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Targeting pancreatic cancer cell stemness by blocking fibronectin-binding integrins on cancer-associated fibroblasts

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Running title:

Blocking fibronectin-binding integrins on CAF in PDAC

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Conflict of Interest Disclosure Statement:

SMW and DAC are consultants and Scientific Founders of Alpha Beta Therapeutics. SJM is CEO of Alpha Beta Therapeutics. HIW received research funding for a lab service agreement from Alpha Beta Therapeutics. A patent was filed by SJM, SMW, and DAC describing the development and use of a bispecific antibody to block cancer-associated fibrosis.

Abstract

Cancer-associated fibroblasts (CAF) generate an extracellular matrix (ECM) which provides a repository for factors that promote pancreatic cancer progression. Here, we establish that CAF contribution to pancreatic tumor initiation, i.e. stemness, depends on fibronectin (FN) as a scaffold required for assembly of a collagen-containing fibrotic ECM with a critical dependence on the FN-binding integrins, α5β1 and αvβ3. CAF matrix assembly can be prevented by knockdown of *FN*, *ITGA5*, or *ITGB3*, or by a bispecific antibody with dual recognition of α5β1 and αvβ3 that can also destabilize a pre-existing matrix. In mice, the ability of CAFs to produce a stiff collagenous matrix and accelerate tumor initiation can be blocked by knockdown of *FN* or FN-binding integrins, or systemic treatment with the α5β1/αvβ3 bispecific antibody. Together, these results reveal that dual targeting of the FN-binding integrins α5β1/αvβ3 can block the ability of CAFs and their matrix from enhancing pancreatic cancer stemness and progression.

Significance

Simultaneous targeting of two integrins that function as receptors for fibronectin, a pro-tumor extracellular matrix protein, can prevent fibroblasts from supporting the malignant behavior of pancreatic cancer cells.

Introduction

Pancreatic adenocarcinoma (PDAC) has a notoriously dense stroma comprising approximately 80% of the tumor mass that is linked to tumor progression, immune suppression, drug resistance, and metastasis[1-4]. This stroma is primarily organized by pancreatic stellate cells that become dysregulated during early tumor development to become cancer-associated fibroblasts (CAFs)[5]. Intercellular communication between tumor cells and CAFs is a critical enabler of tumorigenesis and progression. In principle, strategies to impair the function of CAFs for cancer therapy include normalizing their function, depletion, or attempting to alter the function of various CAF-produced matrix proteins[6]. Yet, effectively targeting CAFs in vivo has proven to be complicated due to the heterogeneity of CAF phenotypes, populations, and functions[7, 8].

Fibronectin (FN) is a particularly important and impactful component of the stroma that contributes to the lethality of pancreatic cancer[9]. Since FN produced by CAFs can assemble on the cell surface to provide a scaffold for the assembly of additional matrix proteins, disrupting FN organization in vitro can prevent the formation of a highly fibrotic extracellular matrix[10, 11]. At the cellular level, integrins clustered on the surface of fibroblasts are receptors that selectively recognize and bind to certain matrix protein ligands, producing a physical link that allows intracellular contractile machinery to impose forces on the matrix proteins[12]. Once activated in tumors or fibrotic tissues, fibroblasts gain the expression of two particular FN-binding integrins, α5β1 and αvβ3, that are absent from most normal cell types in the body[12-14]. Previous work has established that selective and specific engagement of monomeric FN molecules by integrins αvβ3 and α5β1 is able to transmit mechanical forces that expose cryptic domains required for fibronectin polymerization and the formation of "biologically active fibrils"[15-17]. In turn, these fibrils act as a scaffold for the generation of an elaborate matrix that contains a variety of extracellular matrix proteins, proteoglycans, and soluble factors that tumor cells can exploit.

During tumor initiation at the primary or metastatic sites, individual tumor cells are forced to overcome a variety of challenging cellular stresses, referred to as isolation stress[18, 19], as they undergo tumorigenesis and attempt to form new proliferative colonies in non-permissive, inhospitable locales. CAFs provide such tumor cells with nurturing ECM footholds, access to

activating secreted factors, and opportunities for intercellular communication. CAFs not only influence tumor cells within the primary tumor but can gain access to the circulatory system to promote the survival and subsequent growth of circulating tumor cells and facilitate the establishment of new tumor cell outposts by providing a haven for individual tumor cells to seed and survive at metastatic sites[20-22].

Despite recent efforts to characterize the various dysregulated stromal cell types and subtypes, strategies to disempower CAFs have yet to realize success as cancer therapeutics. Our goal was to understand how a pancreatic tumor cell exploits CAFs to facilitate tumor initiation and tumorigenesis to design new strategies to interrupt this cell biological process. Our studies reveal how preventing CAFs from assembling FN fibers can perturb collagen fibril formation and thereby prevent tumor cells from surviving long enough to "initiate" a new tumor colony. In preclinical PDAC models, genetically or pharmacologically disrupting FN or FN-binding integrins can prevent CAFs from supporting pancreatic cancer cells at different steps of pancreatic cancer progression. Such strategies may have broad application to disempower the activated fibroblasts that exacerbate the progression of other types of cancer and fibrotic disease.

Materials and Methods

Reagents, chemicals, and commercial antibodies: Primary antibodies used in this study include FN (E5H6X) (Cell signaling #26836, RRID: AB_2924220, 1:1000 for western blots, 1:200 for immunohistochemistry), FN (DH1) (Novus Biologicals NBP1-51723, RRID: AB_11059914, 1:200 for immunofluorescence staining), COL1A1 (E8F4L) (Cell Signaling #72026, RRID: AB_2904565, 1:1000 for western blots; 1:200 for immunofluorescence staining), CTGF (D8Z8U) (Cell Signaling #86641, RRID: AB_2800085, 1:1000 for western blots), vinculin (Boster #MA1103, RRID: AB_3082541, 1:15,000 for western blots), GAPDH (D16H11) (Cell Signaling #5174, RRID: AB_10622025, 1:3000 for western blots), anti-α5 integrin antibody (P1D6) (EMB Millipore MAB1956Z, RRID: AB_94455, 10ug/ml for flow cytometry), and CD31 (R&D Systems #AF3628, RRID:AB_2161028, 1:40 for immunohistochemistry). Anti-αvβ3 integrin antibody (LM609) (10μg/ml for flow cytometry) was produced in the Cheresh lab and is also commercially available (Millipore MAB1976, RRID: AB_2296419). Anti-α5 was purchased from Millipore (MAB1956). Predesigned siRNAs used in this study were purchased from Millipore Sigma. Each siRNA combo is mixed with two distinct siRNAs (siRNA1 and siRNA2, 1:1 mixture) targeting different gene regions of the gene of interest. The siRNA IDs are listed in **Supplementary Table S1**.

Novel antibodies: Novel integrin antibodies provided by Alpha Beta Therapeutics and benchmark controls are summarized in **Supplementary Table S2**. ABT-101 and ABT-701 are hIgG4-S228P monoclonal antibodies that recognize human integrins αvβ3 and α5β1, respectively. ABT-601 (referred to as "BsAb" in this work) is a bispecific antibody designed for dual recognition of FN-binding integrins αvβ3 and α5β1. ABT-601 is comprised of the antigen-recognizing Fab domains of ABT-101 and ABT-701 with the same hIgG4-S228P Fc domain.

Cells: PANC1 human pancreatic cancer cells were obtained from the American Type Culture Collection (ATCC CRL-1469, RRID: CVCL_0480and cultured using DMEM. Cell line authentication by STR analysis was performed for the PANC1 cells in April 2015 and November 2024. KP4 human pancreatic cancer cells were obtained, along with STR cell line authentication in January 2023, from the Riken BioResource Research Center (BRC) Cell Bank (RRID: CVCL_1338) and cultured using RPMI. Dr. Andrew Lowy (UC San Diego) provided low-passage stock vials of immortalized CAF cell lines hPCF1299 (CAF-1299) and hPCF1424

(CAF-1424) in June 2022 that were previously derived[23] from fresh surgical specimens of human PDAC tissue. All cells were expanded upon receipt, tested for mycoplasma using PCR to detect the 16S rRNA gene from the M. mycoides cluster (Forward 5'-CGA AAG CGG CTT ACT GGC TTG TT-3'; Reverse 5'-TTG AGA TTA GCT CCC CTTCAC AG-3'), and cryopreserved as low-passage stocks[24]. For each experiment, stock vials were thawed, cultured for no more than 30 passages, and tested biweekly for mycoplasma.

Cell-free ECM generation and cell-based assays: 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. Decellularization was performed as previously reported[25]. Samples were washed twice with Hanks' Balanced Salt Solution (HBSS), incubated for 15-20 minutes at 37°C in lysis buffer (8mM Na_2HPO_4, 1% NP-40, pH 9.6), rinsed three times with wash buffer (10mM Na_2HPO_4, 300mM KCl, pH 7.5) and three times with sterile deionized water, then stored in HBSS. Cell-free ECM was then used for immunofluorescence staining or other cell-based assays. For cell-based assays, pancreatic cancer cells were seeded atop the cell-free ECM in the presence of 10% or 2% FBS-containing media and grown for 24 hours before cell harvest for western blots analysis.

Quantitative RT-PCR: RNA was isolated using the RNeasy RNA Purification Kit (Qiagen, 75144) following the manufacturer's instructions. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 4368814), and RT-PCR was performed using CFX96 (Bio-Rad) with SYBR Green (Bio-Rad, 1725272). The custom primer sequences are listed in **Supplementary Table S3**.

Mouse study approval: 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. All animals were housed under standard conditions, i.e. given unrestricted access to food and water, housed in standard cages, and rooms regulated to control temperature and light cycles. Cell lines used for in vivo experiments confirmed negative for a panel of human pathogens.

Subcutaneous xenograft model: The number of PANC1 cells injected varies greatly among studies[26-28], and this is likely influenced by the use of various strains of immune-compromised mice. Since nu/nu mice have a relatively less compromised immune system than NOD-SCID or NSG strains, we selected an injection amount of 0.5 million cells for the experiment in Fig. 5A. $5x10^5$ human PANC1 cells were mixed with or without an equal number of human CAF-1299 cells transfected with different siRNAs for 72 hours. Knockdown was verified via western blot. 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 #088, RRID: IMSR_CRL:088). 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 is counted.

Orthotopic pancreatic cancer model: To determine an injection number that would allow us to assess changes in tumor initiation at a relatively early timepoint, we performed a pilot study to compare orthotopic injections of 5, 1, or 0.2 million KP4-luc cells. While no tumors were detected by bioluminescence imaging after 5 weeks, imaging at 10 weeks revealed tumors in 3/3 mice that received 5 or 1 million KP4-Luc cells but only 1/3 mice for the 0.2 million cell group (**Supplementary Fig. S1A**). We reasoned that orthotopic implantation of 1 million KP4-Luc cells should produce tumors in all mice that are detectable after 5-10 weeks, and furthermore

that co-injection with CAFs should accelerate tumor initiation (i.e., detection at timepoints less than 5 weeks). For the experiment shown in Figure 1A, $1x10^6$ human KP4 cells stably transfected with luciferase lentivirus (KP4-Luc) were mixed with or without the equal number of human CAF-1299 cells transfected with different siRNAs for 72 hours. Knockdown was verified via western blot. 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 #088, RRID: IMSR_CRL:088). 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). Immunoblotting to assess the duration of siRNA-mediated knockdown of FN1 for CAFs (**Supplementary Fig. S1B**) shows that FN knockdown remained effective after 6 days in culture, but significantly declined by Day 14.

Immunofluorescence staining and confocal microscopy: CAFs were seeded on an eight-well chamber slide (Nuc Lab-Tek chamber slide, Thermo Scientific) overnight. The next day, cells were treated with siRNA targeting the genes of interest or antibodies (10 µg/mL) for 72 hours and then fixed with 4% paraformaldehyde for 15 minutes at room temperature. To target pre-existing matrix, CAFs were seeded and allowed to deposit matrix for 72 hours. At 72 hours, antibody was added (10 µg/ml) and allowed to incubate for an additional 72 hours. The cells and matrix were then fixed with 4% paraformaldehyde for 15 minutes at room temperature. All instances were followed by 45 minutes of incubation with a blocking buffer containing HBSS or HBSS-Tween 20 (0.2%) supplemented with 5% horse serum. Cells were then incubated with primary antibodies at a dilution of 1:200 (anti-FN or COL1A1) overnight at 4°C, followed by secondary antibodies at a dilution of 1:1000 for 1 hour and DAPI staining for 5 minutes at room temperature. Images were acquired by Nikon Eclipse Ti C2 confocal microscope with multiple Z-stack images and analyzed with NIS-Elements Viewer 5.21. The fluorescent signal was quantified as % area fraction using ImageJ.

Immunoblotting: Immunoblotting was performed as previously described[29]. Briefly, cells were washed twice with HBSS before lysing with either 1X RIPA buffer containing protease and phosphatase inhibitors, 2X sample buffer containing 1X reducing agent, or for conditioned supernatant, 4X sample buffer containing 1X reducing agent. (BioRad #1610737 and #1610747) A BCA assay (Thermo, 23227) was performed, and the lysates were normalized. Sample buffer (NuPAGE LDS Sample Buffer 4X, Sigma #NP0007) and reducing agent (NuPAGE™ Sample Reducing Agent, Sigma #NP0009) were added to the cell lysates. All samples were heated at 95°C for 5 min. 10 µg of protein or 30 µl of each sample containing Laemmli buffer was loaded onto an SDS-PAGE gel. Blocking was performed in 5% BSA in TBS-T, and probing was performed in 5% BSA in TBS-T buffer.

Flow cytometry: Cell pellets were washed with PBS, blocked with 1% BSA in PBS for 30 min at room temperature, and stained with or without indicated primary antibodies with fluorescently labeled secondary antibodies. Cells were incubated with live/dead fixable blue dead cell stain kit (Invitrogen, L23105). Flow cytometry was performed on a BD Fortessa X-20 (BD) analyzer, and the data were analyzed using FlowJo (Treestar) software.

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 human FN, low-pH antigen retrieval (Invitrogen, 00-4955-58) was performed for 40 minutes at 95ºC. For CD31, high-pH antigen retrieval (Invitrogen, 00-4956-58) was performed for 40 minutes at 95ºC. Trichrome staining was performed using Abcam AB150686. Picrosirius Red staining was performed using Abcam AB246832. The slides were imaged using an Olympus VS200 Slide Scanner (Olympus) at The UCSD School of Medicine Microscopy Facility (funded by core grant NINDS P30NS047101).

Scanned images were analyzed for protein expression as area fraction per area of tumor tissue calculated using QuPath Open Software for Bioimage Analysis[30].

Atomic force microscopy (AFM): AFM measurements were performed using an MFP-3D atomic force microscope (Asylum Research). 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 20µm-thick OCT-embedded frozen human PDAC tissue sections that were thawed and equilibrated to room temperature by immersion in HBSS for 5 minutes. Indentation tests for the specimens were carried out at 2µm per second loading rate to generate 16 force curves across equally distributed regions of 20µm x 20µm. The Young's Moduli of the samples were determined by fitting force curves with the Hertz model using a Poisson ratio of 0.5.

Data availability statement: The data generated in this study are available upon request from the corresponding author.

Results

Tumor cells benefit from CAF-produced fibronectin to overcome isolation stress

FN plays a critical role in ECM assembly and remodeling during a diverse range of physiological and pathological responses.   To gauge the importance of CAF-produced FN during tumor initiation, KP4 human PDAC cells were injected orthotopically into the pancreas in immune-compromised nu/nu mice alone or at a 1:1 ratio with PDAC-derived CAFs to evaluate tumor initiation in the pancreas (**Fig. 1A**). After 3 weeks, bioluminescence imaging revealed bona fide tumors (luciferase signal >1e+06) in 5/5 mice, compared to 0/5 mice injected with KP4 cells alone, validating the CAF-dependence of tumor initiation for this model. In contrast, CAFs with siRNA-mediated knockdown of FN could enable tumor initiation, a hallmark of tumor stemness, in only 1 out of 5 mice, suggesting that CAF-produced FN could largely account for the contribution of CAFs during this process. Since the siRNA-mediated knockdown efficiency begins to decline 6 days after transfection (**Supplementary Fig. S1B**), this data suggests that CAF-produced FN is critical to enable the early steps of tumor initiation in vivo.

CAF interaction with fibronectin provides a critical scaffold for the deposition of collagen fibers

Previous work has identified FN as a scaffold that coordinates the assembly and incorporation of additional proteins and secreted factors into the ECM[11]. Indeed, CAFs isolated from PDAC patients generate a fibrotic ECM in which FN and collagen (COL) fibers clearly show a high degree of co-patterning (**Fig. 1B and Supplementary Fig. S2A**). As evidence for FN's role as a critical scaffold for the construction of additional matrix proteins, siRNA-mediated knockdown of FN1 prevents CAFs from generating collagen fibers (**Fig. 1B**). This reduced assembly of collagen into insoluble fibers is consistent with increased content of "soluble collagen" in conditioned media collected from CAFs with FN knockdown compared to scramble control (**Fig. 1C**). In contrast, knockdown of the type I collagen alpha 1 chain (*COL1A1*) does not affect FN fiber assembly (**Supplementary Fig. S2B**), highlighting the unique role of FN as a critical base scaffold upon which additional ECM components such as collagen are layered. Together, these findings not only demonstrate that FN is required for CAF-mediated assembly of collagen into fibers, but the genetic approach to disrupt this process establishes proof-of-principle for target tractability.

FN-binding integrins are critical for fibrotic matrix assembly

Previous studies have established that activated fibroblasts (myofibroblasts) use cell surface integrins as anchors to apply tension to fibronectin molecules, revealing cryptic sites required for polymerization into FN "fibers" that, in turn, provide a physical scaffold and repository for the assembly and recruitment of additional pro-cancer factors to support the invasive behavior of cancer cells[10, 15-17]. Although several integrins can serve as receptors for FN, integrins αvβ3 and α5β1 are absent on normal cells but become upregulated on a variety of activated cell types, including myofibroblasts[31-33]. Indeed, CAFs isolated from human PDAC tumors show robust protein expression of both the α5 and β3 subunits (**Fig. 2A**). Similar to knockdown of *FN1*, collagen fibrillogenesis can be reduced by knockdown of the integrin β3 subunit (*ITGB3*, whose expression is the limiting factor for the formation of the αvβ3 heterodimer) or the integrin α5 subunit (*ITGA5*, whose expression similarly dictates α5β1) (**Fig. 2A**). Imaging permeabilized cells reveals that knockdown of FN or FN-binding integrins does not eliminate the ability of CAFs to produce collagen (as evidenced by the presence of intracellular collagen), but rather that the ability of CAFs to *assemble* extracellular collagen into fibers has been largely eliminated (**Fig. 2A**). Together, these findings support the notion that FN-integrin interactions on the surface of CAFs are required for the formation of the FN fibers that function as a scaffold for other fibrotic matrix proteins, and furthermore suggest that targeting integrins α5β1/αvβ3 may provide an opportunity to significantly impair the contribution of CAFs to cancer progression. Since αvβ3 and α5β1 integrins are generally absent on normal cell types but highly expressed on CAFs, targeting their function may provide an opportunity to selectively suppress the pathological fibrosis that supports PDAC progression.

Integrin-blocking antibodies prevent and reverse CAF assembly of ECM

To simultaneously target both integrins required for ECM production by CAFs using a single agent, we designed a novel bispecific antibody (bsAb) for dual monovalent recognition of the integrin α5β1 and αvβ3 heterodimers (**Fig. 3A**). We first compared this bsAb to its two parental control bivalent monoclonal antibodies (mAbs) that individually recognize integrins αvβ3 or α5β1. Commercially available antibodies recognizing integrin αvβ3 (LM609) and integrin α5 (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. 3B-3C**).

Consistent with the effects of genetic knockdown of either β3 or α5, treating CAFs with the mAbs recognizing αvβ3 or α5β1 shows a variable ability to reduce the assembly of FN and COL fibers that may reflect the relative expression level of these integrins in a given cell population, while the bsAb with dual recognition of integrins αvβ3/α5β1 produces a more complete blockade of FN and COL fibril formation than either monoclonal antibody alone (**Fig. 4A**). Immunostaining CAFs for FN and actin reveals punctate FN expression in the antibody-treated cells that is no longer aligned with actin fibers, confirming that this antibody does not impact the matrix adhesion and cytoskeleton of these cells (**Supplementary Fig. S3A**). Decellularization (i.e., removal of CAFs) after 72 hours of treatment leaves behind a "CAF-ECM" substrate that clearly illustrates the significant effect of the bsAb on both FN and COL fibers (**Supplementary Fig. S3B**). A similar effect of the bsAb is observed for primary hepatic stellate cells isolated from patients with liver fibrosis or primary lung fibroblasts isolated from patients with fibrotic lung disease (**Supplementary Fig. S4**), suggesting this antibody may have the potential for broad use as a general anti-fibrotic agent to target activated fibroblasts that depend on FN and integrins α5β1/αvβ3 for matrix assembly. Consistent with a change in matrix assembly but not production, treating CAFs with the BsAb does not alter mRNA expression of either *FN1* or *COL1A1* genes (**Supplementary Fig. S5**).

To directly compare the BsAb to a combination of monospecific mAbs in vitro, fibronectin immunostaining was evaluated for CAFs subjected to antibody treatment for 72 hours. Under these in vitro conditions for which cells are exposed to a saturating antibody doses, all of the test antibodies equivalently block CAF production of FN compared to the IgG isotype control (**Supplementary Fig. S6**). Interestingly, while the αvβ3 mAb produces a significant reduction in FN levels, it does not induce the unique FN puncta as observed for the α5β1 mAb, BsAb, and combination of α5β1/αvβ3 mAbs.

Integrins exist in a dynamic equilibrium of conformational states such that binding events are continuously being formed and dissociated. While small peptide Arg-Gly-Asp (RGD)-based competitive inhibitors cannot easily reverse integrin-ligand binding, a function-blocking β1 antibody was reported to increase the dissociation rate of integrin-FN complexes and act allosterically[34]. To test if the bsAb may be able to interfere with a pre-existing matrix, we allowed CAFs to deposit ECM proteins for 72 hours in culture before adding integrin-targeted antibodies. Indeed, over the span of 3 days, the bsAb was able to disrupt pre-existing FN and COL fibers produced by CAFs (**Fig. 4B**), suggesting that its ability to allosterically interfere with continuously cycling on/off states of FN-integrin binding can exert a significant anti-fibrotic effect by shifting the equilibrium to a non-adhesive state.

As one biological readout for how PDAC cells respond to CAFs, we considered the ability of CAF-produced ECM to upregulate tumor cell expression of connective tissue growth factor (CTGF), a matricellular protein produced by both stromal and tumor cells that mediates their crosstalk, promotes fibrosis, and enhances tumor initiation in pancreatic cancer[35, 36]. CAFs were cultured for 72h 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 CTGF in the PDAC cells, and this is prevented in the presence of the bsAb (**Fig. 4C**). This assay demonstrates how blocking the two primary FN-binding integrins, αvβ3 and α5β1, can prevent tumor cell upregulation of CTGF in response to CAF-produced ECM.

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

To evaluate the ability of the αvβ3/α5β1 bsAb to disrupt tumor initiation in vivo, we utilized a subcutaneous xenograft model to readily detect the earliest emergence of tumor initiation over time. As for the orthotopic model (**Fig. 1A**), a suboptimal number of PDAC cells was injected so that initiating a tumor depended on the co-injection of CAFs. 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. 5A**). That is, palpable tumors formed at 12 out of 12 injection sites co-injected with PANC1 cells with CAFs, validating this model as a readout for CAF-dependent tumor initiation.

As observed for the orthotopic model (**Fig. 1A**), the "boost" in CAF-dependent tumor initiation at a subcutaneous site could be prevented using CAFs with siRNA-mediated knockdown of *FN1* (si-FN), while CAFs with *ITGA5* knockdown (si-α5) produced tumors at 1 out of 11 injection sites. Since the siRNA knockdowns are transient, these results suggest that the CAF contribution to tumor initiation occurs within the first several days after co-injection when tumor cells exploit CAFs to overcome isolation stress as they colonize 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.

Accordingly, we asked if the therapeutic bsAb designed for dual targeting of integrins αvβ3/α5β1 could exert a similar activity to block tumor initiation. 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 <u>human</u> CAFs co-injected with <u>human</u> 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. 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. 5A**), suggesting that the boost in tumor initiation offered by CAFs can be targeted therapeutically. Since the effects of the bispecific antibody are mimicked by knockdown of FN or α5 in the CAFs, we propose that blocking integrin function on CAFs has the ability to prevent tumor initiation.

BsAb treatment reduces the fibrotic effect of human CAFs co-injected with tumor cells

To evaluate the mechanism(s) of action for the knockdown and blockade strategies, all palpable tumors were harvested at the 8-week endpoint of the experiment. Compared to untreated tumors, analysis of H&E-stained sections show far less stroma and but display extensive necrosis in the two tumors that formed in the bsAb-treated group (**Fig. 5B**). Previous studies have documented that PDAC tumors are highly stiff due primarily to the extensive stroma and ECM deposited within the tumor, and that stiffness correlates with PDAC progression in mice and man[37, 38]. Consistent with this, mice treated with the bsAb show significantly lower tissue stiffness throughout the entire tumor as measured by atomic force microscopy (**Fig. 5C**).

Analysis of the tumor microenvironment also supports the notion that the co-injection of CAFs produces a highly fibrotic and reactive tumor environment that can be targeted therapeutically. Staining tumors formed by co-injection of PANC1+CAFs using an antibody that recognizes human FN (hFN), but not mouse FN, reveals areas of intense fibrillar hFN staining (**Fig. 6A**), suggesting that the co-injected CAFs represent a significant producer of tumor stroma in this model relative to the host (i.e., mouse) fibroblasts. Tumor areas with dense hFN staining also show significant fibrosis (collagen polymerization), as evaluated by Masson's trichrome (**Fig. 6B**) and picrosirius red staining for collagen fibers (**Fig. 6C**). In comparison, tumors from mice treated with the bsAb show a complete absence of hFN, while collagen fibers appear both fewer and smaller. Since the bsAb utilized in this study does not recognize mouse antigens, it can identify only the injected human PDAC cells and human CAFs in this xenograft model. This particularity suggests that interactions between co-injected PANC1 cells and CAFs mediate the earliest steps of tumor initiation before any mouse stromal cells are recruited into the tumor microenvironment. Analysis of serial sections of tumors stained for hFN and collagen suggests that the human CAFs contribute to the production of highly fibrotic matrix, while mouse host cells that are insensitive to the bsAb may be the source of less developed collagen networks.

In addition to the observed suppression of CAF-generated FN, we predict that in man, the bsAb might gain additional anti-tumor efficacy by acting on activated cell types within the tumor that are known to gain expression of αvβ3/α5β1, including angiogenic endothelial cells and tumor-associated macrophages. In the xenograft model, we observed a reduction in immunostaining for CD31, an endothelial cell marker used to assess tumor angiogenesis. It is therefore possible that the bsAb may exert an indirect anti-angiogenic effect on the mouse vascular compartment (**Fig. 6D**), by inhibiting the accumulation of stimulatory factors and ECM proteins within the tumor microenvironment that stimulate vascular cells to generate new blood vessels[39, 40].

Discussion

CAFs are dysregulated stromal cells that exert a massive influence on the biology of tumor cells to facilitate the progression of many solid tumor types, especially PDAC with its notorious dense

and reactive stroma. Despite efforts to characterize various CAF types and subtypes[41], strategies to "disempower" CAFs have yet to realize success as cancer therapeutics. In addition to their pro-fibrotic role within the primary tumor microenvironment, CAFs can gain access to the circulatory system to promote the survival and subsequent growth of circulating tumor cells and facilitate the establishment of new tumor cell outposts by serving as the "soil" or "niche" that creates a friendly environment for individual tumor cells to seed and survive at distant sites[20, 42]. Pancreatic cancer is notorious for its dense and stiff stroma that supports tumor progression and hinders the delivery of therapeutic drugs[2]. While tumor cells within this oasis benefit from a plethora of growth factors and stimulatory signals that allow their uncontrolled growth and survival, we focus on how CAFs may nurture individual tumor cells during tumor initiation, a situation which may also occur during metastatic colonization of distant sites where tumor stroma has yet to form. We consider how crosstalk between CAFs and tumor cells represents a limiting factor required for tumor cells to survive the challenges encountered during what we refer to as "isolation stress"[18]. This not only relates to the ability of limited numbers of cells to establish tumor colonies within the pancreas, but also 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.

Our work demonstrates that preventing CAFs from generating cell surface FN fibers can have significant effects, akin to destroying the foundation of a building. Similar effects have been reported for agents such as the functional upstream domain "FUD" peptide[43] (and a PEGylated form developed to slow its rapid renal clearance) that binds to the N-terminal domain of FN to sterically inhibit the polymerization of FN monomers into fibrils. While such agents that block FN fibrillogenesis have been shown to produce significant anti-fibrotic activity, pharmacokinetic stability issues have limited their development for use in man. In contrast, we have devised an antibody therapeutic to disrupt FN fibrillogenesis by interfering with the anchoring of FN to FN-binding integrins, thereby preventing the tensile forces that reveal cryptic sites required for FN polymerization. We show that dual blockade of αvβ3/α5β1 or integrin knockdown can inhibit CAFs from producing fibrillar FN and collagen, and this prevents stem-like reprogramming of tumor cells, measured here as tumor initiation in mice. Since the bsAb selectively targets human (but not mouse) cells, it provides a unique mechanistic tool that could be used in future studies to dissect the benefits of perturbing integrin function on CAFs alone, tumor cells alone, or both cell types.

Our work reveals that CAF-mediated assembly of FN and COL fibers is especially enabling and enhancing when PDAC cells are challenged with "isolation stress", a state that occurs during various aspects of tumor initiation and progression. We show that disrupting the ability of CAFs to organize FN fibers results in the loss of COL fibril formation and thus provides an opportunity to interfere with intercellular communication and prevent tumor cell survival long enough to "initiate" a new tumor colony (**Fig. 7**). Though not addressed directly in this study, it is tempting to speculate that tumor initiation at a metastatic site would similarly depend on CAF-produced matrix. We show that disrupting tumor initiation can be achieved by knocking down the expression of FN or its receptors, or by utilizing a unique bispecific antibody that targets the two primary receptors CAFs use to organize FN, integrins αvβ3 and α5β1. By perturbing the ability of CAFs to generate the FN fibrils that function as a cornerstone for the construction of additional ECM components, tumor cells are less able to overcome the effects of isolation stress. As such, disrupting FN-integrin binding provides a powerful approach to prevent CAFs from supporting PDAC tumor initiation.

The therapeutic efficacy of blocking FN-binding integrins may be derived from direct effects on CAFs plus indirect effects on various cell types that are sensitive to changes in the tumor

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stroma. Aside from CAFs, αvβ3 and α5β1 are upregulated on several key cell types in a tumor, including mesenchymal tumor cells and angiogenic endothelial cells. For example, the CAF matrix is a rich source of VEGF and other factors that promote tumor angiogenesis[39, 40]. By targeting αvβ3/α5β1 on tumor-associated CAFs, we observed that the level of CD31-positive vascular cells was significantly reduced, representing an example of an indirect effect of the human-specific bsAb that cannot directly recognize mouse vascular cells. By preventing the deposition of a dense, reactive ECM that contributes to an immunosuppressive environment, targeting CAFs with an agent like the αvβ3/α5β1 bsAb may also improve tumor responsiveness to immunotherapy. Reducing the extent of fibrotic tumor stroma may also improve blood flow and the delivery of systemically administered cancer therapeutics to the tumor microenvironment. Since αvβ3 and α5β1 are not expressed on normal (i.e., non-activated) cells, targeting these integrins in man should provide an opportunity to selectively impair the function of multiple activated cell types within the tumor microenvironment, allowing for dose escalation with limited off-target effects and toxicity.

While we found that the BsAb did not functionally outperform the combination of monospecific mAbs in vitro (**Supplementary Fig. S6**), a bispecific antibody provides a more straightforward path for clinical development since it is a single therapeutic agent for which biodistribution, efficacy, and dose-limiting toxicity can be more cleanly determined compared to a combination of two individual monoclonal antibodies that may differ in their PK/PD and safety properties. In terms of quality of life, an additional potential benefit of a bispecific antibody is the ability to inject patients with a single agent instead of two separate agents. Also, two antibodies delivered together may also produce steric hindrance effects, and it becomes complicated to determine an optimal safe and effective dose for each antibody. From a biological perspective, a bispecific antibody may provide a functional benefit if both antigens are present on a single cell. To evaluate the biological importance of targeting α5β1 +/- αvβ3 in vivo, we used the approach to knock down fibronectin itself, as well as integrins α5 and β3, in CAFs before injection into mice. This strategy allowed us to selectively perturb FN and integrin function on the CAFs without impacting tumor cells that express these proteins, and avoided complications associated with delivering multiple agents in vivo that may have significantly different PK profiles.

Our study approaches CAF-tumor crosstalk from a less mainstream angle that focuses on how CAFs might enable stem-like abilities of tumor cells. In contrast to the long-term impact of CAFs on tumor progression, our study relates to the ability of CAFs to provide individual tumor cells with supportive factors and ECM that allow them to become more stem-like to overcome "isolation stresses" (e.g., loss of cell-cell and cell-matrix contacts, nutrient or oxygen scarcity, or immune surveillance). This relationship may enhance the ability of a single tumor cell to gain cancer stem cell-like stress tolerance and invasive behavior that facilitates the establishment of new colonies within the pancreas, in the circulation, or at metastatic outposts. This point of view is unique compared to studies that explore the contributions of CAFs during later stages of cancer when cancer cells are already surrounded by a well-developed tumor microenvironment.

While approaches to impair tumor initiation may not be appreciated when the growth of a primary tumor is the primary readout, our in vitro and in vivo models include challenges that mimic the critical steps involved in tumor initiation. Our findings agree with previous studies identifying FN as a key component of CAF function, where knockdown of FN, β3, or α5 produced a significant effect on tumor cell invasion through ECM proteins in vitro[44]. Indeed, CAFs produce ECM and soluble factors that strongly influence a tumor microenvironment, and integrins are ECM receptors utilized by all cell types to interact with and respond to specific forms of ECM[4, 45]. In contrast to these studies, our new data shows how CAFs can contribute to a somewhat different step of tumor progression, i.e. the ability of individual cancer cells to

overcome isolation stress during tumor initiation or establishment of a growing tumor colony within an otherwise normal tissue environment that does not contain aberrant ECM. We propose that CAF-enhancement of tumor initiation is relevant for scenarios that lack available therapeutic interventions. For example, circulating tumor cells in the bloodstream or disseminated tumor cells during metastasis must find a way to survive within environments that are inhospitable and prone to recognition by immune cells. As such, we propose that the contribution of CAFs to promote tumor initiation will involve a unique set of soluble factors and secreted matrix proteins that might not overlap with the role of CAFs that has been reported to support cancer cell growth in 2D culture or invasion through ECM matrices in vitro.

While the focus of our work is currently limited to pancreatic cancer, a disease for which few targeted therapeutics have produced clinical benefit, our findings might extend to additional cancer types, since an individual circulating or disseminated tumor cell may utilize tissue-resident fibroblasts in the liver, lung, or lymph nodes during colonization of these sites during metastatic spread of pancreatic cancer. Similarly, other pathological conditions that involve fibrosis might respond well to blockade of FN-integrin interactions that provide a scaffold for the escalation of a chronic fibrotic response. Indeed, we show that targeting integrins $\alpha v\beta 3/\alpha 5\beta 1$ with the bsAb can suppress the generation of fibrotic ECM by primary fibroblasts isolated from patients with chronic lung or liver fibrosis.

Acknowledgements

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Figure Legends

Figure 1: Tumor cells utilize CAF-produced fibronectin to overcome isolation stress

1A: CAFs enhance tumor initiation via FN (orthotopic pancreas cancer model)

> Luciferase-expressing KP4 PDAC cells were orthotopically injected (with or without CAF-1299) to the pancreas of nu/nu mice. After 3 weeks, luciferase imaging was performed to survey tumor establishment in the pancreas.

1B: Fibronectin knockdown prevents CAF assembly of collagen fibers

> CAF-1299 and CAF-1424 cells were treated with siRNA for a scramble control vs. FN. After 72 hours, immunofluorescence staining shows FN and COL content. Images are representative of at least 3 independent experiments.

1C: Knockdown of FN in CAFs increases soluble COL in media

> CAF-1299 cells were cultured for 72 hours, then the conditioned media and cell lysates were collected and processed for immunoblot to confirm fibronectin knockdown and assess the level of soluble collagen secreted by the cells into the media.

Figure 2: FN-binding integrins mediate ECM production

2A: Knockdown of FN or FN-binding integrins in CAFs prevents the formation of FN and COL fibers

> CAF-1299 were treated with siRNA for a scramble control vs. FN, ITGA5, or ITGB3. After 72 hours, immunofluorescence staining shows FN and COL content. The top set of images was not permeabilized. Images are representative of at least 3 independent experiments. Blots confirm knockdown.

Figure 3: BsAb improves targeting of FN-binding integrins on CAFs

3A: Bispecific antibody (bsAb) for dual recognition of αvβ3 and α5β1 heterodimers

> Schematic depicts design of novel bispecific antibody with monovalent recognition of two antigens, integrin αvβ3 and integrin α5β1. The Fab domains for the bispecific antibody are identical to the "control" bivalent (i.e. monospecific) monoclonal antibodies recognizing αvβ3 (derived from etaracizumab) and α5β1 (derived from volociximab).

3B: BsAb binding to cells compared to commercial and control antibodies

> Flow cytometry plots show binding of each antibody to CAF-1299 and CAF-1424.

3C: BsAb shows improved binding to CAFs compared with constituent mAbs

> Graph shows median fluorescence intensity (MFI) for antibody binding to CAF-1299 and CAF-1424 cells. Bars and error bars represent standard deviation from n=3 independent experiments.

Figure 4: Dual blockade of integrins αvβ3/α5β1 can prevent and reverse CAF-ECM assembly

4A: Function blocking antibodies targeting FN receptors prevent CAF assembly of FN/COL fibers

> CAF-1299 were incubated with control IgG vs. indicated antibodies for 72 hours, and then processed for immunostaining to examine FN and COL. Images are representative of at least 3 independent experiments. Graph shows the quantification of staining as mean+/-SD for each marker that was measured as % area for each experiment, then normalized to IgG control. *P<0.05 using one-sample t-test.

4B: Integrin-targeted antibody can disrupt pre-existing CAF-produced ECM

> CAF-1299 were plated and allowed to produce ECM for 72 hours before adding control IgG vs. indicated antibodies for an additional 72 hours. Samples were then processed for immunostaining to examine FN and COL. Graph shows the mean ± SD staining for each marker measured as %area in each experiment and normalized to IgG control. *P<0.05 using one-sample t-test.

4C: BsAb prevents activation of YAP, a mechanosensor that promotes stemness

> CAF-1299 were plated and allowed to produce ECM for 72 hours then treated with antibody for another 72 hours before cells were removed to leave behind a cell-free ECM atop which PANC1 cells were then plated. After 24 hours, the PANC1 cells were lysed and prepared for immunoblotting to detect protein expression of the YAP target, CTGF. Blots are representative of at least 3 independent experiments.

Figure 5: Disrupting FN-binding integrins prevents the ability of CAFs to enhance tumor initiation

5A: CAFs enhance tumor initiation via FN and FN-binding integrins (subcutaneous xenograft model)

> PANC1 human PDAC cells were injected subcutaneously (with or without CAF-1299) to the flank areas of nu/nu mice. Mice were monitored twice weekly to detect the earliest emergence of palpable tumors. Graph shows tumor take rate vs. time for 10-12 mice per group, using a volume of $100mm^3$ (computed as length x $width^2$) as the threshold for tumor take. At the endpoint of the experiment (Day 56), tumors were harvested and prepared for histological analysis.

5B: Bispecific antibody treatment reduces tumor stroma and increases necrosis

> Tumors sections were stained using H&E. Areas of stroma (S) and necrosis (N) are noted.

5C: Bispecific antibody treatment reduces tumor stiffness

> Cryosections of tumors were analyzed using atomic force microscopy to evaluate tissue stiffness. Dots depict the mean value for each 20μm x 20μm region of interest (ROI). 15 ROIs were evaluated per tumor, for 2 tumors per group.

Figure 6: BsAb treatment reduces fibrotic effect of human CAFs co-injected with tumor cells

6A-D: BsAb-treated tumors contain less CAF-produced fibronectin, fibrosis, and angiogenesis

Serial FFPE sections of tumors were processed for immunohistochemical detection of human-specific FN (hFN) (A) or CD31 (D), shown in brown. Trichrome (B) and picrosirius red (C) histological stains were used to visualize collagen. Graphs depict the quantification of IHC staining using QuPath, with each dot representing the mean value for each tumor slice examined. P-values were computed using Student's t-test.

Figure 7: CAF-produced fibronectin boosts tumor initiation (summary schematic)

Cancer-associated fibroblasts produce a dense and reactive stroma that supports tumor initiation and progression. This project establishes the link between cell surface integrins and FN as a lynchpin for the construction of pro-tumor ECM that can account for the ability of CAFs to support tumor initiation for multiple in vitro and in vivo models. Knockdown or antibody blockade of integrins $\alpha v\beta 3$ and/or $\alpha 5\beta 1$ can disrupt FN fiber assembly. FN fibers are known to function as a scaffold that mediates the assembly of other ECM proteins and incorporation of pro-tumor secreted factors. Knockdown of $\alpha v\beta 3/\alpha 5\beta 1$ or their dual blockade using a novel bispecific antibody (bsAb) can disrupt this cascade by preventing the initial assembly of FN fibers. This approach has broad potential as a novel strategy to target the aberrant fibrosis that exacerbates the progression of cancer and fibrotic disease.

Figure 1

Figure 2

A

Human
PDAC CAFs
(CAF-1299)
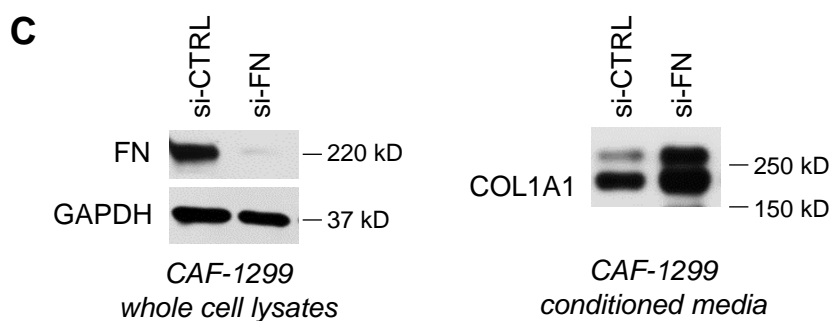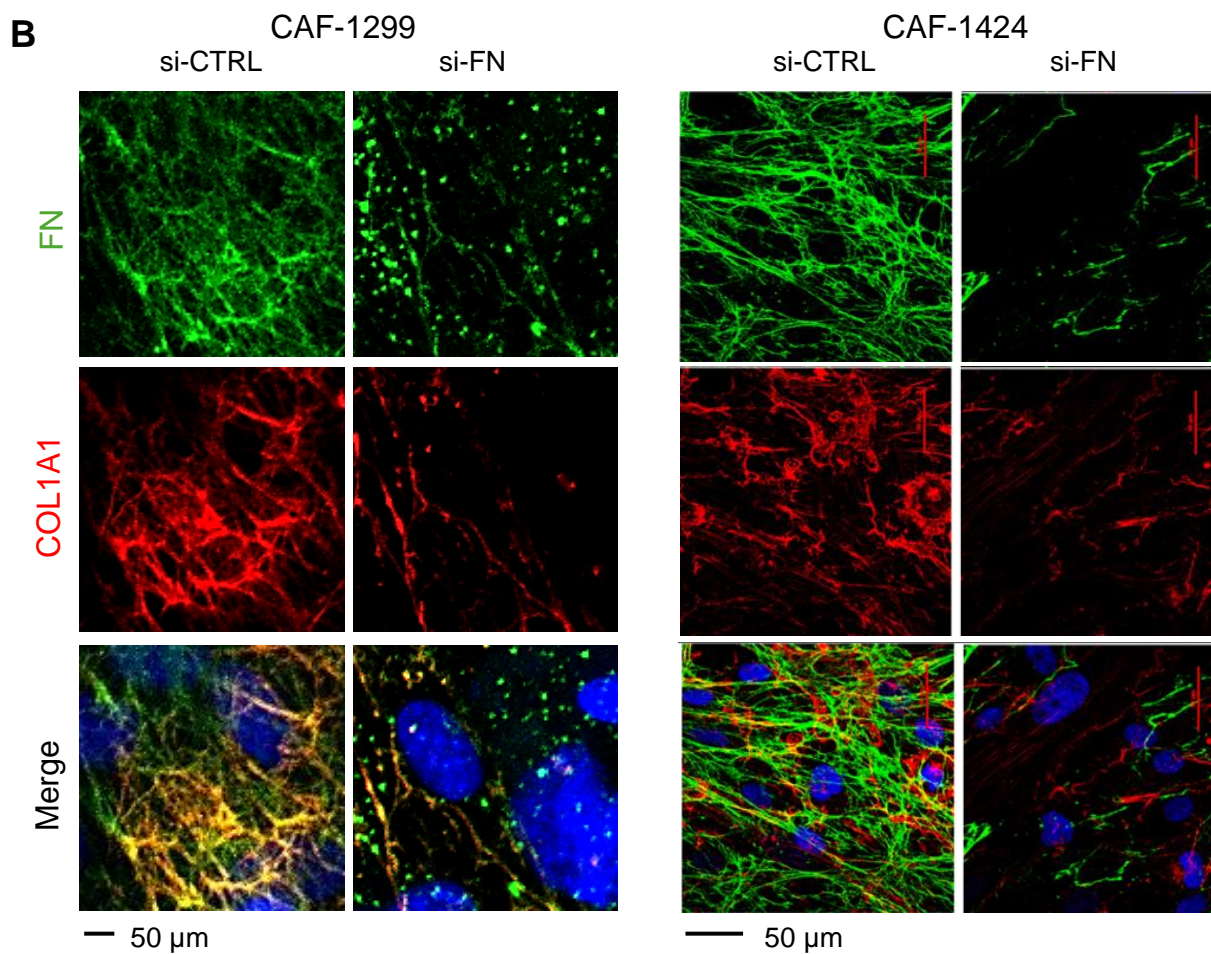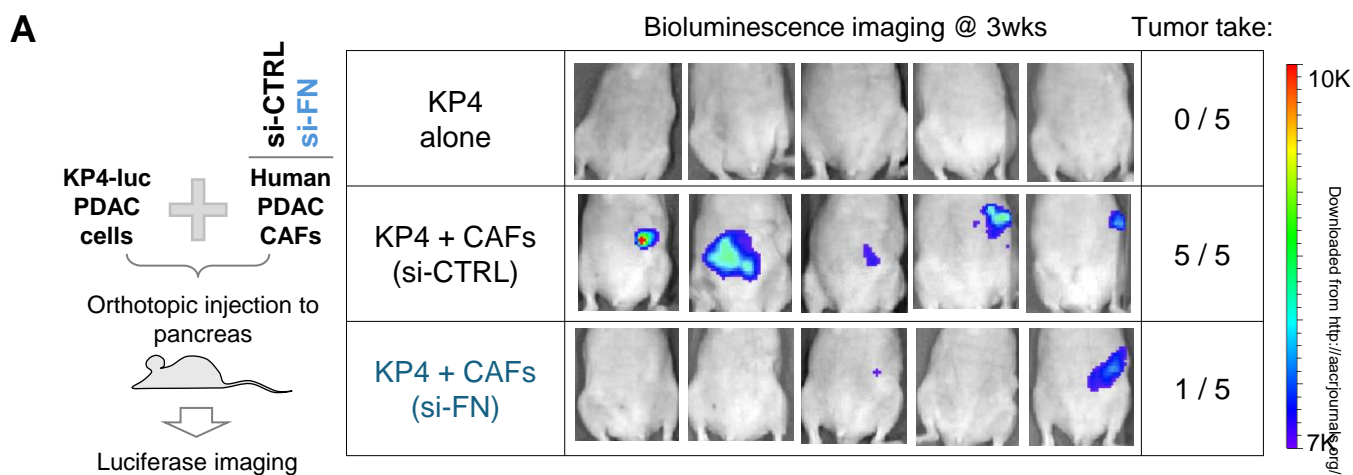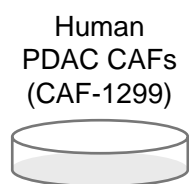
⟹ Analyze
72 hrs "CAF-ECM"

CAF-1299   si-CTRL si-FN si-α5 si-β3

FN

α5

β3

GAPDH
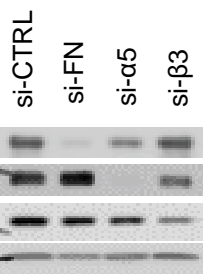
| si-CTRL | si-FN | si-integrin α5 | si-integrin β3 |

100 μm

FN / COL1A1 / DAPI (not permeabilized)
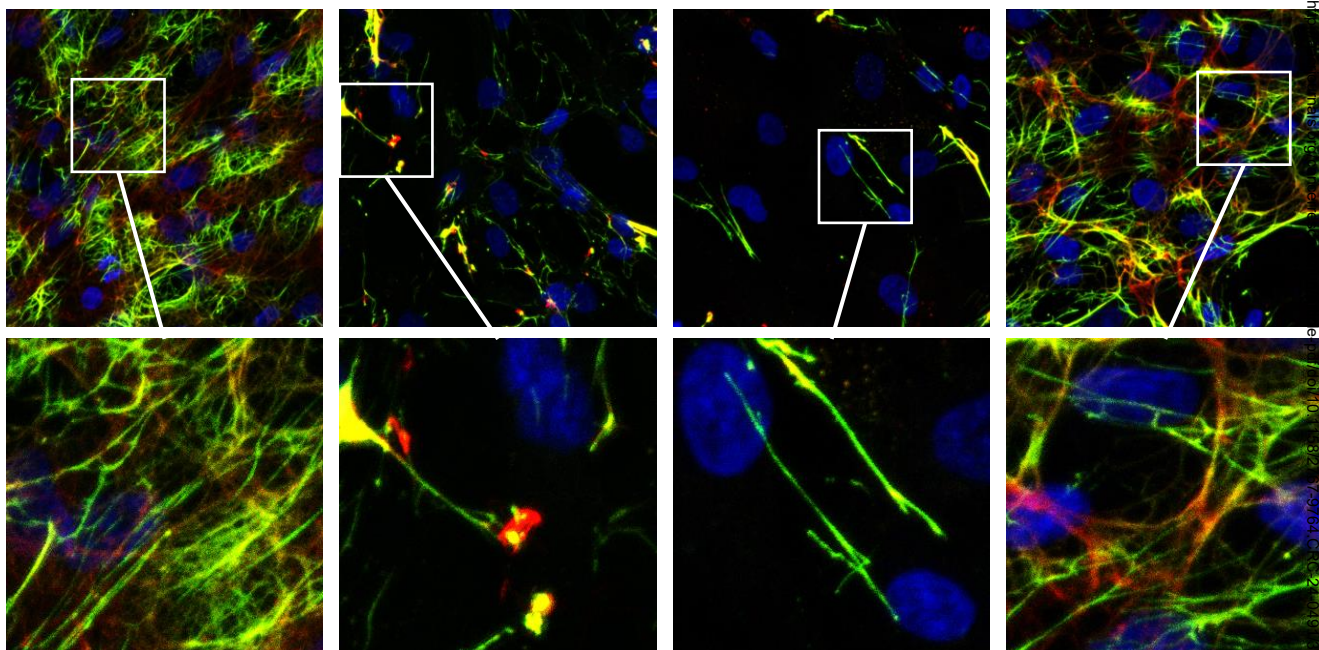
| si-CTRL | si-FN | si-integrin α5 | si-integrin β3 |

FN / COL1A1 / DAPI (permeabilized)

Figure 3

A

Human αvβ3   Human α5β1

Fab   Fab

BsAb

Fc

B

CAF-1299   CAF-1424

10 µg/ml

2° Ab only (1 µg/mL)

Isotype control

Benchmark: LM609

Anti-αvβ3

Benchmark: P1D6

Anti-α5β1

BsAb

AF647   AF647

C

CAF-1299   CAF-1424

Antibody binding (MFI)

Isotype Ctrl, Anti-αvβ3, Anti-α5β1, BsAb, Isotype Ctrl, Anti-αvβ3, Anti-α5β1, BsAb

Figure 4

A

CAFs + Ab

⇩ *72 hrs*

Analyze "CAF-ECM"

● FN   ● COL

Area fraction (%)

IgG   αvβ3   α5β1   BsAb

Control IgG   Anti-αvβ3   Anti-α5β1   Bispecific Ab

FN / COL1A1 / DAPI

50 µm

B

CAFs

⇩ *72 hrs*

Antibody Tx

⇩ *72 hrs*

Analyze "CAF-ECM"

● FN   ● COL

Area fraction (%)

IgG   αvβ3   α5β1   BsAb

Control IgG   Anti-αvβ3   Anti-α5β1   Bispecific Ab

FN / COL1A1 / DAPI

C

CAFs + Ab

⇩ *72 hrs*

Remove CAFs…
Add PANC1 cells
in 2% FBS to "CAF-ECM"

⇩ *24 hrs*

Analyze YAP targets

IgG4   BsAb

CTGF

Vinculin

Figure 5

A

CAF siRNA:		Tumor take *(Day 56)*
PANC1 alone	-	0 / 12
si-CTRL		10 / 10
si-FN		0 / 10
si-α5		1 / 11
si-CTRL + BsAb		**2 / 12**

PANC1 PDAC cells **+** Human PDAC CAFs

Subcutaneous injection

± BsAb

Palpable tumors

B

PANC1 + CAFs

PANC1 + CAFs + BsAb

100 µm   100 µm

C

Tumor cryosection

⬇

Atomic force microscopy:
- 20µm x 20µm ROIs
- 15 ROIs per tumor
- 2 tumors per group

Figure 6

200 μm

Figure 7

1. CAFs secrete **FN monomers**

PDAC CAF

2. αvβ3/α5β1 required for **FN fiber assembly**

α5β1
αvβ3

3. **FN** is a scaffold for **collagen** & **soluble factors**

α5β1
αvβ3

4. CAF-produced ECM supports **tumor initiation & progression**

Isolation stress
Tumor cell
Stress tolerance
α5β1
αvβ3
Tumor initiation

αvβ3/α5β1 BsAb blocks CAF interaction with FN to prevent the construction of a pro-tumor ECM and sensitize PDAC cells to the effects of isolation stress