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Patient-Derived Xenograft Models for Pancreatic Adenocarcinoma Demonstrate Retention of Tumor Morphology through Incorporation of Murine Stromal Elements

Daniel Delitto,* Kien Pham,† Adrian C. Vlada,* George A. Sarosi,*‡ Ryan M. Thomas,*‡ Kevin E. Behrns,* Chen Liu,† Steven J. Hughes,* Shannon M. Wallet,§ and Jose G. Trevino*

From the Departments of Surgery, Pathology,† and Periodontology and Oral Biology§ and the North Florida/South Georgia Veterans Health System,‡ Colleges of Medicine, Dentistry, and Public Health and Health Professions, University of Florida Health Science Center, Gainesville, Florida*

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Address correspondence to Jose G. Trevino, M.D., Department of Surgery, University of Florida College of Medicine, Shands Hospital, 1600 SW Archer Rd, Room R6116, Gainesville, FL 32610. E-mail: jose.trevino@surgery.ufl.edu.

Direct implantation of viable surgical specimens provides a representative preclinical platform in pancreatic adenocarcinoma. Patient-derived xenografts consistently demonstrate retained tumor morphology and genetic stability. However, the evolution of the tumor microenvironment over time remains poorly characterized in these models. This work specifically addresses the recruitment and incorporation of murine stromal elements into expanding patient-derived pancreatic adenocarcinoma xenografts, establishing the integration of murine cells into networks of invading cancer cells. In addition, we provide methods and observations in the establishment and maintenance of a patient-derived pancreatic adenocarcinoma xenograft model. A total of 25 histologically confirmed pancreatic adenocarcinoma specimens were implanted subcutaneously into nonobese diabetic severe combined immunodeficiency mice. Patient demographics, staging, pathological analysis, and outcomes were analyzed. After successful engraftment of tumors, histological and immunofluorescence analyses were performed on explanted tumors. Pancreatic adenocarcinoma specimens were successfully engrafted in 15 (60%) of 25 attempts. Successful engraftment does not appear to correlate with clinicopathologic factors or patient survival. Tumor morphology is conserved through multiple passages, and tumors retain metastatic potential. Interestingly, despite morphological similarity between passages, human stromal elements do not appear to expand with invading cancer cells. Rather, desmoplastic murine stroma dominates the xenograft microenvironment after the initial implantation. Recruitment of stromal elements in this manner to support and maintain tumor growth represents a novel avenue for investigation into tumor-stromal interactions. *(Am J Pathol 2015, 185: 1297–1303; http://dx.doi.org/10.1016/j.ajpath.2015.01.016)*

A recent analysis of phase 1 cancer trials conducted from 2001 to 2012 revealed an overall objective response rate in only 3.8% of patients.[1] At some point, most agents included in this analysis demonstrated efficacy in xenograft models derived from cancer cell lines. The poor predictive value of cancer cell lines *in vivo* has been directly investigated and largely attributed to genetic instability resulting from primary culture.[2,3] In contrast, patient-derived xenografts implant viable sections of cancer tissue directly into an immunocompromised host, thus avoiding the cell culture process altogether. In addition, patient-derived xenotransplantation demonstrates up to 10 times the success rate of cell line derivation from cancer specimens.[4,5] Therefore, patient-derived xenografts represent a greater proportion of human malignancies, demonstrate reliable genetic stability, and are more predictive of clinical outcomes.[6,7]

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In particular, reliable preclinical models are desperately needed in pancreatic cancer (PC). PC is the fourth leading cause of cancer death in the United States, projected to be second only to lung cancer by 2030.[8] Cytotoxic chemotherapy represents the major treatment modality in 80% of patients presenting with PC, extending survival to only 5 to 7 months,[9,10] and the addition of recently approved targeted therapies, such as erlotinib or nab-paclitaxel, only prolongs survival by an estimated 1 to 2 months.[11,12] Thus, annual death rates from PC continue to increase, underlining a global need for more representative preclinical models in the development of novel therapies.[13]

Notably, a small series investigating patient-derived xenografts in PC indicated a high degree of genetic stability when compared to the original PC specimen.[14] We sought to expand on this method in a cohort of 23 patients with PC, including two patients with metastatic lesions. Indeed, our results indicate that xenotransplantation of patient-derived PC specimens into immunocompromised mice successfully generated tumor grafts in most cases. Patient-derived xenografts retain morphological characteristics of the original PC specimen as well as metastatic potential from the implantation site. Furthermore, our results indicate that murine stroma is integrated into networks of expanding PC cells. Implications from this model are globally applicable to investigations into new pharmacological agents against PC, specifically agents that target tumor-stromal interactions.

Materials and Methods

Murine Xenograft Experiments

A viable 2 × 2-mm portion of tissue was immediately isolated from a surgically resected primary PC specimen with minimal ischemia time. PC tissue was then implanted subcutaneously into an 8-week-old female nonobese diabetic severe combined immunodeficiency mouse (Jackson Laboratory, Bar Harbor, ME). Xenografts were allowed to grow to a maximum diameter of 1.5 cm before passage. Herein, we define a passage as explantation of a PC xenograft and implantation into the flank of a new host. Tumor dimensions were measured three times per week using calipers. Tumor volumes were calculated using the following equation: $v = (xy)^2/2$, where v is volume, x is tumor length, and y is tumor width. Final growth rate was determined using the amount of time taken to reach 1.5 cm in maximum diameter. Histological analysis of specimens was performed using hematoxylin and eosin staining.

Cells were isolated from the blood of tumor-bearing mice by cardiac puncture and subjected to purification using a Ficoll Paque gradient (Sigma-Aldrich, St. Louis, MO). Live cells were then cultured in RPMI 1640 medium, 10% fetal calf serum, 20 ng/mL epithelial growth factor, and antibiotic antimycotic solution (Sigma-Aldrich) for 2 months before fixation and staining.

Patient Data and Statistical Analysis

A review of an institutional review board–approved, prospectively maintained PC database at the University of Florida (Gainesville) was performed. Clinicopathologic data were analyzed for patients who underwent pancreatic resection for PC. All statistical analysis was performed using SPSS version 22.0 (IBM SPSS Statistics for Windows; IBM Corp., Armonk, NY). All clinical data were tested for normality using the Shapiro-Wilk test and displayed nonparametric distributions ($P < 0.05$). U-tests and χ^2 coefficients were, therefore, used to determine significance between groups for continuous and categorical variables, respectively. Kaplan-Meier survival curves and a Cox proportional hazards model examined the respective effects of successful engraftment and xenograft growth rate on overall survival. The log-rank test was used to determine survival differences between groups in Kaplan-Meier analysis. $P < 0.05$ was considered statistically significant.

Antibodies and Immunofluorescence Analysis

Immunohistochemical staining of tissue specimens was performed by the University of Florida's Molecular Pathology Core Facility. Briefly, patient tumors, xenografts, and murine organ specimens were analyzed in formalin-fixed, paraffin-embedded sections (5 μm thick). Hematoxylin and eosin stains were performed on all specimens, and subsequent stains were performed in serial sections (5 μm thick). Primary antibodies against human-specific vimentin and human leukocyte antigen (HLA)-A (Abcam, Cambridge, UK) were used after antigen retrieval with citrate buffer (pH 6.0) in tissue immunohistochemical analysis. Immunocytochemistry was similarly performed after methanol fixation of cells in culture.

Results

Patient-Derived PC Xenografts Demonstrate Reliable Growth Patterns in Immunocompromised Mice

In total, 15 (60%) of 25 patient-derived PC specimens were successfully engrafted subcutaneously into nonobese diabetic severe combined immunodeficiency mice. Wounds healed by postoperative day 7. Tumor invasion into muscular tissue was typically observed by week 2, palpable growth was observed by week 3, and growth end point (1.5 cm) was reached within 8 to 16 weeks (Figure 1A). Because each xenograft represents a specific patient, we hypothesized that clinically significant markers would correlate with successful engraftment and subsequent growth kinetics. Interestingly, all examined clinicopathologic parameters displayed no correlation with successful engraftment or PC xenograft growth rate (Tables 1 and 2 and Figure 1B). Accordingly, neither successful engraftment nor growth rate correlated with overall patient survival (Figure 1C). Although the

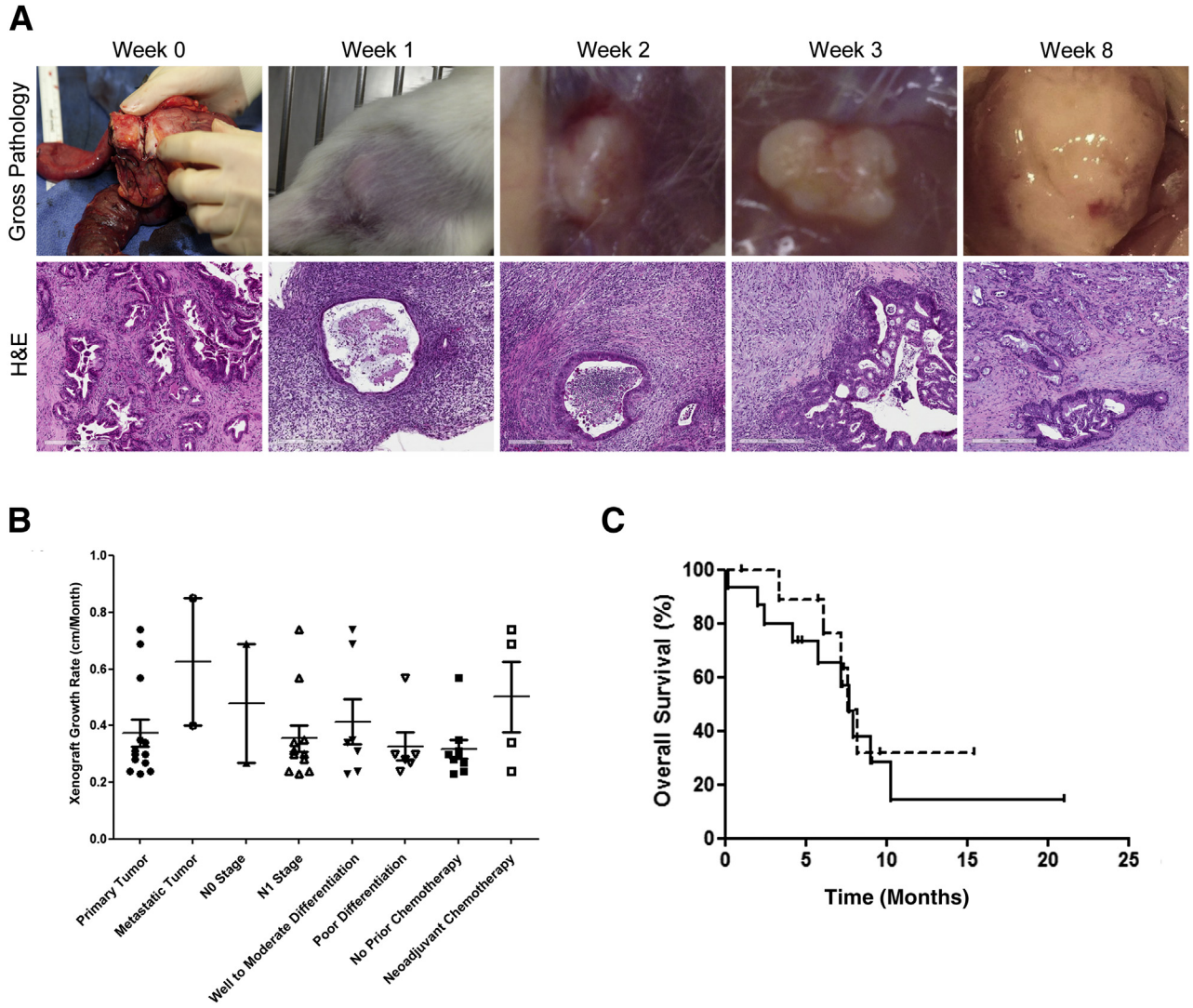

Figure 1 Patient-derived pancreatic cancer (PC) xenografts demonstrate rapid growth on subcutaneous implantation into immunocompromised mice. **A:** PC engraftment process is shown chronologically with representative histological correlates at the time of surgical resection and implantation (week 0), weeks 1 through 3, and growth end point (week 8). The subcutaneous wound heals by week 1, and malignant invasion of murine tissues is present as early as week 2. **B:** Clinicopathologic parameters do not correlate with xenograft growth rate. Tumor site, stage, grade, and neoadjuvant chemotherapy were assessed for effects on xenograft growth rate (cm per month) among successfully engrafted PC specimens. Statistical comparisons were performed using the Wilcoxon rank sum test. **C:** Successful engraftment does not correlate with patient survival. Patients were followed up after engraftment, and Kaplan-Meier survival curves were generated (median ± SEM). Patient survival was similar between both failed (dashed line) and successful (solid line) engraftment groups (median overall survival, 7.6 ± 0.6 versus 7.7 ± 0.6 months; $P = 0.60$). In addition, a Cox proportional hazards model reveals no correlation between xenograft growth rate (cm/month) and survival (hazards ratio, 0.63; $P = 0.77$). $n = 15$ (**B**). Original magnification, ×10 [hematoxylin and eosin (H&E) images, **A**]. Scale bars: 300 μm (**A**).

precise conclusions from these data are limited, we speculate that successful PC engraftment is likely dependent on technical, rather than patient, factors. Specifically, the reduction of tumor ischemia time to an absolute minimum is critical to successful engraftment.

Patient-Derived PC Xenografts Retain Tumor Morphology and Metastatic Capacity in Murine Tissue

Patient-derived PC xenografts maintained architectural characteristics of the original PC specimen after continuous passaging. Our results demonstrated retention of tumor morphology, specifically with regard to tumor differentiation and glandular formation (Figure 2A). Desmoplastic stromal elements persisted through multiple passages of PC xenografts into new mice. We next asked whether human tumor cells circulated systemically in mice bearing patient-derived PC xenografts. To address this, cells were isolated from the blood of tumor-bearing mice and cultured. Subsequent immunofluorescence analysis revealed positive human HLA-A staining, indicating that these circulating cells were of human origin (Figure 2B). In addition, the metastatic potential of PC xenografts is demonstrated in this model, which is similarly maintained

Table 1 Clinicopathologic Parameters Do Not Correlate with Successful Tumor Engraftment

Parameter	Successful engraftment		*P* value
	No ($n = 10$)	Yes ($n = 15$)	
Age, years*	67.2 ± 3.8	67.2 ± 2.6	0.765
Tumor location†			
Pancreas	10 (100)	13 (87)	0.500
Hepatic metastasis	0	2 (13)	
N1 stage†	8 (80)	11 (85)	1.00
Positive lymph node ratio*	0.22 ± 0.07	0.14 ± 0.03	0.522
Poor tumor differentiation†	4 (40)	6 (46)	1.00
Tumor size (cm)*	3.05 ± 0.44	3.59 ± 0.27	0.563
CA 19-9 (U/mL)*	1514 ± 994	1437 ± 775	0.546
Neoadjuvant therapy†	2 (20)	4 (31)	0.660
R1 resection†	4 (40)	5 (39)	1.00

Clinicopathologic variables were assessed for effects on successful pancreatic cancer engraftment using χ^2 coefficients for categorical variables or the Wilcoxon rank sum test for continuous variables because of nonparametric distributions.

*Data are given as means ± SEM.

†Data are given as number (percentage).

through multiple passages. We have observed hepatic, pulmonary, and splenic metastases in mice bearing patient-derived xenografts, confirmed by hematoxylin and eosin examination (Figure 2C). This model, therefore, incorporates desmoplastic architectural features generated from tumor-associated stromal elements in the microenvironment. Furthermore, human cells originating in these tumors circulate systemically and metastasis continues to occur in murine tissue. More important, this phenomenon was not observed in all patient-derived PC xenografts. Metastatic patterns in murine tissue are displayed for xenografts derived from six different patients with PC (Table 3). Subcutaneous human PC tumors display a propensity to metastasize to pulmonary tissue in immunosuppressed mice. However, our preliminary observations demonstrate no specific correlation between clinicopathologic parameters and metastasis in murine tissue.

Murine Stromal Elements Are Incorporated into Expanding Patient-Derived PC Xenografts

Tumor-stromal interactions in the microenvironment represent a current focus in PC. We, therefore, asked whether stromal incorporation into expanding PC xenografts was of human origin. To answer this question, we incorporated immunohistochemical and immunofluorescence staining using human-specific antibodies. Each method reveals that human stromal elements actively produce vimentin in PC specimens (Figure 3, A and E). However, on incorporation into a PC xenograft, a transition area is consistently observed surrounding a 2-mm core of tissue representing the implanted PC specimen. Positive staining for human-specific vimentin diminishes in this area (Figure 3, B–D). Peripheral sampling of a PC xenograft confirms the presence of human HLA-A containing PC cells and the absence of human-specific vimentin-positive stroma (Figure 3F). Taken together, these results indicate that PC xenografts incorporate murine stromal elements. Therefore, despite retention of tumor-stromal morphology, stromal elements in continuously passaged patient-derived PC xenografts are actually of murine origin.

Discussion

We sought to examine the feasibility of patient-derived PC xenotransplantation and its potential for wider application in preclinical investigations. We describe initial results with respect to patient-derived PC xenograft morphology, metastatic ability, and incorporation of murine cellular components. Our data indicate that these xenografts are an effective method of investigating a representative population of PC specimens. We demonstrate that xenografts maintain the architecture of the original tumor, particularly with respect to desmoplastic elements that are common in PC. Remarkably, murine stromal elements are incorporated into expanding xenografts, representing an important consideration for further preclinical work with this model.

It is important to recognize that patient-derived xenografts have been extensively characterized in other models. In fact, success rates of up to 70% to 100% have been reported in multiple series.[4,5,14] This leads us to conclude that successful engraftment in PC is likely more dependent on technical, rather than clinicopathologic, factors, particularly the reduction of ischemia time to an absolute minimum. Regarding PC, replacement of human stroma has been suggested by species-specific genomic analyses.[7,15] This work demonstrates the replacement of human stroma by murine elements, because these elements are mostly undetectable beyond the original implanted specimen. Interestingly, desmoplastic elements are retained in continuously passaged xenografts, indicating that an important component of tumor-stromal interactions may not be species specific.

Our work contrasts with the work of other groups in that we demonstrate metastatic potential from subcutaneous patient-derived xenografts.[4,16] It is important to

Table 2 Among Successfully Engrafted Tumors, Clinicopathologic Parameters Do Not Correlate with Xenograft Growth Rate

Parameter	Correlation (Spearman's ρ)	*P* value
Age (years)	−0.254	0.362
Positive lymph node ratio	−0.008	0.979
Tumor size (cm)	−0.025	0.936
CA 19-9 (U/mL)	0.284	0.326

Age, positive lymph node ratio, tumor size, and preoperative CA 19-9 were compared with xenograft growth rate (cm/month growth in maximum diameter) among successfully engrafted xenografts ($n = 15$) using Spearman's rank order correlation.

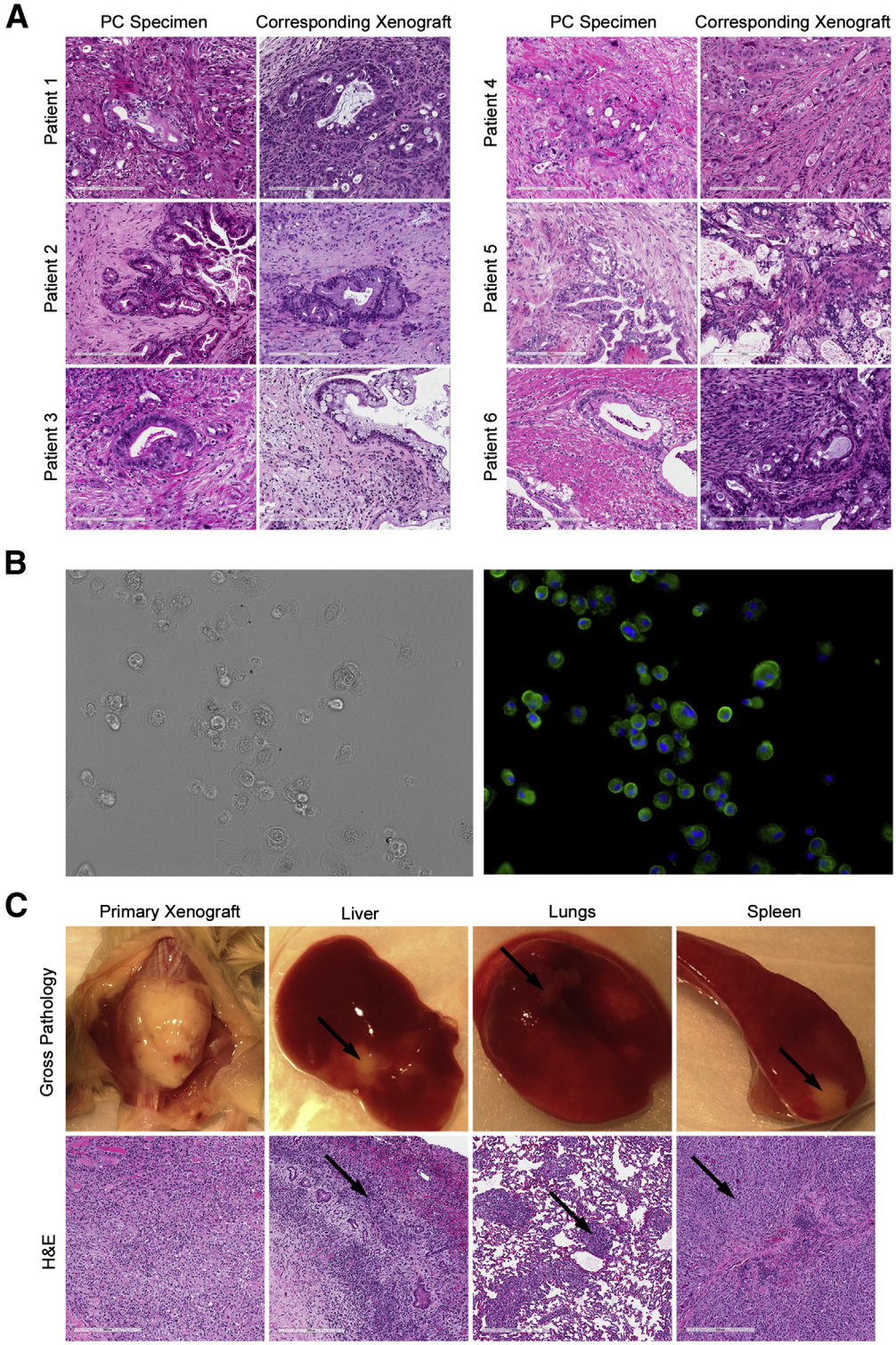

Figure 2 Patient-derived pancreatic cancer (PC) xenografts retain tumor morphology and metastatic ability. **A:** Hematoxylin and eosin (H&E) analysis of six separate PC specimens and corresponding patient-derived xenografts reveals maintenance of tumor differentiation and perpetuation of the desmoplastic tumor microenvironment. Notably, tumors maintain their differentiation on engraftment and passage in mice. **B:** Human cells circulate in the blood of mice bearing patient-derived PC xenografts. Immunofluorescence analysis of cells isolated from the blood of tumor-bearing mice and cultured cells reveals the expression of human leukocyte antigen-A (green), indicating the human origin of cultured cells. Nuclei are stained with DAPI (blue). **C:** A subcutaneous, patient-derived, PC xenograft was explanted after 2 months of growth. Postmortem laparotomy and thoracotomy reveal gross metastatic disease to the liver, lungs, and spleen. Tumor metastasis was confirmed by H&E analysis. **Arrows** indicate metastatic foci. Original magnifications: ×20 (H&E images, **A**); ×10 (**C**). Scale bars: 200 μm (**A**); 300 μm (**C**).

Table 3 Metastatic Patterns of Patient-Derived PC Xenografts from Six Patients

Patient no.	Stage	Differentiation	Neoadjuvant chemotherapy	Overall survival (months)	Metastatic sites in mouse
1	T3N0M0	Moderate to poor	No	7.6	None
2	T3N1M0	Moderate	No	Alive at 15 months	None
3	T3N1M0	Moderate to poor	No	4.1	None
4	T3N1M0	Poor	No	2.4	Lungs
5	T3N1M0	Moderate	Yes	Alive at 7 months	Lungs, liver, and spleen
6	T3N1M1	Poor	No	7.9	Lungs

Xenografts from six different patients were examined for metastatic patterns. Organs were harvested from mice bearing tumors at size end points and analyzed via hematoxylin and eosin examination. Clinicopathologic characteristics of each patient are displayed as well as metastatic patterns of xenografts.
PC, pancreatic cancer.

recognize that these cases typically displayed direct invasion into musculoskeletal tissue. However, we have observed systemically circulating human cells and metastatic spread to multiple intra-abdominal organs in the absence of peritoneal extension of the primary tumor. Notably, subcutaneous xenografts have been widely criticized due to a lack of observed metastatic potential. However, in contrast to direct orthotopic implantation into the murine pancreas, subcutaneous xenografts provide major advantages with respect to the technical expertise required and ease of monitoring, enabling a wider application and higher throughput. Our findings reinforce the validity of subcutaneous xenografts as a preclinical model.

In summary, we demonstrate that the generation of a patient-derived PC xenograft model is feasible with a high rate of successful engraftment. We additionally reveal two important considerations for future preclinical investigations. First, murine stromal elements are incorporated into expanding xenografts early after implantation. Second, PC xenografts demonstrate metastatic potential from implantation sites. These findings support the investigations of

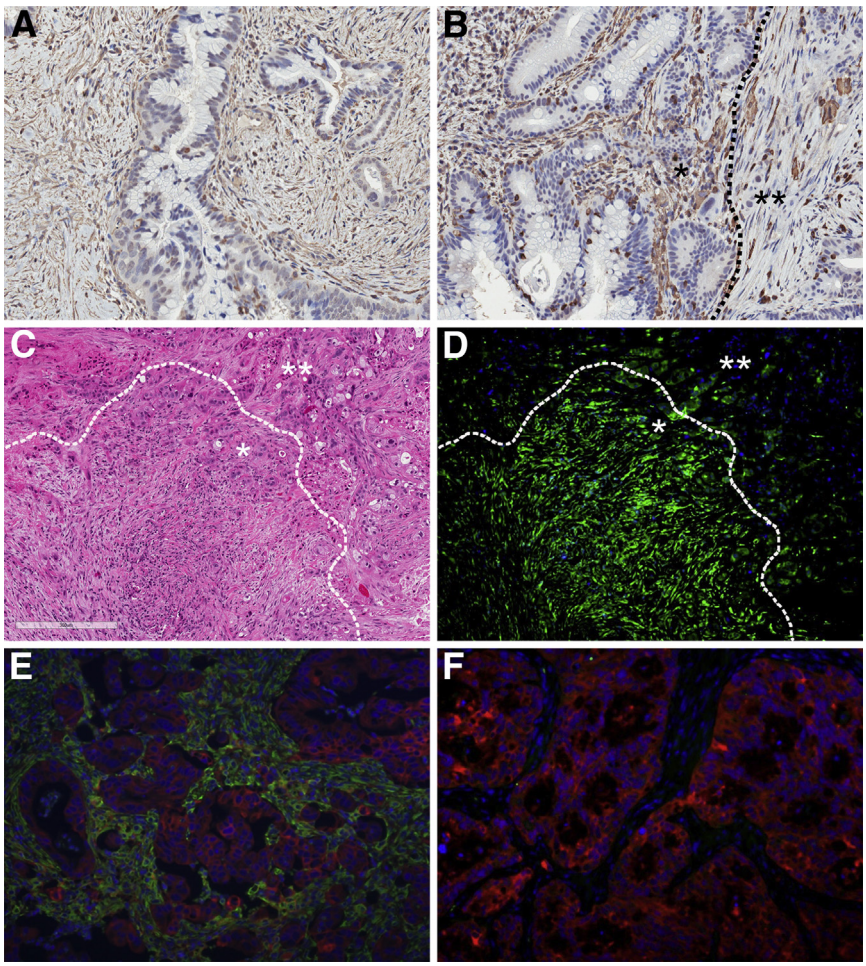

Figure 3 Murine stromal elements are incorporated into expanding patient-derived pancreatic cancer (PC) xenografts. **A:** Immunoperoxidase staining for human-specific vimentin demonstrates diffuse stromal staining in a representative human PC specimen. **B:** The resultant PC xenograft reveals conserved human stroma in the original 2-mm core of tissue representing the implanted human PC specimen. Human-specific vimentin staining diminishes significantly beyond the initial xenograft invasion front (**black dotted line**). **C:** Hematoxylin and eosin staining of a separate patient-derived xenograft demonstrates continued desmoplasia at the invasion front (**white dotted line**). **D:** A serial section stained for human vimentin (green) again demonstrates relatively few human stromal cells beyond the initial invasion front (**white dotted line**). **E:** Similarly, dual immunofluorescence staining for human-specific vimentin (green) and human leukocyte antigen (HLA)-A (red) demonstrates diffuse HLA-A staining and stromal-specific human vimentin staining in a PC specimen. **F:** Conversely, peripheral sampling of the corresponding PC xenograft reveals human cancer cells displaying HLA-A and undetectable human-specific vimentin staining. **Single asterisks** indicate original PC implants; **double asterisks**, invasive fronts of implanted PC specimens. Scale bar: 300 μm (**C**).

establishing patient-derived tumor xenografts as an emerging preclinical model in PC.

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