycin (Thermo Fisher Scientific). For studies that required the absence of exogenous LPA, Dulbecco's modified Eagle medium (Thermo Fisher Scientific) containing charcoal-stripped fetal bovine serum (Corning) was used to culture cells.

Preparation of LPAR4+ cell-derived ECM

34E+EV or 34E+R4 cells cultured in 6-well tissue culture plates in Dulbecco's modified Eagle medium containing charcoal-stripped fetal bovine serum (Corning) to ensure the absence of exogenous LPA. The cells were allowed to deposit ECM onto the

tissue culture plate for 72 hours. Decellularized ECM was prepared using 20% ammonium hydroxide as previously described by Hellewell *et. al.* [52].

Live Cell Imaging

LPAR4- 34E+EV cells were seeded at a low density of 100,000 cells per well onto the decellularized ECM deposited by LPAR4+ cells and cultured in serum-free Dulbecco's modified Eagle medium to mimic isolation stress conditions. After allowing the cells to adhere overnight, the plates were moved into the incubation chamber of the IncuCyte S3 Live-cell imaging system at the Human Embryonic Stem Cell Core at the Sanford Consortium for Regenerative Medicine. Live cell imaging was performed to capture cell morphology at various time points (0h, 12h, 24h, 48h, 72h). The IncuCyte software was used to measure phase object count and quantify cell confluence over time.

Immunofluorescence Staining

34E+EV, 34E+R4 and CAF1299 cells were seeded onto 8-well chamber slides (Nunc Lab-Tek chamber slide, Thermo Scientific) and allowed to deposit ECM for 72 hours. Decellularization of the ECM was performed as described above. The samples were fixed with 4% formaldehyde for 15 minutes at room temperature, followed by overnight incubation with anti-FN primary antibody (CST #26836, 1:1000) at 4 °C. This was followed by incubation with the Alexa Fluor 568 anti-rabbit secondary antibody (Life Technologies #A10042, 1:2000) for 1 hour and staining with the nuclear stain DAPI for 5 min at room temperature. Images were acquired by Nikon Eclipse C1 confocal microscope and analyzed using the NIS-Elements Viewer 5.21 software.

Preparation of Pure Fibronectin Matrix

6-well non-tissue culture treated plates were coated overnight with 10ug/ml of Human plasma fibronectin (R&D Systems #1918-FN) at 4 °C. The coated plates were blocked with 5% bovine serum albumin (Sigma Aldrich) for 2 hours at room temperature. LPAR4- KP4 cells were seeded at a low density onto this matrix and cultured in serum-free Dulbecco's modified Eagle medium to mimic isolation stress conditions. Cells seeded on tissue culture treated plates were used as a control.

Western Blots

Cell lysates for western blots were prepared using 1X RIPA buffer containing protease and phosphatase inhibitors. Protein concentrations were estimated by BCA assay (Thermo Fisher Scientific). After denaturing the lysates with 4X Laemmli Sample buffer (BioRad) containing a the NuPAGE reducing agent (Invitrogen), 30ug of protein per sample was loaded onto SDS-PAGE gels, followed by transfer onto polyvinylidene difluoride membranes. 5% fat-free milk in TBS-T buffer was used for blocking and probing. The following primary antibodies were used: Vinculin (Boster #MA1103, 1:3000), YAP (CST #4912S, 1:2000), pYAP-S127 (CST #4911S, 1:2000), CTGF (CST #86641S, 1:1000), CYR61 (Proteintech #26689-1-AP, 1:2000). The secondary antibodies used were goat anti-rabbit HRP (BioRad #170-6515) and goat anti-mouse HRP (BioRad #170-6516). ECL reagent (Thermo Fisher Scientific) was used to develop the blots. Vinculin was used as the loading control.

Table 3.1: List of expression vectors used in this study.

Target	Ectopic Expression (GeneCopoeia)
Empty vector (EV) control	EX-M0914-Lv122
LPAR4 (R4)	EX-NEG-Lv122

3.6 Acknowledgements

Chapter 3 contains unpublished data which was acquired with the help of Chengsheng Wu from the Cheresh lab. The thesis author was the primary author of this chapter.

CHAPTER 4: DISCUSSION

Our lab has previously established that pancreatic cancer cells upregulate LPAR4 in response to isolation stress, which results in a cell-autonomous production of the ECM component fibronectin, allowing cells to establish their own tumor-initiating niche [8]. In this study, I report that the ectopic expression of LPAR4 in pancreatic cancer cells induces a stress-tolerant phenotype, characterized by stem-like and mesenchymal features such as formation of migratory projections and colonies, suggesting that the cells undergo epithelial-to-mesenchymal transition (EMT) to survive isolation stress. This adaptive gain of stress-tolerance is driven by the enrichment of FN in the ECM and the enhanced activation of the transcriptional regulator Yes-associated protein (YAP). Activated YAP drives the expression of CTGF and CYR61, which are reliable readouts for cancer stemness. The knockout of FN in LPAR4+ cells significantly hampered the YAP-driven expression of CTGF and CYR61, indicating that FN is necessary to drive the activation of YAP. Furthermore, LPAR4- cells grown on the ECM deposited by LPAR4+ cells under isolation stress conditions gain the same stress-tolerant phenotype exhibited by LPAR4+ cells through ECM-mediated paracrine signaling. LPAR4- cells grown on pure FN-matrix can activate YAP in response to isolation stress, indicating that FN is sufficient to induce YAP-mediated stress-tolerance and cancer stemness.

Apart from its well-established role in promoting epithelial-to-mesenchymal transition (EMT) [31] and resistance to chemotherapy in cancer cells [53], YAP is also crucial for the cells to respond to ECM stiffness through mechanotransduction pathways [35]. This role of YAP is especially important in the pancreatic tumor microenvironment, which is characterized by a desmoplastic reaction leading to the deposition of a dense

and rigid ECM [6]. Thus, YAP and YAP target genes can be potential targets for the development of chemotherapeutic agents. Our lab has also generated preliminary data showing that YAP is activated in LPAR4+ lung cancer cells in response to isolation stress (not shown in this study), indicating that the findings of this study have the potential to be extended to other cancer types.

Since integrins are the primary receptors for various ECM proteins including fibronectin, blocking integrin-FN interactions could be an effective strategy to reduce stress-tolerance in tumor-initiating cells, making them susceptible to chemotherapy. Our lab has established integrin αvβ3 as a marker of cancer stemness [26], and this integrin is also a receptor for fibronectin [25]. Our lab is currently studying the effect of various integrin function-blocking antibodies on YAP activation and cancer stemness.

The ECM is extremely complex in its composition, and fibronectin is known to interact with various other ECM components [54]. It is likely that cancer stemness is driven by the combined effects of various ECM signaling pathways. The ECM is very dynamic and undergoes remodeling in response to various environmental cues in cancer [55]. Exploring other key players in the ECM that contribute to EMT and stress-tolerance is crucial to identify potential targets for cancer therapy.

Thus, the findings of this study establish that pancreatic cancer cells utilize a fibronectin-enriched ECM niche to gain stress-tolerance and drive cancer stemness through the activation of YAP, leading to the expression of YAP targets CTGF and CYR61. Further work is required to study the interactions of FN with different integrins and other ECM components to obtain a complete picture of ECM-mediated cancer stemness and progression.

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