UNIVERSITY OF CALIFORNIA SAN DIEGO

Fibronectin-Binding Integrins Alpha-5 Beta-1 and Alpha-v Beta-3 on Cancer-Associated Fibroblasts Block Fibronectin Collagen Matrix Assembly, Tumor Initiation and Tissue Stiffness

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

in

Bioengineering

by

Diva Sansanwal

Committee in charge:

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

University of California San Diego
2024

DEDICATION

To

My parents, Kavita and Sanjeev
For their unparalleled love and sacrifices. I am grateful for their efforts towards supporting me financially and emotionally throughout my master's journey and always encouraging me to pursue my passion for research.

My brother and my pet, Ikshaan and Coco
For being a constant motivation for me.

My friends,
For giving me a wholesome atmosphere away from home, throughout my journey as a Masters student in a foreign country.

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ABBREVIATIONS

CAF – Cancer associated fibroblasts

COL – Collagen

COL1A1 – Type I collagen alpha 1 chain

ECM – Extracellular matrix

EDA – Extra-domain A

EDB – Extra-domain B

EMT – Epithelial-to-mesenchymal transition

FN – Fibronectin

MSC – Mesenchymal stem cell

PanIN – Pancreatic intraepithelial neoplasia

PaSC – Pancreatic stellate cells

PDAC – Pancreatic ductal adenocarcinoma

RGD – Arginine (R), Glycine (G), and Aspartic acid (D)

STIFMap – Spatially transformed inferential force map

TGF-ß – Transforming growth factor

TME – Tumor microenvironment

VEGF – Vascular endothelial growth factor

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ABSTRACT OF THE THESIS

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Professor David A. Cheresh, Chair
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Pancreatic cancer is characterized by a dense fibrotic stroma, consisting of cancer-associated fibroblasts (CAFs), immune cells, and extracellular matrix (ECM) that promote tumor growth and treatment resistance. CAFs secrete and organize on their surface ECM proteins, including fibronectin (FN) which coordinates the assembly of collagen fibrils that promote matrix stiffness. Since CAFs are a major contributor to fibrosis and the progression of pancreatic cancer, novel approaches to target their function could have a significant impact on patient outcome. This research demonstrates that the ability of pancreatic cancer cells to initiate tumors in mice is greatly enhanced by co-injection of CAFs. Targeting FN via FN-binding integrins α5β1 and αvβ3 that are highly expressed on CAFs disrupts CAF-induced fibrosis, decreases tumor stiffness, and prevents

the initiation of pancreas tumors in mice. These results highlight the therapeutic potential of targeting fibronectin-binding integrins, α5β1 and αvβ3, on CAFs to disrupt the fibrotic stroma in pancreatic cancer.

CHAPTER 1 – INTRODUCTION

1.1. Pancreatic cancer

The term "cancer" refers to more than a hundred forms of disease and almost every tissue in the organism can generate malignancies. Some even yield several types ("How Cancer Arises on JSTOR" n.d.). While each type of cancer has distinct characteristics, this thesis specifically focuses on pancreatic cancer. Pancreatic cancer is the third-leading cause of cancer death in men and women combined (Siegel Mph et al. 2024). The overall survival rate for this type of cancer is only 6% (Puckett and Garfield 2022), and it is projected to become the second leading cause of cancer-related deaths by 2030 (Rahib et al. 2014). Pancreatic cancer is associated with poor prognosis and its low survival rate in patients is attributed to multiple factors, such as the late stage at which most patients are diagnosed. Most patients with this disease are asymptomatic until it develops into an advanced stage, and only 20% of patients are eligible for initial resection (Kamisawa et al. 2016). Tumor biology of pancreatic cancer contributes to early recurrence and metastasis; autopsy series have shown that approximately 90% of cases of pancreatic cancer are complicated by distant metastasis (Kamisawa et al. 1995).

1.2.Pancreatic ductal adenocarcinoma (PDAC)

PDAC is one of the most aggressive types of pancreatic cancer with a high metastatic potential. Most PDAC patients are diagnosed with metastatic disease, for which the five-year survival rate is only 3% (Pereira and Chio 2020). PDAC is the most common pancreatic neoplasm; it arises from precursor lesions known as pancreatic intraepithelial neoplasia (PanIN). PanINs progress in a stepwise process through the acquisition of genetic alterations and culminate in the development of overt PDAC (Mizrahi et al. 2020). PDAC is unique among solid tumors because it is an invasive mucin-producing and gland-forming neoplasm that evokes an intense stromal desmoplastic reaction (Youn et al. 2018; Kamisawa et al. 2016). It has been reported that extensive fibroblastic cell proliferation in PDAC correlates with poorer disease outcome (Pandol et al. 2009).

1.3. The desmoplastic stroma

Desmoplasia containing myofibroblastic pancreatic stellate cells (PaSCs), immune cells, endothelial cells, mesenchymal stem cells (MSCs), normal fibroblasts, and extracellular matrix (ECM) deposited by cancer-associated fibroblasts (CAFs), also known as activated fibroblasts, contribute to a complex tumor microenvironment (TME) in PDAC (Pandol et al. 2009). The desmoplastic stroma in PDAC constitutes up to 90% of the tumor volume (Neesse et al. 2015; Erkan et al. 2008; Hidalgo 2010). CAFs are important for the formation of the desmoplastic stroma, as these cells promote tumor progression, metastasis, chemoresistance, and immunosuppression (Kalluri 2016).

1.4. Cancer-associated fibroblasts (CAFs)

CAFs are characterized by their diverse origins, including the activation of resident fibroblasts and PaSCs, the recruitment and differentiation of bone marrow-derived MSCs, and epithelial-to-mesenchymal transition (EMT). In order to acquire activated phenotypes, quiescent fibroblasts undergo activation through diverse mechanisms in response to growth factors such as transforming growth factor-beta (TGF-ß). The most prominent source of CAFs in PDAC is PaSCs, the resident mesenchymal cells of the pancreas (Vonlaufen et al. 2008). Once PaSCs are activated, they suffer a loss of vitamin A reserves, switch to a contractile and secretory phenotype, and secrete large amounts of ECM components, such as COLs and fibronectin (FN) (McCarroll et al. 2006).

1.5. Cells and the extracellular matrix (ECM)

Cells respond to various types of mechanical stimuli, such as substrate rigidity, hydrostatic pressure, tensile, and shear stress in their microenvironment (Bukoreshtliev, Haase, and Pelling 2013). The cell converts these mechanical cues into biochemical signals through a process known as mechanotransduction (Bukoreshtliev, Haase, and Pelling 2013). Mechanical information is transduced through mechanoreceptors located between a cell and the ECM. Cellular mechanoreceptors interact with the surrounding ECM within their microenvironments through cell surface receptors such as integrins, a family of cell-ECM mechanoreceptors that govern cell proliferation, migration, survival, tissue invasion, and innate immunity (Seetharaman and Etienne-

Manneville 2018). Clusters of integrins connect a cell to the ECM and integrate signals from both sides of the plasma membrane to transduce them bidirectionally (Desgrosellier and Cheresh 2010).

1.6. Integrins

Integrins are composed of non-covalently linked heterodimeric α and ß subunits; eighteen α units can combine with eight ß subunits to form twenty-four different combinations in mammals (Takada, Ye, and Simon 2007). Each integrin subunit possesses an extracellular domain, a single transmembrane domain, and a short cytoplasmic tail, except for ß4. The heterodimeric nature of integrins controls binding of a given integrin to distinct motifs on ligands. For example, a subset of integrins can bind to the Arg-Gly-Asp (RGD) motif found in certain ECM proteins, including FN (Danen 2013). Meanwhile, the cytoplasmic tails of ß subunits bind to components of the actomyosin cytoskeleton, allowing integrins to bi-directionally "integrate" extracellular signals with intracellular signalling pathways (Geiger, Spatz, and Bershadsky 2009).

In the TME, CAFs and cancer cells can communicate through the ECM. ECM remodelling by CAFs creates a favorable stromal environment for cancer cells to invade during tumor progression, and deregulated ECM proteins promote the metastatic cascade (Givant-Horwitz, Davidson, and Reich 2005). The ECM can be remodelled by many processes, including synthesis, contraction and proteolytic degradation. Integrins are the primary ECM receptors mediating ECM remodelling (DeMali, Wennerberg, and Burridge 2003).

1.7. Fibronectin (FN) classification and structure

FN is a large, 440 kDa, glycoprotein composed of two smaller, 230-250 kDa monomers (White and Muro 2011). Every monomeric strand is composed of a combination of type I, II, and III repeats, and each of these repeats generates an anti-parallel beta sheet(White and Muro 2011). Monomers are composed of 12 type I repeats, 40 amino acids each, 2 type II repeats, 60 amino acids each, and 15-17 type III repeats, 90 amino acids each (White and Muro 2011). Splice variation produces differences in FN; Cellular FN differs from plasma FN by a number of additional repeats. Both types of FN have 12 types of I repeats and 2 type II repeats (White and Muro 2011). Plasma FN has 15 type III repeats and cellular FN has 17 type III repeats(To and

Midwood 2011). The most notable of these splice variations are Extra-domain A (EDA, located after the 11th type III repeat) and Extra-domain B (EDB, located after the 7th type B repeat (To and Midwood 2011). These small structural variations have functional implication affecting the role for the EDA as a region for specialized integrin binding. Overall, the process of splice variation results in 20 unique isoforms of FN and there is a great diversity of possible ligand-receptor interactions (White and Muro 2011). Plasma FN lacks EDA and EDB and is secreted by hepatocytes into the blood stream. On the other hand, cellular FN, which differs from plasma FN by the inclusion of the EDA/EDB and IIICS splice sites, has a more robust biological role (Zand et al. 2003). It is produced by a variety of cell types, but most cellular FN originates from fibroblasts (Y et al. 1998).

1.8. Role of FN

FN is a major constituent of the extracellular matrix within the TME. While various cell types including cancer cells and endothelial cells can produce FN, it is primarily produced by cancer-associated fibroblasts (Pankov and Yamada 2002). It is essential for wound healing, development, and maintaining tissue homeostasis (Topalovski and Brekken 2016). For PDAC, high FN expression correlates with a significantly larger tumor size (Hu et al. 2019).

1.9. FN assembly

In cell matrix adhesions, FN monomers are unfolded and assembled into fibrils by a cell-driven process termed fibrillogenesis (Schwarzbauer and DeSimone 2011). Integrins bind to ECM components via their globular head domains and connect to the actin cytoskeleton via adaptor proteins that bind their short cytoplasmic tails (Van der Flier and Sonnenberg 2001; Hynes 2002). Besides mediating adhesion to ECM components, integrins participate in the assembly of the ECM via binding to FN (Van der Flier and Sonnenberg 2001; Hynes 2002). The major receptor of cellular FN, α5ß1 integrin, can be found in different adhesion structures, such as focal complexes, focal adhesions, fibrillar adhesions, and 3D-matrix adhesions (Larsen et al. 2006). The activation of FN is induced by α5ß1 integrin binding to the RGD domain in the type III-10 module along with a synergy sequence located in the adjacent type III-9 module (Pierschbacher and Ruoslahti 1984; Nagai et al. 1991; Sechler, Corbett, and Schwarzbauer 1997). Additional RGD-binding

integrins, including αvß3, can substitute for α5ß1 in FN matrix assembly to form a dense and delicate fibrillar network in vitro (Chuanyue Wu et al. 1995; Wennerberg et al. 1996). FN organized by CAFs requires myosin-II-driven contractility and increased traction forces that are transduced to the ECM through integrin α5ß1 (Erdogan et al. 2017). Certain αv integrins, such as αvß3, are essential for CAF-mediated FN assembly that stimulates colon carcinoma cell invasion (Attieh et al. 2017a).

1.10. Collagen (COL) classification and structure

COLs are the most abundant proteins in mammals. Inside the cell, procollagen proteins form as a triple helix of three polypeptide α chains, numbered with Arabic numerals, that are secreted into the extracellular space (Ricard-Blum 2011). Outside the cell, the procollagen peptides align to form higher level collagen structures, such as fibers or networks. Beyond the existence of 28 COL types, diversity occurs in the COL family because of the existence of several molecular isoforms for the same COL type (Ricard-Blum 2011). Splicing events are sometimes specific to a tissue and/or a developmental stage, and splicing variants modulate COL functions (Ricard-Blum 2011). Several COLs carry glycosaminoglycan chains and are considered also as proteoglycans (Ricard-Blum 2011).

COLI and III have been reported to be the major product of activated PaSCs (Armstrong et al. 2004). COL type I has two α chains (Makareeva and Leikin 2014) and it makes up 90% of the human body, and it is densely packed and used to provide structure to skin, bones, tendons, and ligaments ("Collagen: What It Is, Types, Function & Benefits" n.d.). Meanwhile, COLIII consists of only one α chain (Nielsen and Karsdal 2016) and it is found in muscles, arteries, and organs ("Collagen: What It Is, Types, Function & Benefits" n.d.). Both, COLI and COLIII, are fibril-forming collagens and are secreted into the ECM as precursor molecules, known as procollagens, that require enzymatic removal of the C- and N-propeptides, an essential step in COL fibril assembly (Broder et al. 2013). COLI is a stiff fibrillar protein that gives tensile strength, whereas COLIII produces an elastic network that stores kinetic energy as an elastic rebound (Broder et al. 2013).

1.11. Collagen (COL) in the PDAC ECM

Increased desmoplasia is thought to contribute to disruption of the basement membrane leading to increased exposure of PDAC cells to interstitial collagens and reduction of basement membrane collagens (Weniger, Honselmann, and Liss 2018). COLI and, to a lesser degree, COLIII are among the most abundant ECM proteins in the PDAC stroma and are suggested to be responsible for most of the desmoplastic reaction and have been associated with reduced survival in PDAC patients (Mollenhauer, Roether, and Kern 1987; Imamura et al. 1995). Armstrong et al. demonstrate that COLI confers a survival advantage to pancreatic cancer cells by regulating proliferation and apoptosis.

ECM collagens interact with integrins expressed on the surface of PDAC cells to promote the proliferation and migration of tumor cells (Berchtold et al. 2015). Collagen binding to integrins depends on the integrin specificity of the collagen subtype (Tulla et al. 2001).

1.12. FN-mediated and integrin-mediated collagen (COL) fibrillogenesis

Fibrillar COLs are generally pro-tumorigenic because they provide a scaffold and reservoir for soluble growth factors; their alignment, crosslinking, and remodeling can signal to cancer and stromal cells to promote pro-tumorigenic behavior, such as invasion, proliferation, and metastasis (Kanematsu et al. 2004). They also serve as a nutrient source for cancer cells to scavenge (Kanematsu et al. 2004). Collagen fibrils are largely synthesized and secreted by CAFs, although cancer cells also deposit a small fraction of the total tumor collagen (Olivares et al. 2017).

The cognate FN-COL-binding sites are located at the 3/4-1/4 mammalian collagenase cleavage site on COL and within a region near the N-terminus of FN that contains type I and II module repeats(Owens and Baralle 1986; Kleinman, McGoodwin, and Klebe 1976). Antibody binding to the COL-binding site on FN inhibits COL fibrillogenesis (Li et al. 2003). In one study, inhibition of FN assembly with an anti-α5ß1 integrin antibody completely inhibited COL assembly (Li et al. 2003). It is clear that FN is required for COL fibril assembly (Kadler, Hill, and Canty-Laird 2008).

1.13. ECM stiffness

The desmoplastic stroma plays a vital role in driving and supporting the progression of PDAC (Ferrara et al. 2021). The ECM remodelling process, including the activation of CAFs and the accumulation and crosslinking of FN and COL, determines stiffness in tumors (Mai et al. 2024). In the stages of COL synthesis and fibrillogenesis, crosslinking plays a crucial role, organizing procollagen chains into a cohesive network of COL triple helices (Mai et al. 2024). The crosslinking of COL in the ECM is primarily regulated by the LOX protein family, which consists of LOX-1 and four related enzymes, LOX-like protein (LOXL 1-4) (Kim, Kim, and Kim 2011). LOXL2, in particular, leads to the stiffening of PDAC tissues by promoting the crosslinking of COL fibers and increasing the secretion of related factors (such as exosomes) in primary tumor tissues, which leads to ECM remodeling and enhances matrix stiffening (Alonso-Nocelo et al. 2023; Mai et al. 2024). The stiffness of the ECM significantly influences the malignant behaviors of cancer cells, including morphological changes, proliferation, metabolic reprogramming, EMT, invasion, metastasis, and resistance to chemotherapy (Mai et al. 2024).

It has been reported that elevated ECM stiffness activates the FAK/RhoA/ROCK and PI3K/AKT signaling pathways via integrins, thereby increasing the expression of MMP2 and MMP9, and enhancing the invasiveness of cancer cells (Gao et al. 2020). A stiff ECM alters cellular mechanics, leading to changes in cell shape, cytoskeletal organization, and migration patterns (Wang and Ingber 1994). Furthermore, increased tumor stiffness, caused by a stiff ECM, obstructs the efficient delivery of therapeutic drugs within the tumor, diminishing the efficacy of such treatments (Muñoz et al. 2021). Additionally, increased ECM stiffness enhances aerobic glycolysis in cancer cells via YAP activation, contributing to cancer cell migration (Liu et al. 2020).

1.14. ECM stiffness effect on the yes-associated protein (YAP) pathway

The Hippo/yes-associated protein (YAP) pathway has been recognized o play a critical role in mechano-transduction and in sensing ECM stiffness (Panciera et al. 2017; Ibar and Irvine 2018). YAP is the major downstream effector of Hippo pathway and it has been identified as a potent oncogene (Moroishi, Hansen, and Guan 2015). YAP is a prominent transcriptional coactivator that

translates extracellular physical information into protein expression by translocating to the nucleus and regulating messenger RNA expression (Feng et al. 2021). It has been reported that YAP mediates cellular mechano-responses and inhibition of YAP translocation inhibits EMT (Dupont et al. 2011). Matrix stiffening has been reported to enhance YAP activation in fibroblasts and the generation and maintenance of CAFs correlate strongly with YAP translocation (Feng et al. 2021). One possible way to check for YAP activation is by establishing the presence of CTGF and Cyr61; both genes have been reported to be upregulated upon YAP activation (Zhao et al. 2008).

This thesis investigates the pivotal role of FN-binding integrins α5β1 and αvβ3 in regulating the complex interplay between CAFs, FN, COL fibrillogenesis, and ECM stiffness within PDAC microenvironment. By targeting these integrins with a bispecific antibody, there is a promising avenue to disrupt FN assembly, subsequently inhibiting COL fibrillogenesis and reducing ECM stiffness. This approach holds potential to mitigate the aggressive desmoplastic reaction characteristic of pancreatic cancer, thereby offering new insights into therapeutic strategies aimed at altering TME to impede tumor initiation and progression.

CHAPTER 2 – RESULTS

2.1. CAFs promote tumor initiation via FN

Fibronectin plays a vital role in ECM assembly and remodeling. In order to test the role of CAF-produced FN during tumor initiation, KP4 human PDAC cells were injected orthotopically into the pancreas of immune-compromised nu/nu mice alone or at a 1:1 ratio with PDAC-derived CAFs to evaluate tumor initiation in the pancreas (**Fig. 1**). After 3 weeks, bioluminescence imaging revealed bone fide tumors (luciferase signal >1e+06). While PDAC cells injected alone failed to form detectable tumors at this early timepoint, co-injection of CAFs boosted tumor initiation in 5/5 mice. Meanwhile, only 1/5 tumor formed when CAFs with FN knockdown were co-injected with tumor cells, suggesting that CAFs promote tumor initiation via FN.

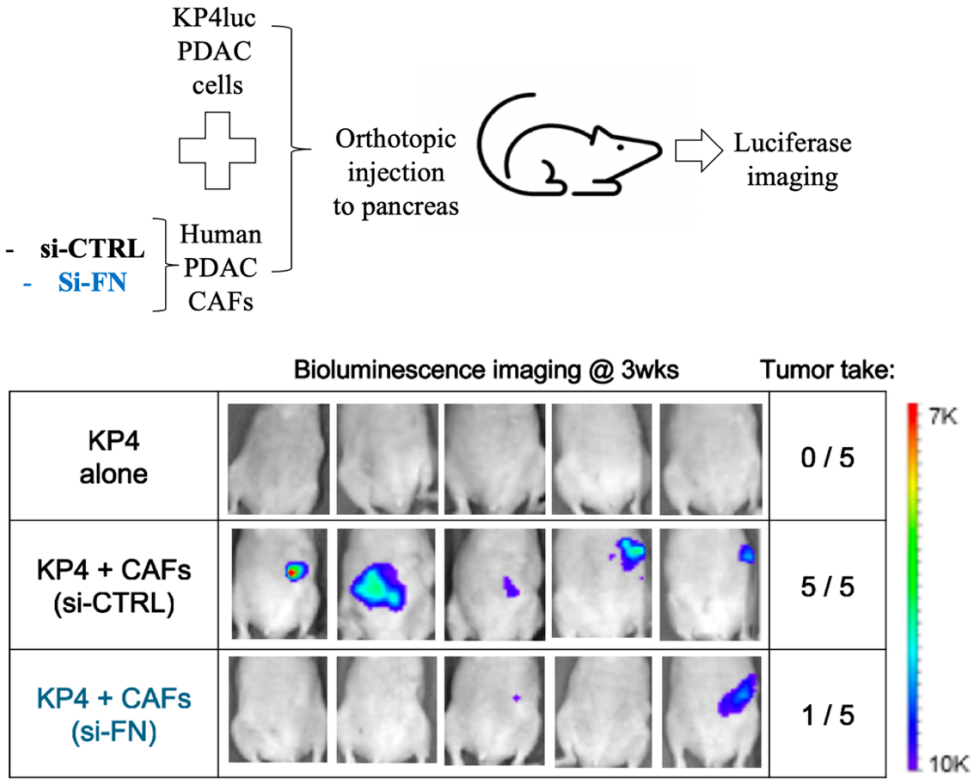


Figure 1. CAFs enhance tumor initiation via FN (orthotopic pancreas cancer model). 1 million KP4-luciferase cells were injected orthotopically into the pancreas of immune-compromised nu/nu mice alone or at a 1:1 ratio with PDAC derived CAF1299 cells (5 mice injected per group: KP4 cells alone, KP4 cells with CAFs, and KP4 cells with CAFs with FN knockdown). Tumor initiation was evaluated for 3 weeks via bioluminescence imaging (Luciferase signal >1e+06).

2.2. FN and FN-binding integrins (αvß3 and α5ß1) are vital for COL fiber assembly

FN has been described as the "master regulator" of ECM assembly (Dalton and Lemmon 2021) and it serves as a scaffold for the construction of additional matrix proteins as the si-RNA mediated knockdown of FN completely prevents the ability of CAFs to generate COL fibers (**Fig. 2, first panel only**). In contrast, knockdown of the type 1 collagen alpha 1 chain (COL1A1) does not affect FN fiber assembly (**Sup. Fig. 1)**. These findings suggest that FN is required for COL assembly, but not vice-versa, and highlight the critical role of FN in the tumor microenvironment.

CAFs use cell surface integrins as anchors to apply tension to FN molecules, revealing cryptic sites required for polymerization into FN "fibers" (Jang and Beningo 2019). Several integrins can serve as receptors for FN, including integrins αvß3 and α5ß1, that are absent from most normal cells but become upregulated in activated fibroblasts (CAFs) (Gasparini et al. 1998; Zhou et al. 2021; Lygoe et al. 2004). Similar to FN knockdown, COL fibrillogenesis can be reduced by knockdown of the integrin ß3 or α5 subunits (*ITGB3* and *ITGA5*, whose expression is vital for the formation of αvß3 and α5ß1 heterodimer formation, respectively). Imaging CAFs with si-RNA mediated knockdown of FN or subunits of FN-binding integrins (αvß3 and α5ß1) eliminates the ability of CAFs to produce and assemble COL fibers (**Fig. 2**). These results highlight the importance of FN-binding integrins in CAF-mediated FN and COL assembly.

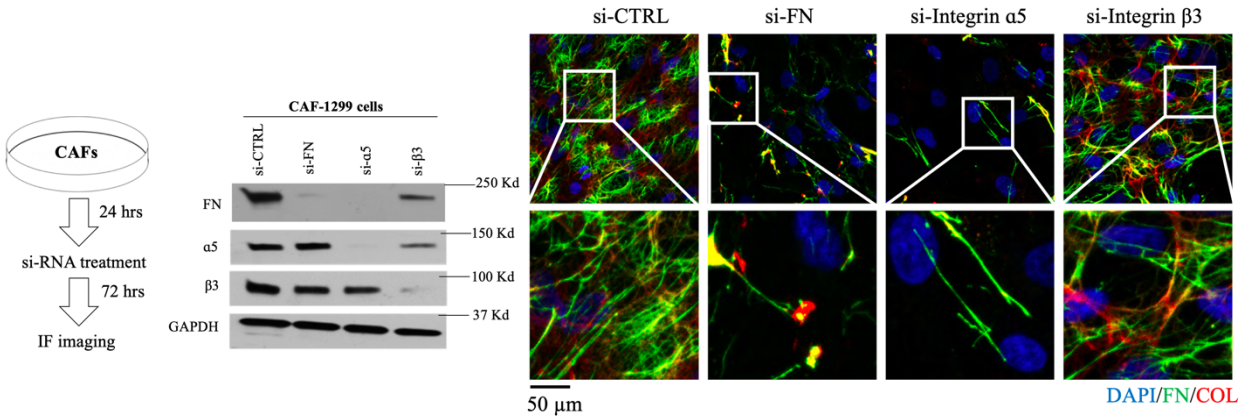


Figure 2. FN and FN-binding integrins in CAFs are critical for fibrillogenesis of FN and COL. CAF1299 cells were cultured for 24 hours in 8 well chamber slides. The cells were treated with non-targeted si-RNA, si-FN, si-α5, and si-β3; after 24 hours wells were stained using antibodies against FN and COL. Each panel shows an image of one representative field from three independent experiments. Blots confirm knockdown.

2.3. Integrin-blocking antibodies disrupt CAF-mediated FN and COL assembly

In order to simultaneously target both integrins (αvß3 and α5ß1) required for ECM production by CAFs using a single agent, a novel bispecific antibody (bsAb) for dual monovalent recognition was proposed (**Table 1**). The bsAb was first compared to its two parental control bivalent monoclonal antibodies (mAbs) that individually recognize integrins αvß3 and α5ß1. Commercially available antibodies recognizing integrin αvß3 (LM609) and α5ß1 (P1D6) were included as additional benchmarks. Flow cytometry analysis was performed to evaluate binding of each antibody (tested at a concentration of 10 μg/mL) to two different PDAC patient-derived CAF lines, CAF-1299 and CAF-1424. For both CAF models, the median fluorescence intensity (MFI) signal for the bsAb was slightly more than the additive sum of its two parental mAbs (**Fig. 3**).

	Antigens	**Parental antibodies**	**Isotype**	**Source**	**description**
bsAb	αvβ3 + α5β1	Etaracizumab + Volociximab	hIgG4-S228P	ABT-601	Novel bispecific Ab
Anti-αvβ3	αvβ3	Etaracizumab	hIgG4-S228P	ABT-101	Control mAbs (for the bsAb)
Anti-α5β1	α5β1	Volociximab	hIgG4-S228P	ABT-701	
LM609	αvβ3		mIgG1	Millipore MAB1976	Benchmark mAbs
P1D6	α5		mIgG3	Millipore MAB1956	

Table 1. Bispecific antibody (bsAb) for dual recognition of αvβ3 and α5β1 heterodimers. Design of the novel bsAb with monovalent recognition of two antigens, integrins αvβ3 and α5β1. The Fab domains for the bsAb are identical to the "control" bivalent monoclonal antibodies recognizing αvβ3 (derived from etaracizumab) and α5β1 (derived from volociximab).

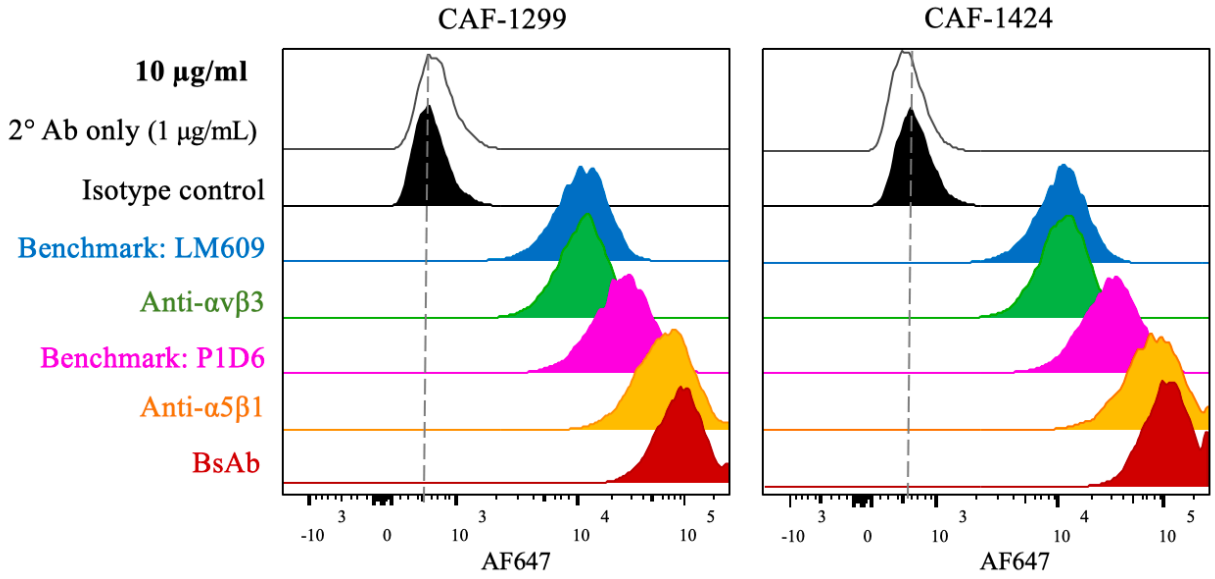


Figure 3. BsAb binding to cells compared to commercial and control antibodies. Flow cytometry plots show binding of each Ab to CAF-1299 and CAF-1424.

Consistent with the effects of genetic knockdown of either β3 or α5 subunits, treating CAFs with the mAbs recognizing αvβ3 or α5β1 can reduce the assembly of both FN and COL fibers, while the bsAb with dual recognition of integrins αvβ3/α5β1 results in the most efficient blockade of FN and COL fibril formation than either monoclonal antibody alone (**Fig. 4**).

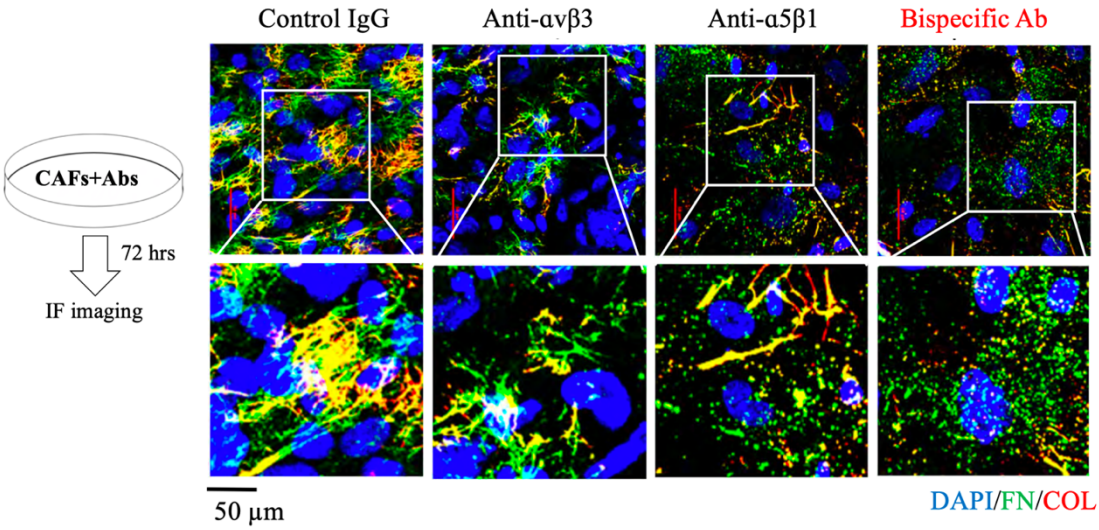


Figure 4. Antibodies targeting FN receptors prevent ECM assembly by CAF1299 cells. CAF1299 cells, in the presence of antibodies, were cultured for 72 hours in 8 well chamber slides to produce an ECM. Wells were then stained using antibodies against FN and COL. Each panel shows an image of one representative field from three independent experiments.

2.4. Tumor cells benefit from CAF-produced ECM

In order to study how PDAC cells can benefit from CAFs, we considered the ability of CAF-produced ECM to trigger stem-like reprogramming of cancer cells, as measured by the activation of Yes-associated protein (YAP), a driver of EMT, stemness, and tumor initiation in pancreatic cancer (Dupont et al. 2011; Mao et al. 2021; Moroishi, Hansen, and Guan 2015; Panciera et al. 2017). CAFs were cultured for 72 hrs to allow for matrix deposition, then were treated for an additional 72 hours with isotype control or the bsAb. CAFs were removed to leave behind "CAF-ECM" upon which PANC1 cells were plated. After an additional 24 hours, PANC1 cell lysates were collected and analyzed by immunoblotting. In this model, CAF-ECM triggers a strong upregulation of the YAP target gene CTGF in the PDAC cells, and this is prevented in the presence of the bsAb (**Fig. 5**). This result establishes that CAF-ECM triggers stem-like reprogramming of cancer cells in an in vitro setting, and demonstrates that this can be prevented by targeting the two primary FN-binding integrins, α5β1 and αvβ3 using the bsAb. It therefore becomes important to confirm these findings in an in vivo setting.

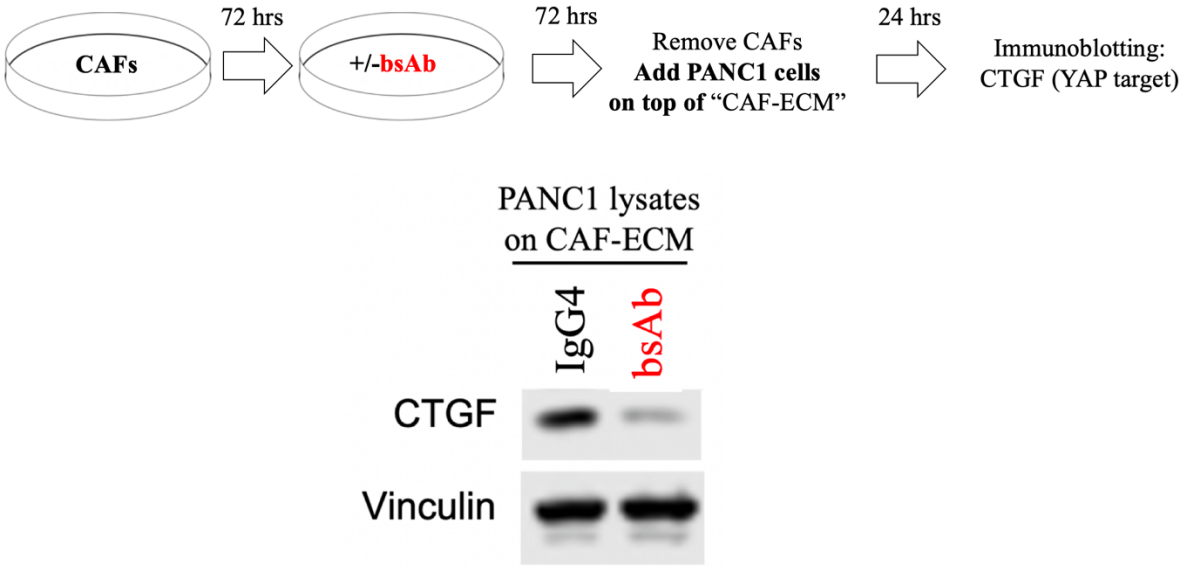
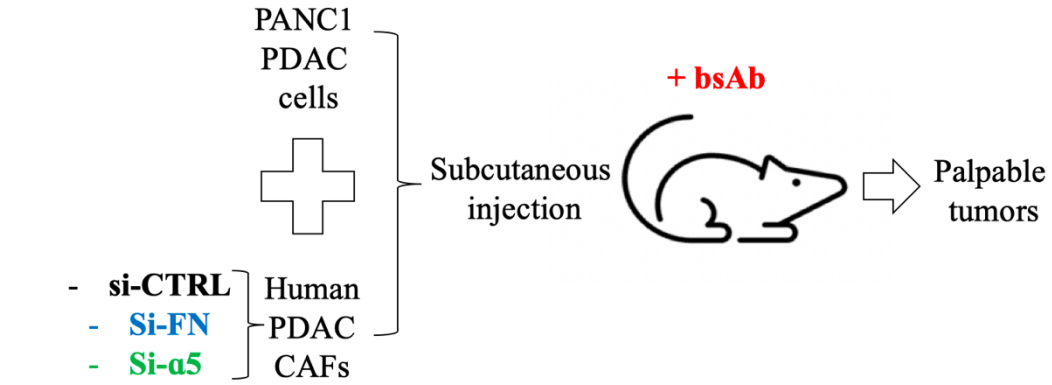


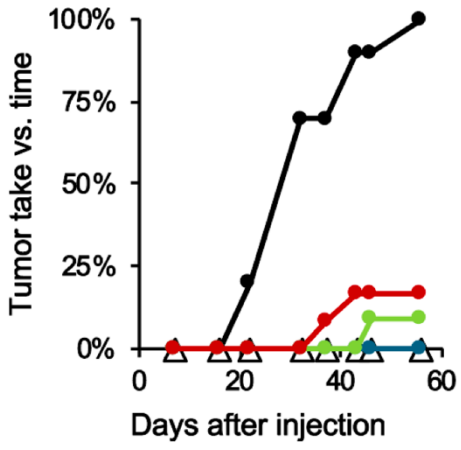
Figure 5. Bispecific antibody prevents activation of YAP, a mechanosensor that promotes stemness. CAF1299 cells were seeded in the presence of the bispecific antibody or IgG isotype control for 72 hours to allow matrix deposition. CAFs were removed to isolate the CAF-ECM upon which PANC1 PDAC cells were plated. The PANC1 cells were harvested and immunoblotting was performed, probing for CTGF, a YAP target, and Vinculin as a loading control.

2.5. Disrupting FN-binding integrins prevents the ability of CAFs to enhance tumor initiation

A subcutaneous xenograft model was used to evaluate CAFs/FN/subunit α5-dependent tumor initiation over time. In this model, no palpable tumors were detected 8 weeks after subcutaneous injection of a limiting number of PANC1 human PDAC cells alone, whereas co-injection of PANC1 cells with CAFs at a 1:1 ratio produced a 100% take rate (**Fig. 6**). Tumor initiation is blocked by FN and subunit α5 knockdown in CAFs (co-injected with the PANC1 cells). Considering that the si-RNA mediated knockdowns are transient, these results suggest that the CAF contribution to tumor initiation occurs within the first few days after co-injection when tumor cells exploit CAFs to overcome isolation stress as they create a tumor-initiating niche. During this critical phase, eliminating CAF expression of FN or integrin α5 is sufficient to completely account for their ability to boost tumor initiation.

In order to see the bsAb treatment effect on tumor initiation, tumor cells and CAFs were premixed with 10 μg/mL. Once the tumor cells were injected, the antibody was then administered systemically by intraperitoneal injection twice weekly for the experiment at a dose of 10 mg/kg. Remarkably, mice treated with the αvβ3/α5β1 bsAb developed palpable tumors at only 2 out of 12 injection sites (**Fig. 6**). Since this bsAb recognizes antigens on most species except mouse, its influence on tumor initiation in this xenograft model can be attributed to its direct binding to its antigens on the human CAFs co-injected with human PDAC cells, but not to the integrins on the surface of mouse stromal or vascular cells. Tumor cells and CAFs were premixed with 10 μg/mL bsAb immediately before injection. This result suggests that the boost in tumor initiation caused by CAFs can be targeted therapeutically.





		CAF	Tx	Tumor take Day 56
PANC1		-	-	0 / 12
PANC1 + CAF		si-CTRL	-	10 / 10
		si-FN	-	0 / 10
		si-a5	-	1 / 11
		si-CTRL	bsAb	2 / 12

Figure 6. CAFs enhance tumor initiation via FN and FN-binding integrins (subcutaneous xenograft model). 500,000 PANC1 cells were injected subcutaneously into the flank areas of immune-compromised nu/nu mice alone or at a 1:1 ratio with PDAC derived CAF1299 cells. Graph shows tumor take rate vs. time for 10-12 mice per group, using a volume of 100 mm^3(computed as length x width^2) as the threshold for tumor take. Tumor initiation was evaluated twice a week for 9 weeks. At the endpoint of the experiment (day 56), tumors were harvested and prepared for histological analysis.

2.6. BsAb treatment reduces tumor size, stroma, and stiffness

To evaluate the mechanism of action for the knockdowns and blockade strategies, tumors were dissected from the subcutaneous xenograft model at the end of the experiment (tumors with volumes over 100 mm^3 were considered) as shown in **Fig. 7**. As observed, the tumor size varies dramatically between the untreated and the bsAb treated group suggesting that the bsAb treatment reduces tumor size.

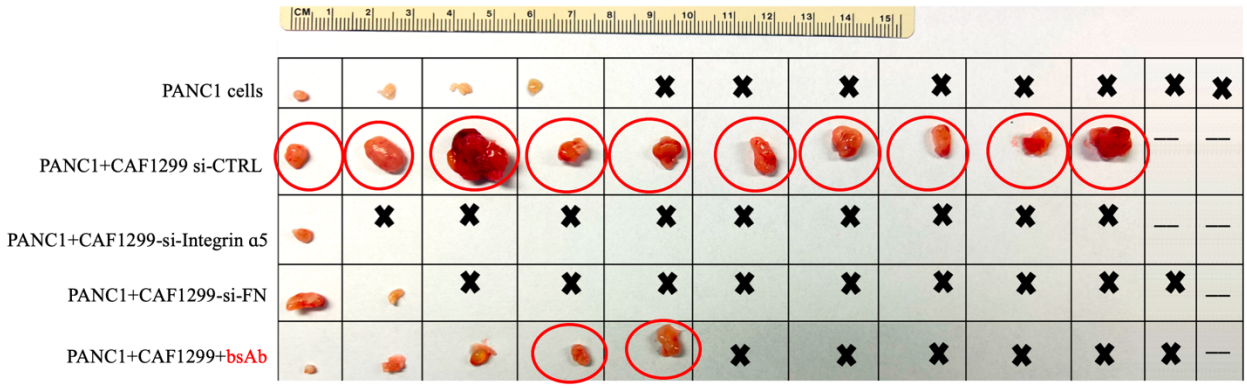


Figure 7. CAFs enhance tumor size via FN and FN-binding integrins (subcutaneous xenograft model). Tumors were dissected from the subcutaneous xenograft model at the end of the experiment and tumors with volumes over 100 mm^3 were considered for further analysis (circled in red).

Compared to untreated tumors, analysis of H&E-stained sections show less stroma and extensive necrosis in the two tumors that formed in the bsAb-treated group (**Fig. 8**).

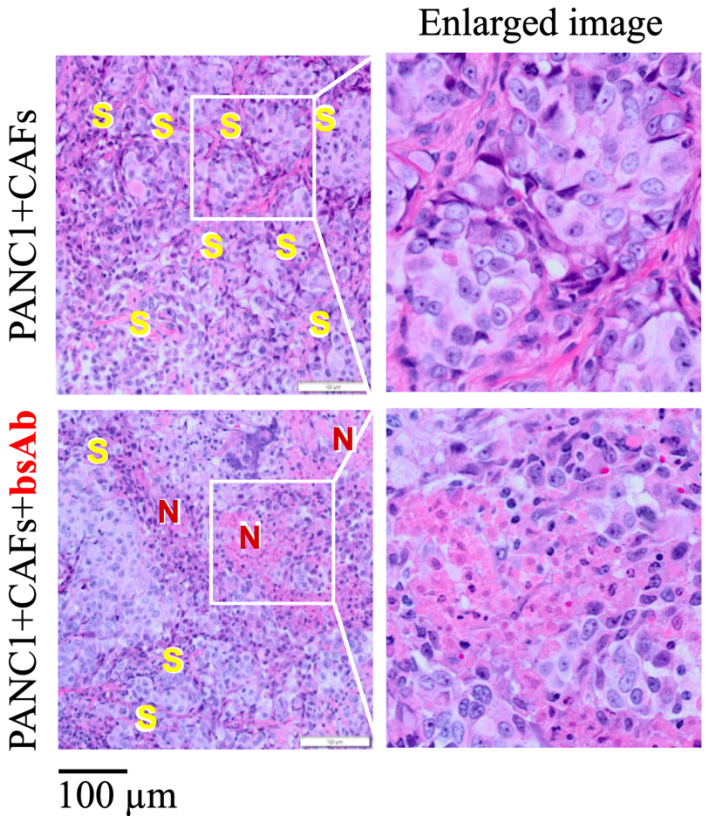


Figure 8. Bispecific antibody treatment reduces stroma (S) and increases necrosis (N). Tumor sections were stained using H&E. Areas of stroma (S) are demonstrates in yellow and necrosis (N) in red.

To detect areas with high human FN (hFN) in the tumor tissues, an antibody that recognizes hFN but not mouse FN was used. This staining reveals areas of intense fibrillar hFN staining in the untreated tumors once compared to the bsAb treated group (**Fig. 9**). This result suggests that the tumor stroma is produced by the human CAFs that were co-injected with the tumor cells and is not relative to the host's fibroblasts (mouse, in this case). Treated tumors also show significant fibrosis (COL polymerization), as evaluated by picrosirius red staining. In comparison, tumors from mice treated with the bsAb show a complete absence of hFN, while collagen fibers appear both fewer and smaller. Interactions between the co-injected PANC1 cells and CAFs mediate the earliest steps of tumor initiation before any mouse stromal cells are recruited into the tumor microenvironment because the bsAb utilized in this study does not recognize mouse antigens, it can identify only the injected human PANC1 cells and human CAFs in this xenograft model. These results suggest that the bsAb treatment prevents FN and COL assembly in the tumor stroma and this is proven in an in vivo setting.

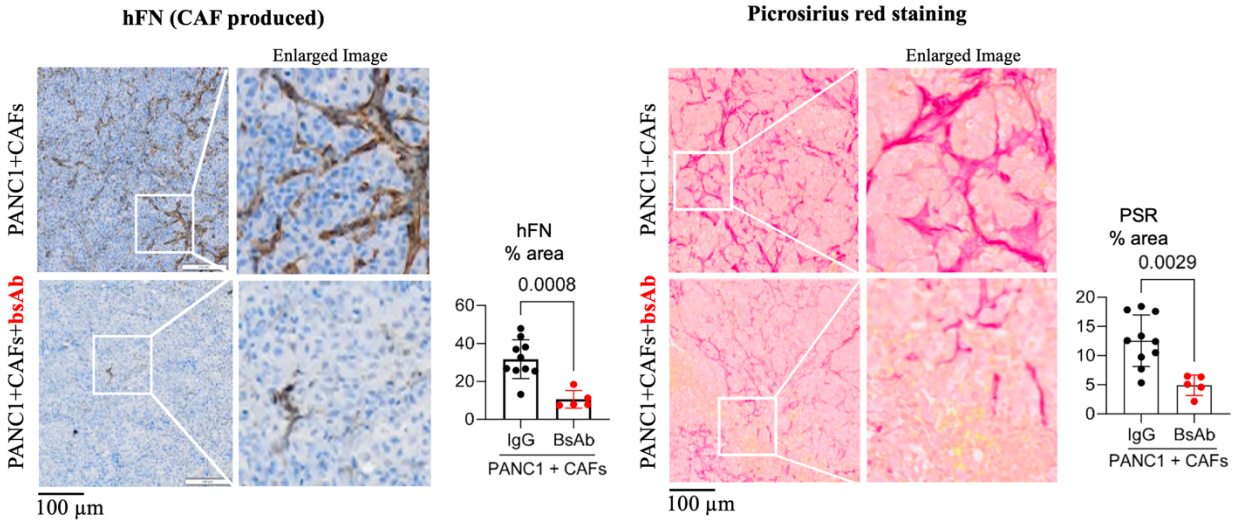


Figure 9. Bispecific antibody treatment reduces fibrotic effect of human CAFs co-injected with tumor cells. FFPE sections of tumors were processed for immunohistochemical detection of human-spefici FN (hFN) and picrosirius red histological stain was used to visualize collagen. The graphs depict the quantification of IHC staining using QuPath, with each dot representing the mean value for each tumor slice examined. P-value was computed using Student's t-test.

Previous studies have documented that PDAC tumors are highly stiff primarily due to the extensive stroma and ECM deposited within the tumor, and that stiffness correlates with PDAC progression in mice and man(Payen et al. 2020). Some studies have also linked COL deposition to stromal stiffness(Stashko et al. 2023). Consistent with this, mice treated with the bsAb show

significantly lower tissue stiffness throughout the entire tumor as measured by atomic force microscopy (**Fig. 10**).

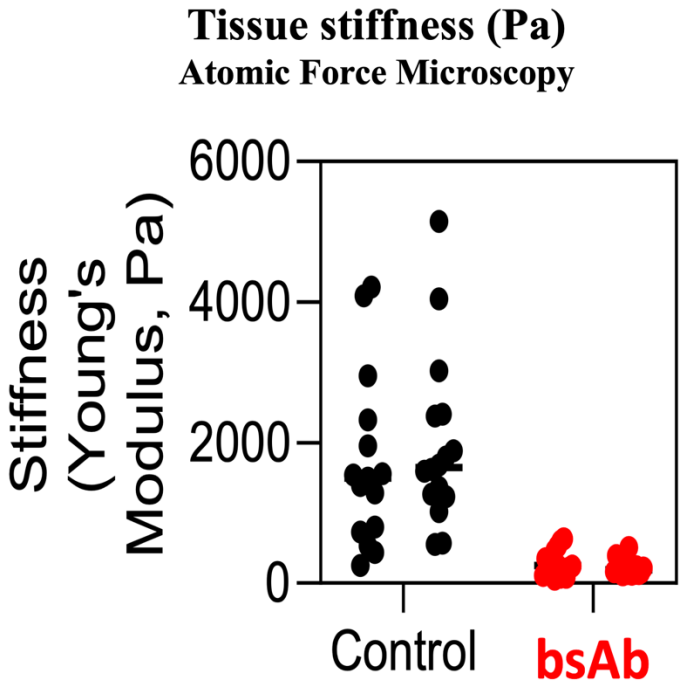


Figure 10: Bispecific antibody treatment reduces tumor stiffness. Cryopreserved tumors from the subcutaneous xenograft model were sectioned into 20 µm thick slices. Their stiffness was evaluated using an MFP-3D atomic force microscope and indentation tests were carried out at 2 µm per second loading rate to generate 15 force curves across equally distributed regions of 20 × 20 μm^2 size (mean value represented by each dot). The Young's Moduli of the slices were determined by fitting force curves with the hertz model using a Poisson ratio of 0.5.

CHAPTER 3 – DISCUSSION

PDAC is considered to be a highly aggressive tumor, largely because of its dense and reactive stroma. CAFs are the main contributors of this fibrotic stroma through their main role which is to deposit and assemble a dense FN and COL matrix via integrins on their cell surface (αvβ3 and α5β1). Various studies have reported CAFs role in shaping the PDAC stroma (Vaish et al. 2021; Brichkina et al. 2023), however the idea of targeting CAFs functions has not been presented as a possible cancer therapeutic. Besides their pro-fibrotic role in the TME, CAFs are also known to be tumor-promoting via the secretion of inflammatory cytokines and growth factors (Bhattacharjee et al. 2021), which confer proliferation, metastasis and chemo resistance of cancer

cells (Zhang et al. 2022). They are also reported to serve as “bio-incubator" by providing favorable "soil" for subsequent growth tumor cells in the circulation during EMT (Mirza et al. 2023).

This study focuses on how CAFs nurture individual tumor cells during tumor initiation. We consider how interaction between CAFs and tumor cells is required for tumor cells to survive the challenges encountered during what we refer to as “isolation stress”(Wu, Weis, and Cheresh 2024). This not only relates to the ability of limited numbers of cells to establish tumor colonies within the pancreas, but can also extend to the situations faced by circulating tumor cells or disseminated tumor cells that need help from extrinsic factors in order to survive in the circulation and to initiate tumor formation at distant sites where they are surrounded by normal tissue and subject to immune surveillance.

There are existing studies that explore how CAFs lead to tumor invasion through integrin-β3 and α5 dependent FN assembly (Attieh et al. 2017b). This work reveals that tumor initiation is promoted by CAFs due to its capability to produce and assemble FN as well (**Fig. 1**). We show that dual blockade of αvβ3/α5β1 using a bsAb can largely prevent CAFs from producing fibrillar FN and collagen (**Fig. 4**), and this prevents stem-like reprogramming of tumor cells and tumor initiation in mice (**Figs. 5 and 6**). Since the bsAb selectively targets human (but not mouse) cells, it could be used in future studies to perturb integrin function on CAFs and prevent tumor cells to take advantage of them in order to overcome “isolation stress”.

Professor Val Weaver’s research proves that there is intratumor heterogeneity in breast cancers, stromal stiffness accompanies cancer progression and they correlate collagen density with stromal stiffness using a spatially transformed inferential force map (STIFMap) (Stashko et al. 2023). We show that disrupting the ability of CAFs to organize FN fibers results in the loss of COL fibril formation, provides an opportunity to interfere with intercellular communicatin, and prevents tumor cell survival long enough to “initiate” a new tumor colony (**Fig. 6 and 7**). By th9e way of the dual blockade of integrins αvβ3/α5β, the tumor size is significantly decreased (**fig. 7**), there is more necrosis and less stroma in the tumor microenvironment (**fig. 8**). We also see fewer collagen deposition in the tumors formed in treated mice (**fig. 9**).

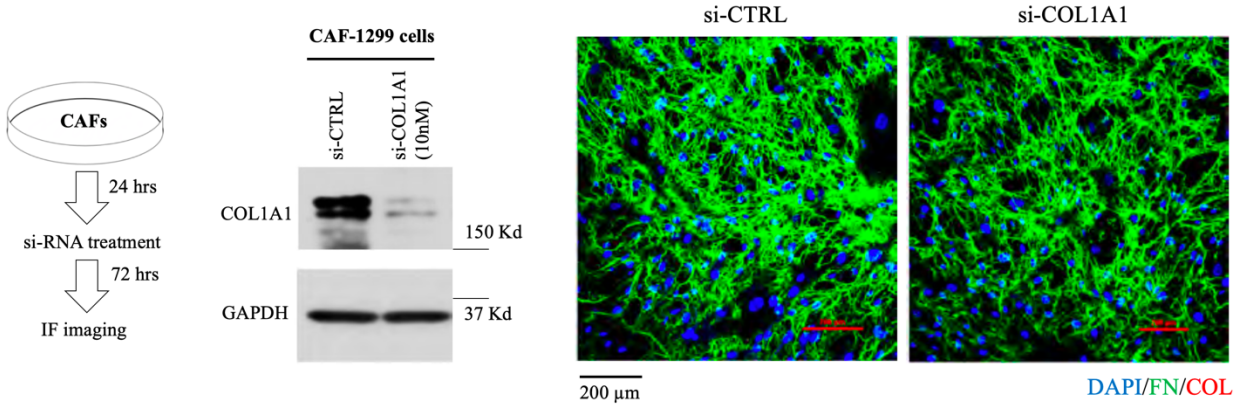
COL fibers are not always crosslinked and are sometimes lying on top of each other without interacting with each other. The STIFMap technology by Prof. Val Weaver's research lab evaluates stiffness based on qualitative data regarding COL distribution in the TME. I believe, the use of a physical method is more efficient when it comes to evaluating stiffness based on COL assembly and crosslinking. Therefore, we implemented Atomic Force Microscopy (AFM) to do so. As seen in **fig. 10**, PDAC tumors possess intratumor heterogeneity same as breast cancer tissues (previously reported by Prof. Val Weaver's group), and this stiffness is significantly reduced by blocking integrins αvβ3 and α5β.

Focusing on stiffness while studying cancer mechanistic is very important. There are studies that show how increasing matrix stiffness upregulates vascular endothelial growth factor (VEGF) expression in cancer cells (LaValley et al. 2017; Ishihara and Haga 2022; Dong et al. 2014). VEGF is a key molecule for angiogenesis meaning that it triggers the development of new blood vessels that supply nutrients and oxygen to cancer cells. This is exactly what we believe helps individual tumor cells to overcome "isolation stress" in order to form tumors.

Studies show that matrix stiffness prevents the penetration of therapeutic agents into the TME (Netti et al. 2000). There is a strong correlation between mechanical stiffness and resistance to movement of macromolecules of tumor tissues. Therefore, if a tool such as the bispecific antibody (targeting integrins αvβ3 and α5β) can reduce stiffness within the TME by reducing FN and COL deposition, other therapeutics can better penetrate the TME and have success for patients.

This work is currently limited to pancreatic cancer, a disease for which few targeted therapeutics have produced clinical benefit. Considering that other studies have linked stromal stiffness to EMT characteristics within tumor cells for other cancer types, such as breast cancer (Stashko et al. 2023), there is potential for this work to extend to other cancer types. Other pathological conditions that involve fibrosis might also respond well to this approach because this work shows that targeting integrins αvβ3/α5β1 with the bsAb can suppress the generation of fibrotic ECM by primary fibroblasts isolated from patients with chronic lung or liver fibrosis.

CHAPTER 4 – SUPPLEMENTAL DATA



Supplemental figure 1. COL knockdown does not alter FN assembly. CAF-1299 were treated with non-targeting si-RNA for control vs. si-COL1A1. After 72 hours, immunofluorescence staining shows FN content. Images are representative of at least 3 independent experiments. Blot confirms COL1A1 knockdown.

CHAPTER 5 – MATERIALS AND METHODS

5.1. Reagents, chemicals, and antibodies

The detailed information for each antibody and siRNA is presented in **Table 1**. Each siRNA combo had been mixed with two distinct siRNAs (siRNA1 and siRNA2, 1:1 mixture) targeting different gene regions of the gene of interest.

Table 2. List of Antibodies used in this study.

Reagent or resource	Source	Identifier
Primary Antibodies		
Fibronectin (Immunoblotting)	Cell signaling	E5H6X
Fibronectin, DH1 (Immunofluorescence staining)	Novus Biologicals	NBP1-51723
COL1A1	Cell Signaling	E8F4L
CTGF	Cell Signaling	D8Z8U
Vinculin	Santa Cruz Biotechnology	H-10
GAPDH	Cell Signaling	D16H11
Anti-αvβ3	Cheresh lab	LM609
Anti-α5	Cheresh lab	P1D6
Human IgG4, Kappa	Biolegend	403702
Secondary antibodies		
Goat anti-rabbit HRP	BioRad	170-6515
Goat anti-mouse HRP	BioRad	170-6516
Alexa Fluor 488 goat anti-mouse IgG H+L	Invitrogen	2465113
Alexa Fluor 568 goat anti-rabbit IgG H+L	Invitrogen	2500544

Table 3. List of siRNAs used in this study.

Predesigned siRNAs	Source	Identifier
Negative control	Millipore Sigma	Universal Negative Control #1
FN1-siRNA1	Millipore Sigma	SASI_Hs01_00203291
FN1-siRNA2	Millipore Sigma	SASI_Hs01_00203292
ITGA5-siRNA1	Millipore Sigma	SASI_Hs01_00058581
ITGA5-siRNA2	Millipore Sigma	SASI_Hs02_00333426
ITGB3-siRNA1	Millipore Sigma	SASI_Hs01_00174219
ITGB3-siRNA2	Millipore Sigma	SASI_Hs01_00174221
COL1A1-siRNA1	Millipore Sigma	SASI_Hs02_00301842
COL1A1-siRNA2	Millipore Sigma	SASI_Hs01_00182242

5.2. Cell culture

Pancreatic cancer cell line PANC1 was obtained from American Type Culture Collection (ATCC). KP4 cells were obtained from the Riken BioResource. Immortalized CAF cell lines hPCF1299 and hPCF1424 (CAF1299 and CAF1424), previously derived from fresh surgical specimens of human PDAC tissue, was obtained from Dr. Andrew Lowy's laboratory (University of California, San Diego). All the cell types were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% Fetal Bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Thermo Fisher Scientific). Cells were occasionally tested for mycoplasma using a MycoScope PCR Mycoplasma Detection Kit (Genlantis, MY01050) and no more than 30 passages were used.

5.3. Preparation of CAF1299 cell-derived ECM

Cells were seeded in eight-well chamber slides, glass coverslips, or tissue-treated cell culture plates for up to 7 days to allow cells to produce and deposit sufficient ECM. The cells were treated with antibodies (10 $\mu g/mL$) and after 72 hours, wells were washed twice with Hanks' Balanced Salt Solution (HBSS), then incubated for 15-20 minutes at 37°C in lysis buffer (8mM Na_2HPO_4, 1% NP-40, pH 9.6). The samples were rinsed three times with wash buffer (10mM Na_2HPO_4, 300mM KCl, pH 7.5) and once with sterile deionized water for 5 minutes. Following this, the cell-free ECM was either stored in HBSS for a 3-4 of days or used for immunofluorescence (IF) staining or other cell-based assays.

5.4. Immunofluorescence (IF) staining

Cells were seeded on an 8-well chamber slide in DMEM with 10% FBS, and 1% penicillin-streptomycin. The cells were treated with siRNA targeting the genes of interest (5pmol) or antibodies (10 $\mu g/mL$) right after seeding, or after being allowed to deposit ECM for 72 hours. The wells were fixed with 4% formaldehyde for 15 minutes at room temperature, washed 3 times with HBSS, and blocked for 60 minutes at room temperature with HBSS and 5% Normal Goat Serum (NGS, Jackson Immunoresearch; #005-00-121). After blocking, the wells were incubated overnight with FN antibody (1:200) and COL antibody (1:200) in HBSS and 5% NGS at 4ºC. The wells were washed 3 times with HBSS, and incubated in secondary antibodies: Alexa Fluor 488 goat anti-mouse IgG H+L (1:200) and Alexa Fluor 568 goat anti-rabbit IgG H+L (1:200) in HBSS for 45 minutes at room temperature in dark. The wells were washed 3 times with HBSS and then incubated for 5 minutes at room temperature in the dark with the nuclear stain DAPI (1:200) in HBSS. The wells were rinsed once with HBSS and mounted using Fluorescense Mounting Medium and covered with glass coverslip to be stored in 4ºC. IF images were acquired using the Nikon Eclipse C1 confocal microscope with multiple Z-stack images and analyzed using the NIS-Elements Viewer 5.21 software. Fluorescent signal was quantified as %area fraction using ImageJ.

5.5. Immunoblotting

PANC1 cells were seeded atop CAF1299 cell-derived ECM using DMEM with 2% FBS, and 1% penicillin-streptomycin. Cell lysates for western blots were obtained 24 hours after cell seeding by adding 2X Laemmli Sample buffer (BioRad, #1610737) containing 1X NuPAGE Sample Reducing Agent (Sigma, NP0009) directly to the cells on the tissue culture plate. The plate, with the lysis buffer, was placed on a shaker for 10 minutes. The lysates were collected in 1.5 mL tubes and heated at 95ºC for 5 minutes followed by centrifuging the lysates at high speed (12,500 RPM for 2 minutes). The lysates were stored at -80ºC. 30 μL was loaded onto an SDS-PAGE gel, followed by transfer onto polyvinylidene difluoride membranes. Blocking and probing was performed in 5% BSA in TBST. The following primary antibodies were used: Vinculin (1:5000), used as a loading control, CTGF (1:1000), CYR61 (1:2000). The secondary antibodies used were goat anti-rabbit HRP (1:4000) and goat anti-mouse HRP (1:4000). ECL reagent

(advantsa, #R-03031-D10) and Peroxide (advantsa, #R-03025-D10) were used to develop the blots.

5.6. Mouse studies

All experiments involving mice were conducted under protocol S05018, approved by the UC San Diego Institutional Animal Care and Use Committee. All experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

1. Subcutaneous xenograft model: 5×10^5 PANC1 cells were mixed with or without the equal number of CAF-1299 cells transfected with different siRNAs for 72 hours (knockdown validated by immunoblotting). For the antibody treatment groups, PANC1 cells or CAF-1299 were pre-mixed with 10μg/mL antibody for 10 minutes before injection. Cells were suspended in a 1:1 mixture of HBSS and Phenol Red-free Basement membrane matrix (BD Biosciences) (total volume is 100 μL per injection) and injected subcutaneously in 6-to-8-week-old female immune-compromised nu/nu mice (Charles River Labs). Fresh antibody (10 mg/kg) was injected intraperitoneally twice a week, and mice were examined twice weekly for palpable tumors. A tumor larger than 100 mm^3 in volume was counted.
2. Orthotopic pancreatic cancer model: $1x10^6$ KP4 cells stably transfected with luciferase lentivirus (KP4-Luc) were mixed with or without the equal number of CAF-1299 cells transfected with different siRNAs for 72 hours (knockdown validated by immunoblotting). Cells were suspended in HBSS and injected into the pancreas of 6-to-8-week-old female immune-compromised nu/nu mice (Charles River Labs). The tumor growth was monitored twice a week by using non-invasive bioluminescence imaging using an IVIS Spectrum system (Perkin-Elmer). All mice were imaged 10 minutes after being injected with D-luciferin (L9504, Sigma-Aldrich).

5.7. Immunohistochemistry

Immunohistochemical staining was performed on formalin-fixed paraffin-embedded slides using an ImmPRESS Excel Staining Kit (Vector, MP-7602) following the manufacturer's instructions. For integrin β3, low-pH antigen retrieval was performed for 20 minutes at 95ºC. The

slides were imaged using an Olympus VS200 Slide Scanner (Olympus). Scanned images were analyzed for protein expression as area fraction per area of tumor tissue calculated using QuPath Open Software for Bioimage Analysis.

5.8. Atomic Force Microscopy (AFM)

AFM measurements were performed using an MFP-3D atomic force microscope (Asylum Research, Prof. Engler's Lab, UCSD). Silicon nitride cantilevers were used with a normal spring constant of 0.08 Nm^{-1} and a 200 μm length (Nano World, PNP-TR-50). Cantilevers were calibrated using the thermal fluctuation method and verified by probing glass of known elasticity. The specimens used were 20 μm thick, OCT-embedded frozen human PDAC tissue sections, thawed and equilibrated to room temperature by immersion in HBSS for 5 minutes. Indentation tests for the specimens were carried out at 2 μm per second loading rate to generate 16 force curves across equally distributed regions of $20 \times 20\ \mu m^2$ size. The Young's Moduli of the samples were determined by fitting force curves with the hertz model using a Poisson ratio of 0.5.

CHAPTER 6 – FINAL ACKNOWLEDGEMENTS

Chapters 2 and 4, have been submitted for publication: co-authors: Chengsheng Wu, and Tami Von Schalscha. The dissertation/thesis author was the primary investigator and author of this material.

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