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Orthotopic Patient-Derived Pancreatic Cancer Xenografts Engraft Into the Pancreatic Parenchyma, Metastasize and Induce Muscle Wasting to Recapitulate the Human Disease

Kristina L. Go, MD¹, Daniel Delitto, MD, PhD¹, Sarah M. Judge, PhD², Michael H. Gerber, MD¹, Thomas J. George Jr., MD³, Kevin E. Behrns, MD, FACS¹, Steven J. Hughes, MD, FACS¹, Andrew R. Judge, PhD², Jose G. Trevino, MD, FACS¹

¹Department of Surgery, College of Medicine, University of Florida Health Science Center, Gainesville, FL 32610

²Department of Physical Therapy, University of Florida Health Science Center, Gainesville, FL 32610

³Department of Medicine, College of Medicine, University of Florida Health Science Center, Gainesville, FL 32610

Abstract

Objective: Limitations associated with current animal models serve as a major obstacle to reliable preclinical evaluation of therapies in pancreatic cancer (PC). In an effort to develop more reliable preclinical models, we have recently established a subcutaneous patient-derived xenograft (PDX) model. However, critical aspects of PC responsible for its highly lethal nature, such as the development of distant metastasis and cancer cachexia, remain underrepresented in the flank PDX model. The purpose of this study was to evaluate the degree to which an orthotopic patient-derived xenograft (PDX) model of pancreatic cancer (PC) recapitulates these aspects of the human disease.

Methods: Human PDX-derived PC tumors were implanted directly into the pancreas of NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice. Tumor growth, metastasis and muscle wasting were then evaluated.

Results: Orthotopically implanted PDX-derived tumors consistently incorporated into the murine pancreatic parenchyma, metastasized to both the liver and lungs and induced muscle wasting directly proportional to the size of the tumor, consistent of the cancer cachexia syndrome.

Conclusions: Through the orthotopic implantation technique described, we demonstrate a highly reproducible model that recapitulates both local and systemic aspects of human PC.

Keywords

Pancreatic Cancer; Patient-Derived Xenografts; PDX; Orthotopic Model; Cachexia

*Corresponding Author: Jose G. Trevino, Department of Surgery, University of Florida College of Medicine, PO Box 100286, 1600 SW Archer Rd, Gainesville, FL 32610. jose.trevino@surgery.ufl.edu.

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Introduction

Pancreatic cancer (PC), associated with a dismal 8% 5-year survival rate, is projected to be the second leading cause of cancer deaths by 2030.^{1,2} Approximately 80% of patients present with unresectable locoregional or systemic disease, excluding the possibility of curative therapy.³ Additionally, cancer cachexia is present in the majority of patients diagnosed with PC.⁴⁻⁶ further limiting therapeutic options. To compound this issue, current treatments demonstrate limited efficacy. Cytotoxic chemotherapy and recently approved targeted therapies extend median survival by only weeks to months in the majority of patients.⁷⁻⁹ Thus, despite numerous preclinical breakthroughs associated with PC, few therapies demonstrate efficacy in clinical trials.

The discrepancy between antitumor activity in preclinical models and therapeutic efficacy in patients suggests that current experimental models may not accurately reflect the human disease. To address the known limitations of highly passaged murine and human PC cell lines in recapitulating the human disease, we have recently developed and validated a patient-derived xenograft (PDX) model by implanting fresh surgical specimens into the flanks of immunocompromised mice.¹⁰ Due to a relatively high engraftment rate and the preservation of a desmoplastic microenvironment, the flank PDX model was an important step toward a more representative preclinical model of human PC. However, the development of systemic metastasis and the cancer cachexia syndrome, two key clinical components of PC, are poorly represented by the flank PDX model. Though our subcutaneous model demonstrated metastatic potential in some tumors, others have shown limited metastatic disease, and none have reported the development of cachexia solely from flank PDX implantation.¹¹

Key elements of the tumor microenvironment in subcutaneous xenograft models may differ from orthotopic counterparts, which may contribute to the limited weight loss and metastasis observed in flank models of PC. Therefore, we hypothesized that an orthotopic PDX model would more effectively recapitulate the degree of metastasis and cachexia associated with human PC. Indeed, the orthotopic implantation technique presented here resulted in a 100% success rate of engraftment, reliable invasion into the murine pancreatic parenchyma, universal distant metastasis and significant muscle wasting. Thus, through a relatively uncomplicated procedure, we demonstrate a reproducible model that recapitulates both local and systemic aspects of clinical PC.

Material and Methods

Orthotopic PDX Model.

All animal studies were performed with approval from the University of Florida Institutional Animal Care and Use Committee. Human PC specimens representing tumors from four patients were expanded in a flank PDX model for one passage as previously described.¹⁰ Initial implantation was chosen for greater expansion of tumor tissue, as mice with subcutaneous xenografts can sustain large tumors with minimal morbidity. Upon reaching an endpoint of 1.5 cm in maximum diameter, PDX tumors were harvested and minced into 2 mm pieces. Orthotopic implantation was then performed in NOD.Cg-*Prkdc*^{scid}

Il2rg^{tm1Wjl}/SzJ (NSG) mice as described in the results section. Abdominal ultrasound was performed weekly using the LOGIQ™ e Vet model and measurements taken with the internal software package (General Electric, Fairfield, CT). Pancreatic tumors were allowed to engraft and grow until one cm in maximum diameter. At the time of euthanasia, tumors, liver, lungs and bilateral tibialis anterior (TA) muscles were harvested for histological analysis. Weights were recorded for whole mice, tumors and TA muscles.

Histological Analysis.

Staining of tissue specimens was performed by the University of Florida's Molecular Pathology Core Facility. Briefly, tumors and murine organ specimens were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin. Glass slides containing 5 µm-thick sections were generated and hematoxylin and eosin (H&E) stains were performed.

Patients and Statistical Analysis.

Attainment of all biospecimens was compliant with an institutional review board-approved (IRB) protocol at the University of Florida (UF). Informed consent was obtained from all patients. Pathologic diagnosis of pancreatic adenocarcinoma was confirmed for patients who underwent pancreatic resection. All statistical analysis was performed using GraphPad Prism Version 7 (San Diego, CA). Correlations were performed between continuous variables using Pearson product-moment correlation coefficients. Correlations demonstrating $p < 0.05$ were considered statistically significant.

Results

Orthotopic transplantation of patient-derived xenografts

Tumors from four different patients were harvested from a single round of expansion in a flank PDX model¹⁰, sectioned into 2 × 2 mm viable portions, and placed in sterile phosphate-buffered saline with minimal ischemia time (Fig. 1A). Eight-week old NSG mice were anesthetized with 2–2.5% vaporized isoflurane in oxygen using an induction chamber. The surgical area was denuded; the mouse was placed in the right lateral decubitus position; and a left subcostal incision was made. The pancreas was located just medial to the spleen and mobilized toward the incision using a rotational motion with a sterile Q-tip (Fig. 1B). 7–0 Prolene® suture was secured first to a tumor fragment (Fig. 1C–D), then to the body of the pancreas (Fig. 1E–G) such that the pancreas and tumor fragment were connected by one continuous suture (Fig. 1H). The suture was then tied, securing the pancreas to the tumor fragment (Fig. 1I–J). Abdominal contents were reduced (Fig. 1K), and both the abdominal wall fascial layer and skin were closed with three wound clips (Fig. 1L). Clips were removed two weeks postoperatively and tumor growth was monitored by ultrasound

Orthotopic PDX tumor fragments from one patient's tumor were implanted into a total of 13 mice. Abdominal ultrasound was performed weekly to evaluate tumor size and all xenografts were harvested when the first intra-abdominal tumor reached one centimeter in maximum diameter. Tumors were typically palpable by one month and reached growth endpoint at three months. Sonographic analysis demonstrated expanding hypoechoic tumors at the approximate location pancreatic tail, just anterior to the left kidney, over monthly intervals

postoperatively (Fig. 2). Harvested tumors demonstrated an expected pattern of growth centered around the implantation site (Fig. 3A) adjacent to the spleen (Fig. 3B) and the retained Prolene® suture was observed in the center of the tumor mass (Fig. 3C). Likewise, in PDX tumors originating from each of four patients, we observed gross metastatic disease in the liver (Fig. 3D) and lungs (Fig. 3E), common clinical sites of metastasis in patients with PC.

Orthotopic PDX Tumors Invade into Murine Pancreatic Tissue and Metastasize

Similar to our flank PDX model, orthotopic PDX tumors demonstrated conserved morphology compared to corresponding primary tumors (Fig. 4) and preserved a desmoplastic microenvironment (Fig. 5A). However, orthotopic tumors incorporated into the murine pancreatic microenvironment consistently demonstrated a remnant of intact pancreatic parenchyma largely replaced by tumor (Fig. 5B–C). Additionally, duodenal invasion, commonly observed in clinical T3 disease, could be observed on the medial aspect of implanted xenografts (Fig. 5D). Unlike our previous observations with orthotopic cell line xenografts, abdominal wall or diaphragmatic invasion was not present. All mice (13/13) demonstrated distant metastatic disease to the lungs (Fig. 5E–G). However, unlike metastatic patterns observed in the human disease, liver metastasis was only observed in 1/13 mice (Fig. 5H). These results suggest that malignant cells of human origin not only invaded murine pancreatic tissue, but also circulated through the portal and systemic circulatory systems to form distant metastatic lesions.

Orthotopic PDX Tumors Induce Muscle Wasting in Murine Hosts

In a survey of patients with advanced cancer, Sun et al. noted that cachexia occurred most frequently in PC patients at an incidence greater than 80%.⁶ However, despite the presence of 2 cm tumors at endpoint, the flank PDX model of PC poorly recapitulates a cachectic phenotype in our hands. We reasoned that, given the same parent tumor, a reliable model of cancer cachexia should demonstrate muscle wasting in proportion to disease burden. To assess surrogates of cancer cachexia in the orthotopic PDX model, tibialis anterior (TA) muscles were harvested from each mouse at endpoint and, together with tumor-free body weights, were correlated to tumor size and metastatic burden. Indeed, in orthotopic PDX tumors from a representative donor, we found the degree of TA muscle wasting was inversely proportional to increasing tumor weight (Pearson $r = -0.82$; $p = 0.0006$) (Fig. 6A) and metastatic burden ($r = -0.59$; $p = 0.035$) as measured by the number of micrometastatic lung lesions per high powered field (Fig. 6B). As expected, tumor-free body weight was proportional to TA weights, confirming that body weight loss in tumor-bearing mice was associated with global muscle wasting ($r = 0.81$; $p = 0.0007$) (Fig. 6C). Accordingly, tumor-free body weight loss also correlated with tumor weight ($r = -0.77$; $p = 0.002$) (Fig. 6D) and metastatic burden ($r = -0.57$; $p = 0.043$) (Fig. 6E). Finally, tumor weight was directly proportional to metastatic burden, further supporting the use of these parameters to estimate disease burden ($r = 0.59$; $p = 0.035$) (Fig. 6F). Taken together, these data suggest the orthotopic PDX model may recapitulate both the metastatic and cachexia-inducing behavior commonly observed in human PC.

Discussion

In this work we sought to develop a PDX model of PC that recapitulated critical local and systemic aspects of the human disease, namely a desmoplastic microenvironment, consistent metastatic spread and the onset of cancer cachexia. The flank PDX model accomplished some of these goals, demonstrating a fibrotic microenvironment similar to the original tumor. However, consistent metastasis and muscle wasting was not observed in this model despite the presence of large subcutaneous tumors. Using orthotopic transplantation of human PDX-derived tumors directly into the pancreas of NSG mice, we establish a xenograft model that maintains a fibrotic state, results in frequent clinically similar metastases and induces muscle wasting characteristic of the cancer cachexia syndrome.

The use of PDX models to predict therapeutic efficacy and personalize cancer treatments is expanding rapidly.^{12–14} While PDX models in PC have not yet resulted in novel therapies, a recent analysis by Thomas et al. demonstrated that PDX growth in a flank model could predict recurrence rate in patients with PC, suggesting some degree of clinical utility presently.¹⁵ However, we have previously demonstrated limitations to the flank PDX model¹⁰ and therefore aimed to develop methodology for an orthotopic model. Fu et al. noted the feasibility of orthotopic implantation in 1992, successfully transplanting five PC specimens into nude mice.¹⁶ Furukawa et al. further demonstrated that orthotopic implantation may be performed with a previously passaged flank PDX to generate larger groups of orthotopically implanted mice for experimentation¹⁷, which is the technique we have chosen to employ in our studies. Hiroshima et al. have recently incorporated the orthotopic PDX model to explore a variety of promising translational aims, including fluorophore-guided resection^{18,19}, genetically modified bacteria with anticancer activity^{20,21} and bisphosphonate therapy.²² Suetsugu et al. implanted patient-derived PC xenografts sequentially into nude mice expressing different fluorescent proteins to evaluate stromal infiltration, demonstrating recruitment of murine stromal elements as we have demonstrated in the flank model.^{10,23} The model presented here adds to the existing literature by detailing the surgical procedure such that any investigator may incorporate orthotopic PDX experiments into their work. Further, we demonstrate for the first time the consistent relationship between tumor growth, systemic metastasis and the onset of cancer cachexia.

It has not escaped our attention that the congenitally athymic (nude) mouse has been exclusively utilized in the aforementioned orthotopic PDX investigations. However, this strain possesses some degree of T cell functionality, leading to potential inconsistencies in engraftment and subsequent growth rates.²⁴ In fact, the use of this strain by Stutman et al. in the 1970s resulted in the false conclusion that T cells had no effect on the development of cancer^{25,26}, which was not rectified until the early 2000s with the development of RAG2 deficient mice.²⁷ Here we demonstrate that engraftment, metastasis and muscle wasting occurs in the NSG mouse, a cousin of the NOD-SCID with an IL2R knockout.^{28,29} These mice demonstrate a complete lack of T cell, B cell and natural killer cell function. Compared to other immune deficient strains, engraftment of human cells was recently shown to be most efficient in the NSG and these mice have largely provided the foundation for current humanized models.^{28,30} This work therefore establishes an orthotopic PDX model that may

efficiently be transitioned into a humanized model for investigations into immunotherapeutic approaches.

Similar to findings reported in a PDX model of breast cancer developed by DeRose et al., orthotopic transplantation resulted in metastases to sites similar to those observed in patients with breast cancer, including consistent spread to the lungs and a less frequent rate of metastasis to the liver.³¹ Of importance is our observation that our model resulted in universal lung metastasis but rarely liver metastasis, which is not typically observed in human PC. This finding may represent poor tropism of this particular tumor to the liver, as this PDX originated from a distal pancreatic tumor that demonstrated local recurrence rather than liver metastasis. Alternatively, the lack of hepatic metastasis may represent a technical complication. Suture placement may disrupt the splenic vasculature posterior to the pancreas, which would compromise the portal venous route to the liver commonly thought to carry metastatic PC cells. Metastatic patterns of orthotopic PDX tumors from additional patients with PC is an active area of investigation in our laboratory and should provide further insights to this discrepancy between the orthotopic PDX and human disease.

Our work possesses several limitations. The engraftment of PDX tumors from a small sample set may not be representative of all patients with PC. Expansion of the orthotopic model to include specimens from a larger and diverse patient population will be necessary to confirm these findings. However, the data presented here provide a robust proof of concept for the orthotopic PDX model to better represent the common clinical features of PC. Another limitation is not unique to this PDX model, namely the sacrifice of tumor-immune interactions despite the use of human tissue. Indeed, the NSG mice lack any form of adaptive immunity and demonstrate deficient innate immune function, thought to represent a major limitation in a time when immune modulation is experiencing unprecedented efficacy in cancer treatment and translational research. This orthotopic PDX model does not help resolve this limitation. However, incorporation of immunocompetent hosts necessitates the use of murine PC cell lines. Despite the capacity to engineer known human driver mutations in the target organs these mice, resultant murine tumors do not recapitulate the genetic diversity or mutational burden of the human disease.³² Further, the opportunity to personalize treatments remains impossible with syngeneic murine studies. As a promising alternative, humanization of the immune system in NSG mice represents a rapidly advancing field, with data to suggest feasibility in PDX models.^{33,34} The development of this orthotopic PDX model in PC therefore represents an important achievement in parallel to the development of humanized models.

In conclusion, we demonstrate and report here a reproducible, orthotopic PDX model for PC that recapitulates critical aspects of the human disease. Orthotopic PDX tumors demonstrated a conserved desmoplastic microenvironment, pancreatic invasion and highly metastatic behavior. Orthotopic PDX tumors were further associated with the onset of cancer cachexia in tumor-bearing hosts that was directly proportional to systemic disease burden. Thus, findings from our model support the use of orthotopic implantation of patient-derived PC xenografts as more robust model and promising tool to advance translational PC personalized research.

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Figure 1.

Orthotopic implantation of patient-derived PC xenografts. (A) Flank PDX tumors were minced into 2 mm fragments. (B) The pancreas of an anesthetized NSG mouse was exposed. (C-G) 7-0 Prolene® suture was placed through the tumor fragment and then through the exposed pancreas. (H-J) The tumor fragment was tied to the exposed pancreas. (K) Abdominal contents with the attached tumor fragment were reduced. (L) Clips were used to close both the abdominal wall fascial layer and the skin.

Figure 2.

Sonographic analysis of orthotopic patient-derived PC xenografts. (A-B) Sagittal (A) and transverse (B) views of early orthotopic tumors taken four weeks postoperatively. (C-D) Transverse (C) and sagittal (D) views of intermediate orthotopic tumors taken eight weeks postoperatively. (E-F) Sagittal (E) and transverse (F) views of orthotopic tumors taken twelve weeks postoperatively. Abbreviations: T, tumor; L, liver; RK, right kidney; LK, left kidney.

Figure 3.

Gross analysis of orthotopic patient-derived PC xenografts. (A) Orthotopic PDX tumor at endpoint, demonstrating attachment to the pancreas medially (arrow). (B-C) Orthotopic PDX tumor demonstrating abutment of the spleen laterally (B, arrow) and a retained Prolene® suture from implantation (C, arrow). (D) Liver metastasis from an orthotopic PDX tumor (arrow indicates metastatic lesion). (E) Lung metastasis from an orthotopic PDX tumor (arrow indicates metastatic lesion).

Figure 4. Orthotopic patient-derived PC xenografts retain morphologic features of the original tumor. Hematoxylin and eosin (H&E) stains demonstrate four PC specimens (*top*) and their corresponding orthotopic PDX tumors (*bottom*). Scale bars indicate 300 μm.

Figure 5.

Orthotopic patient-derived PC xenografts maintain a desmoplastic microenvironment, invade into pancreatic parenchyma and metastasize systemically. (A) Hematoxylin and eosin (H&E) stain of an orthotopic patient-derived PC xenograft (gross tumor depicted in inset) demonstrating a desmoplastic microenvironment. (B-C) Histology of the primary orthotopic PDX tumor demonstrating (B) invasion and (C) eventual replacement of the pancreatic parenchyma. (D) H&E demonstrating invasion of the orthotopic PDX tumor into the duodenum. (E-G) Representative lung metastases observed in all mice bearing orthotopic PDX tumors. (H) Liver metastasis from a mouse with an orthotopic PDX tumor. Black scale bars indicate 200 μm . White scale bars indicate 300 μm .

Figure 6.

Orthotopic patient-derived PC xenografts demonstrate consistent muscle wasting in proportion to systemic disease burden. (A) Orthotopic PDX tumor weight was inversely proportional to tibialis anterior (TA) muscle weight. (B) TA weights were also inversely proportional to metastatic burden, defined as the number of lung metastases per high powered field (HPF). (C) TA weights were directly proportional to tumor-free body weight, defined as the weight of the mouse at the time of harvest minus the weight of the explanted tumor. (D-E) Tumor-free body weight was inversely proportional to both (D) tumor weight and (E) metastatic burden. (F) Metastatic burden was directly proportional to tumor weight. Correlations were performed using Pearson coefficients (r) and significance was considered for $p < 0.05$. *, $p < 0.05$.