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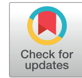
Compartment resolved proteomics reveals a dynamic matrisome in a biomechanically driven model of pancreatic ductal adenocarcinoma



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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is characterized by a severe fibrotic component that compromises treatment, alters the immune cell profile and contributes to patient mortality. It has been shown that early on in this process, dynamic changes in tissue biomechanics play an integral role in supporting pancreatic cancer development and progression. Despite the acknowledgement of its importance, a granular view of how stromal composition changes during the course of PDAC progression remains largely unknown. To mimic the quasi-mesenchymal phenotype and pronounced desmoplastic response observed clinically, we utilized a genetically engineered mouse model of PDAC that is driven by a $Kras^{G12D}$ mutation and loss of Tgfbr2 expression. Application of compartment resolved proteomics revealed that PDAC progression in this KTC model is associated with dynamic stromal alterations that are indicative of a wound healing program. We identified an early provisional matricellular fibrosis that was accompanied by markers of macrophage activation and infiltration, consistent with the inflammatory phase of wound healing. At 20 weeks a proliferative phenotype was observed with increased fibroblast markers, further collagen deposition and loss of basement membrane and native cell markers.

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1. Introduction

A spectrum of distinct pancreatic malignancies that exhibit distinct histological and molecular features have been observed and described.[1] Pancreatic ductal adenocarcinoma (PDAC) is the most common pancreatic neoplasm, accounting for >85% of all pancreatic tumor cases. Additionally, more than 95% of all pancreatic cancers arise from exocrine (i.e. duct where digestive enzymes are produced) elements, while cancers arising from endocrine elements (i.e. neuroendocrine tumors and islet cell tumors) account for <5% of cases. Pancreatic cancer is one of the most lethal malignancies and has a median survival of <6 months and a 5-year survival rate of 7%.[2,3]

The fibrosis that develops during PDAC progression compromises drug delivery, alters immune cell accessibility and promotes disease aggression and therapy resistance.[4–6] Targeting of various stromal components and pathways is considered a promising strategy to biochemically and biophysically enhance therapeutic response. However, none of the efforts have yet led to efficacious and approved therapies in patients. The extracellular matrix (ECM) specifically has been shown to modify almost every hallmark of cancer and is considered an enabling characteristic of solid tumor growth and invasion.[7–9] Paradoxically, others have shown that the same desmoplastic stroma that confers drug resistance might also reduce the ability of PDAC cells to invade and metastasize. In mice, PDACs that are depleted of alpha smooth muscle actin positive myofibroblasts at the early or late stages formed more invasive tumors with reduced overall survival.[10,11] Taken together, these findings illustrate the complex role of dynamic reciprocity between cellular and stromal compartments to influence PDAC progression. As such, a more detailed understanding of the tumor microenvironment in tumor pathogenesis and therapy resistance is needed to optimize strategies that target stromal components.

Pancreatic tumors share common genetic alterations in a handful of genes including Kras, p53, Smad4, and p16.[12] Importantly, these genetic events, combined with accompanying histopathological alterations (i.e. fibrosis), suggest a sequential

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transformation roadmap of pancreatic cancer from normal pancreatic epithelium to increasing grades of pancreatic intraepithelial neoplasia (PanIN) to, ultimately, invasive PDAC. However, tumors with identical germline mutations can exhibit diverse stromal phenotypes that predict tumor aggressiveness[13–15] supporting further study of the tumor microenvironment including the ECM. In support of this, recent studies have demonstrated that the genotype of PDAC tunes epithelial tension to regulate fibrosis and accelerate PDAC progression in mice.[15] Pathological fibroblasts (i.e. myofibroblasts) are generally thought to be the precursors to increases in matrix deposition, stiffness and enhanced biomechanical signaling.[16,17] Although the biological impact of pancreatic cancer stroma on tumor cells has been investigated for some time,[18] the molecular mechanisms that underlie the desmoplastic response are not well understood. In part, this is because there is a lack of methods aimed specifically at characterizing covalently cross-linked and highly insoluble extracellular matrices. Here, we investigate a biomechanically driven model of PDAC at early and late timepoints to age matched normal pancreas using compartment resolved proteomics to better understand the stromal remodeling that occurs with disease progression.

2. Materials and methods

2.1. Reagents

Reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Sodium chloride was from Acros Organics (part of Thermo Fisher). Microcentrifuge tubes and other consumables were from Axygen Inc. (Union City, CA) and RINO Screw Camp Tubes from Next Advance (Averill Park, NY). Formic acid (FA), and hydroxylamine (NH_2OH) hydrochloride were from Fluka (Buchs, Switzerland). Anhydrous potassium carbonate, guanidine hydrochloride, sodium hydroxide, and acetonitrile (LC-MS grade) were from Fisher Scientific (Pittsburgh, PA). Trypsin (sequencing grade, TPCK treated) was from Promega (Madison, WI).

2.2. Sample preparation

All mice were maintained in accordance with University of California Institutional Animal Care and Use Committee guidelines under protocol number AN105326-01D. Three biological replicates of normal pancreas early (5 weeks), normal pancreas late (20 weeks), KTC PDAC early (5 weeks) and KTC PDAC late (20 weeks) were harvested from either normal (C57bl/6) or from a genetically engineered KTC (*Kras*$^{LSL-G12D/+}$*Tgfbr2*$^{flox/+}$*Ptf1a*-Cre) mice (n = 3 each group), as previously described.[15] An equal number of male and female mice were used and matched between the time points. The early tumors visually present as normal pancreas tissue (250 mg) and late tumors take over most of the pancreas (300–500 mg). Tissues were flash frozen in liquid nitrogen and milled to a fine powder using a ceramic mortar and pestle. Tissue was dried overnight in a lyophilizer and weighed tissue (approximately 1 mg for each sample) was homogenized in freshly prepared high-salt buffer (50 mM Tris-HCl, 3 M NaCl, 25 mM EDTA, 0.25% w/v CHAPS, pH 7.5) containing a 1x protease inhibitor cocktail (Halt Protease Inhibitor, Thermo Scientific) at a concentration of 10 mg/mL. Homogenization took place in a bead beater (Bullet Blender Storm 24, Next Advance, 1 mm glass beads) for 3 min at 4 °C. Samples were then spun for 20 min 18,000 × g at 4 °C, and the supernatant removed and stored as Fraction 1. A fresh aliquot of high-salt buffer was added to the remaining pellet at 10 mg/mL of the starting weight, vortexed at 4 °C for 15 min, and spun for 15 min (18,000 × g at 4 °C). The supernatant was removed and stored as Fraction 2. This high-salt extraction was repeated once more to generate Fraction 3, after which freshly prepared guanidine extraction buffer (6 M guanidinium chloride adjusted to pH 9.0 with NaOH) was added at 10 mg/mL and vortexed for 1 h at room temperature. The samples were then spun for 15 min, the supernatant removed, and stored as Fraction 4 (sECM). Fractions 1, 2, & 3 (Cellular) were combined and all fractions were stored at −20 °C until further analysis.

2.3. Hydroxylamine (NH_2OH) digestion

The remaining pellets from each tissue, representing insoluble ECM proteins, were digested with hydroxylamine as previously described.[19] Briefly, after chaotrope extraction pellets were treated with freshly prepared hydroxylamine buffer (1 M NH_2OH-HCl, 4.5 M guanidine-HCl, 0.2 M K2CO3, pH adjusted to 9.0 with NaOH) at 10 mg/mL of the starting tissue weight. The samples were briefly vortexed, then incubated at 45 °C with vortexing for 16 h. Following incubation, the samples were spun for 15 min at 18,000 × g, the supernatant removed, and stored as Fraction 5 (iECM) at −80 °C until further proteolytic digestion with trypsin. The final pellet was stored at −80 °C until further analysis.

2.4. Trypsin digestion

For each sample, 100 µL of the Cellular fraction, and 200 µL of the sECM and iECM fractions, respectively, were subsequently subjected to reduction, alkylation, and enzymatic digestion with trypsin. A filter-aided sample preparation (FASP) approach, as well as C18 cleanup, was performed as previously described.[20]

LC-MS/MS Analysis – Samples were analyzed on an Q Exactive HF mass spectrometer (Thermo Fisher Scientific) coupled to an EASY-nanoLC 1000 system through a nanoelectrospray source. The analytical column (100 µm i.d. × 150 mm fused silica capillary packed in house with 2.7 µm 80 Å Cortex C18 resin (Phenomenex; Torrance, CA)). The flow rate was adjusted to 400 nL/min, and peptides were separated over a 120-min linear gradient of 4–28% ACN with 0.1% FA. Data acquisition was performed using the instrument supplied Xcalibur™ (version 2.1) software. The mass spectrometer was operated in positive ion mode. Full MS scans were acquired in the Orbitrap mass analyzer over the 300–2000 *m*/*z* range with 60,000 resolution. Automatic gain control (AGC) was set at 1.00E+06 and the fifteen most intense peaks from each full scan were fragmented via HCD with normalized collision energy of 28. MS2 spectra were acquired in the Orbitrap mass analyzer with 15,000 resolution with AGC set at 1.00E+05. All replicates of each tissue were run sequentially and pre-digested yeast alcohol dehydrogenase standard (nanoLCMS Solutions LLC, Rancho Cordova, CA) was run between tissue groups to monitor drift in analytical performance.

2.5. Database searching and protein identification

MS/MS spectra were extracted from raw data files and converted into.mgf files using MS Convert (ProteoWizard, Ver. 3.0). Peptide spectral matching was performed with Mascot (Ver. 2.5) against the Uniprot mouse database (release 201701). Mass tolerances were ± 10 ppm for parent ions, and ± 0.2 Da for fragment ions. Trypsin specificity was used for cellular and sECM fractions, allowing for 1 missed cleavage. For iECM fraction, C-terminal N and trypsin were used, allowing for 1 missed cleavage. Met oxidation, Pro hydroxylation, protein N-terminal acetylation, and peptide N-

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