Development of an orthotopic model of invasive pancreatic cancer in an immunocompetent murine host

William W. Tseng 1,2*, Daniel Winer 1*, Justin A. Kenkel 1, Okmi Choi 1, Alan H. Shain 1, Jonathan R. Pollack1, Randy French3, Andrew M. Lowy 3, Edgar G. Engleman 1

1Department of Pathology, Stanford University, 3373 Hillview Avenue, Palo Alto, CA 94304
2Department of Surgery, University of California at San Francisco, 513 Parnassus Avenue, Room S-321 San Francisco, CA 94143-0470
3Department of Surgery, Division of Surgical Oncology, University of California at San Diego, Moores Cancer Center, 3855 Health Sciences Drive, ML0987, La Jolla, CA 92093-0987

*These authors contributed equally to this work

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REPRINTS REQUEST: Edgar G. Engleman, M.D., Stanford Blood Center, 3373 Hillview Avenue, Palo Alto, CA 94304; e-mail: edengleman@stanford.edu

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Immunocompetent Mouse Model of Pancreatic Cancer
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STATEMENT OF TRANSLATIONAL RELEVANCE

The most common preclinical models of pancreatic adenocarcinoma utilize human cells or tissues that are xenografted into immunodeficient hosts. In these models, the tumor microenvironment bears little resemblance to the natural milieu in which tumors typically develop and metastasize in humans. Several immunocompetent, genetically engineered mouse models of pancreatic cancer exist; however, tumor latency and disease progression in these models are highly variable. Using tumor cells obtained from mice with targeted Kras and p53 mutations, we have developed a rapid and predictable orthotopic model of invasive pancreatic cancer in immunocompetent mice. The tumors that develop in this model mimic the human disease histologically and recapitulate important clinical features of disease progression. This model may further our understanding of the biology of pancreatic cancer and prove useful for the preclinical evaluation of new therapeutic agents.
Immunocompetent Mouse Model of Pancreatic Cancer

ABSTRACT

**Purpose.** The most common preclinical models of pancreatic adenocarcinoma utilize human cells or tissues that are xenografted into immunodeficient hosts. Several immunocompetent, genetically engineered mouse models of pancreatic cancer exist; however, tumor latency and disease progression in these models are highly variable. We sought to develop an immunocompetent, orthotopic mouse model of pancreatic cancer with rapid and predictable growth kinetics.

**Experimental Design.** Cell lines with epithelial morphology were derived from liver metastases obtained from Kras$^{G12D/+;LSL-Trp53^{R172H/+};Pdx-1-Cre}$ mice. Tumor cells were implanted in the pancreas of immunocompetent, histocompatible B6/129 mice, and the mice were monitored for disease progression. Relevant tissues were harvested for histological, genomic and immunophenotypic analysis.

**Results.** All mice developed pancreatic tumors by 2 weeks. Invasive disease and liver metastases were noted by 6-8 weeks. Histological examination of tumors demonstrated cytokeratin-19-positive adenocarcinoma with regions of desmoplasia. Genomic analysis revealed broad chromosomal changes along with focal gains and losses. Pancreatic tumors were infiltrated with dendritic cells, myeloid-derived suppressor cells, macrophages and T lymphocytes. Survival was decreased in RAG$^{-/-}$ mice, which are deficient in T cells, suggesting that an adaptive immune response alters the course of disease in wild-type mice.

**Conclusions.** We have developed a rapid, predictable orthotopic model of pancreatic adenocarcinoma in immunocompetent mice that mimics human pancreatic cancer with regard to genetic mutations, histological appearance and pattern of disease progression. This model highlights both the complexity and relevance of the immune response to invasive pancreatic cancer and may be useful for the preclinical evaluation of new therapeutic agents.
INTRODUCTION

Ductal adenocarcinoma of the pancreas has the worst prognosis of all common epithelial malignancies, with an overall 5-year survival rate of less than 5% (1). Surgical resection offers the only hope for cure; however, less than 20% of patients are eligible for surgery and most patients eventually develop recurrence following resection (2). Gemcitabine and, more recently, Erlotinib, an epidermal growth factor-receptor tyrosine kinase inhibitor, are the only FDA-approved agents used in the treatment of advanced pancreatic cancer. Unfortunately, these therapies typically prolong survival by only a few months and all patients ultimately succumb to progressive disease (2). The availability of appropriate animal models is therefore essential for the preclinical evaluation of novel therapeutic agents.

Most preclinical mouse models of pancreatic adenocarcinoma utilize immunodeficient hosts into which resected human tissues or established human cell lines (e.g. MiaPaCa2, PANC-1, BxPC3) are xenografted (3,4). A major drawback of these models is the lack of an intact immune system in the host animal, which alters the tumor microenvironment and creates potentially significant differences from the human disease. This may in part explain the poor track record of immunodeficient models in predicting response to therapy in patients with pancreatic cancer (2). Immune cells that infiltrate tumors significantly impact the course of tumor growth and progression (5). Infiltrating immune cells have the potential to mount an effector response to limit tumor growth; however, this response is often overcome by a combination of multiple factors, including the release of immunosuppressive cytokines by the tumor (e.g. IL-10) and the recruitment of immunosuppressive cell types (e.g. Gr-1+, CD11b+ myeloid-derived suppressor cells, tumor-associated macrophages and FoxP3+ regulatory T cells) (5). Immune cells can also influence the formation and maintenance of tumor-supporting stroma, which in the case of pancreatic cancer can make up the bulk of the tumor and affect tumor resistance to chemotherapy and radiation therapy (6). Immunocompetent rat and hamster models of pancreatic cancer have been reported (7,8); however, compared to the mouse, few reagents are available for identifying and isolating immune cells from these species.
Immunocompetent Mouse Model of Pancreatic Cancer

In 2003, Hingorani et al. described a genetically engineered mouse model of pancreatic cancer in which expression of oncogenic Kras-G12D was limited to pancreatic tissue (9). The authors utilized a pancreas-specific promoter (Pdx-1) to drive expression of Cre-recombinase, which recognizes and excises a Lox-flanked stop repressor element upstream of the mutant Kras-G12D allele. As a result, these mice express mutant Kras-G12D driven by its native promoter resulting in the gradual development of pancreatic intra-epithelial neoplasia (PanIN) that slowly progresses to invasive ductal adenocarcinoma (9). In 2005, the authors reported that progression to pancreatic cancer could be accelerated in mice by using the same Pdx-1-driven, Cre-Lox system to simultaneously induce expression of Kras-G12D and a mutant form of the p53 tumor suppressor gene (R172H) (10). 96% of these mice, designated LSL-KrasG12D+/-;LSL-Trp53R172H+/+;Pdx-1-Cre, developed pancreatic cancer and 63% were noted to have liver metastases (10); however, the time to the development of invasive disease varied widely, from 47 to 355 days (10). Tumor cells derived from liver metastases found in these mice were initially used to develop cell lines for mutational analysis (10). These cells manifested mixed morphology in culture: some cells were predominantly epithelioid while others appeared mesenchymal. Utilizing subclones with pure epithelial morphology (hereafter referred to as LM-P), we have developed and characterized an immunocompetent, orthotopic mouse model of pancreatic cancer in which disease develops consistently and with rapid and predictable growth kinetics.

MATERIALS AND METHODS

Animals. Eight to ten-week-old immunocompetent female B6/129 mice, histocompatible (H-2b) with the tumor cells used in this study, were obtained from Jackson Laboratories (Bar Harbor, ME). Age-matched, immunodeficient female RAG-/- mice on the identical hybrid background were also obtained from Jackson. All procedures were approved by the Institutional Animal Care and Use Committee of Stanford University.
Immunocompetent Mouse Model of Pancreatic Cancer

Isolation of LM-P Cells from Liver Metastases and In Vitro Culture. Metastatic tumors were isolated from the livers of Kras^{G12D/+};LSL-Trp53^{R172H/+};Pdx-1-Cre mice and cell lines were derived as previously described (10). Cells were grown in DMEM (high glucose 4.5g/L) supplemented with 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, 1X non-essential amino acids (Gibco), and 1X Pen/Strep (100 U/ml Penicillin, 100 ug/ml Streptomycin). Cells were plated at low density (~50,000 cells/100 mm tissue culture dish (Fisher)) in the above growth medium. Individual colonies displaying uniform epithelial morphology were cloned and subcultured. Individual colonies were then recloned for two more rounds.

Purified cells (LM-P) were amplified in T75 flasks and vials of cells were stored at -125 C. In preparation for in vivo implantation, frozen cells were thawed and cultured in growth medium at 37 C in a humidified, 10% CO₂ incubator. Subconfluent cells were detached with 0.25% trypsin and cell viability was assessed by trypan blue exclusion. LM-P cells were tested every 6 months for microbial contamination (including Mycoplasma) using a polymerase chain reaction-based analysis and found to be negative.

Tumor Implantation and Monitoring. To establish subcutaneous tumors, 10⁶ LM-P cells were resuspended in Hanks Balanced Salt Solution (HBSS) and injected under the skin overlying the right flank. To establish orthotopic tumors, mice were first anesthetized with a single intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and then the pancreas was exposed through an abdominal incision (laparotomy). Two techniques were used for orthotopic tumor implantation: 1) direct injection of a single cell suspension of LM-P cells into the pancreas or 2) transplantation of a subcutaneous LM-P tumor fragment onto the pancreas. For direct injection, 10⁶ cells were resuspended in 50 uL of HBSS and injected into the pancreas using a 30G needle (BD). Leakage occurred in less than 5% of mice, and only mice without leakage during injection were included in experiments. For subcutaneous tumor transplantation, 1 cm tumors were harvested and minced into 3-4 mm fragments. One tumor fragment was secured to the pancreas using 7-0 Prolene suture. After tumor implantation (with either technique), the pancreas was carefully returned to the peritoneal cavity and the abdomen was closed with 4-0 Vicryl suture. After tumor implantation, all mice were monitored at least twice weekly for disease.
progression by abdominal palpation and for overall signs of morbidity such as ruffled fur, hunched posture and immobility. Moribund mice were euthanized by CO₂ inhalation. For survival studies, mice were followed until death or euthanized by a blinded observer when signs of morbidity were evident.

**Tumor Progression.** A separate cohort of mice that had undergone either LM-P cell injection (n = 3) or LM-P tumor fragment transplantation (n = 3) underwent repeat laparotomy to directly measure tumor size at 2, 4, and 6 weeks after implantation. Tumor volume was calculated based on the formula for ellipsoid volume, \( V = \frac{4}{3} \pi \frac{(a/2)(b/2)(c/2)} \) where a, b, and c represent the maximal length, width, and height, in millimeters, respectively.

**Tumor Implantation using PDA Cell Lines.** PDA1-1 and PDA3-5 cells were derived from primary tumors found in the pancreas of Kras<sup>G12D</sup>+/;LSL-Trp53<sup>R172H</sup>+/;Pdx-1-Cre mice, using a similar protocol for isolation, purification and microbial testing as described above for LM-P cells. Orthotopic tumor implantation into immunocompetent B6/129 mice was performed and disease progression and tumor histology were assessed.

**Histological Analysis.** Pancreatic tumors, livers and lungs were harvested from mice at 3 and 6 weeks after orthotopic tumor implantation. Tissues were placed in 10% phosphate-buffered formalin and 24-72 hours later embedded in paraffin. 4-5 μm sections were dewaxed in 3 changes of xylene and brought to water through graded alcohols. Representative sections were stained with H&E. For immunohistochemistry, sections were antigen-retrieved in rodent decloaker antigen retrieval solution (Biocare Medical) inside of a pressure cooker. Peroxidase activity was blocked using 3% hydrogen peroxide (Lab Vision). Sections were treated for 20 minutes with serum-free protein blocker (Dako), then incubated for 2 hours with polyclonal rabbit anti-CK19 (AbCam) at 1/200, followed by detection using a Rabbit on Rodent Polymer detection kit (Biocare Medical), which consists of an anti-rabbit HRP-conjugated polymer, for 30 minutes. Color was developed with DAB solution (Vector Labs), and sections were mounted with Faramount aqueous mounting medium (Dako). Microscopic images were taken with a V2.4 Nuance Multispectral Imaging VIS Flex Camera system (CRI) using an Olympus.
BX51 microscope (Olympus America Inc.). Magnification for all images was recorded at the eyepiece with a 10X ocular objective.

**Comparative Genomic Hybridization.** Tumor genomic DNA was isolated using a Qiagen DNA/RNA Allprep Mini kit. Array comparative genomic hybridization (aCGH) was performed according to the manufacturer’s protocol on catalog Agilent 44K murine whole genome arrays. Briefly, 3 µg of genomic DNA from each test sample was random primer-labeled with Cy5 and co-hybridized to the microarray along with 3 µg of Cy3-labeled normal pancreas reference DNA from syngeneic mice. Tumor/normal log2 ratios were extracted using Agilent software (CGH Analytics), and gains and losses were called using the circular binary segmentation algorithm (11).

**Immune Cell Isolation and Surface Marker Staining.** Pancreatic tumors were minced using a sterile scalpel blade and single cell suspensions were generated using a combination of enzymatic digestion (600 U/mL collagenase IV, Worthington) and mechanical dissociation (GentleMACS Tissue Homogenizer, Miltenyi Biotec). Tumor debris was removed using 70- and 100-micron filters (BD Falcon). Peri-pancreatic draining lymph nodes were harvested and processed into single cell suspensions by dissociation over filters. Pancreas and draining lymph nodes from tumor-naïve mice were used as controls for comparison with tissue obtained from tumor-bearing mice. Cells from all tissues were washed with Phosphate Buffered Saline (PBS) containing 2% FBS, Fc blocked (1:50 dil, BioLegend), and then stained using a cocktail of anti-mouse antibodies against CD45.2 (PacBlue or Alexa700); lineage markers – CD19, NK1.1, TCR (PE); B220 (PETxR); CD11c (PECy7); CD11b (PacBlue); Gr-1 (APCCy7); I-Ab (APC); CD3 (FITC); CD4 (PECy7); CD8 (Alexa700). All antibodies were purchased from BD Biosciences or BioLegend. Isotype-matched antibodies were used as negative controls when appropriate. Surface marker expression was determined by multi-color flow cytometry on an LSR II flow cytometer, and data were analyzed using FlowJo 8.7.1 software.

**Intracellular Staining for FoxP3.** Tumor-infiltrating T cells were identified with labeled antibodies to T cell surface markers and then permeabilized and fixed using the Mouse Regulatory T cell Staining Kit.
Immunocompetent Mouse Model of Pancreatic Cancer

Intracellular staining was done using anti-mouse FoxP3 (APC) versus isotype control antibody, as part of the Staining Kit.

Survival of Immunocompetent vs. Immunodeficient Mice. After establishing growth kinetics with $10^6$ and lower cell numbers, B6/129 (n = 6) and immunodeficient RAG-/- mice (n = 5) were orthotopically injected with $10^5$ LM-P cells and then monitored for survival.

Treatment of LM-P tumors with Gemcitabine and and LY364947. For evaluation of in vitro cytotoxicity, LM-P cells were first plated in quadruplicate ($2.5 \times 10^3$ cells/well) onto 96-well plates and allowed to grow for 24 h. Escalating concentrations of gemcitabine (courtesy of Clinical Pharmacy, Stanford Hospital) or a TGF-β receptor-1 kinase inhibitor (LY364947, Calbiochem) were then added and MTT assay (R&D Systems) was performed 48 h later, as per the manufacturer’s protocol. For in vivo studies, orthotopic tumor implantation was performed using later passaged (P8), more aggressive LM-P cells. At 2 weeks, mice with similarly sized pancreatic tumors (determined by palpation) were randomized to receive PBS, gemcitabine alone, or gemcitabine plus the TGF-β receptor-1 kinase inhibitor. Gemcitabine was given at 100 mg/kg by intraperitoneal (IP) injection on days 0,3,6, and 9 after randomization. The TGF-β receptor-1 kinase inhibitor was given at 1 mg/kg IP on days 0,2,4,6, and 8.

Statistical Analysis. Tumor volume comparisons were analyzed using the paired Student’s t-test. The frequency of tumor uptake and metastases between groups was analyzed using the Fischer exact test. Survival differences between groups were assessed with the log-rank test using GraphPad Prism 5.02 software. A p-value of <0.05 was considered statistically significant.

RESULTS

Pancreatic tumor uptake is uniform and disease progression occurs predictably. After either 1) injection of suspended LM-P cells into the pancreas or 2) implantation of a subcutaneous LM-P tumor fragment onto the pancreas, tumors were noted as early as two weeks and progressed predictably by four weeks (Figure 1A). Comparison of average tumor volumes at two and four weeks demonstrated no
Immunocompetent Mouse Model of Pancreatic Cancer

statistically significant differences, suggesting similar growth kinetics with either technique (Figure 1B).

By six weeks, tumors in some mice became difficult to measure owing to their extensive, locally invasive nature and poorly defined borders (Figure 1C, left). At this stage in disease progression, liver metastases were also frequently noted (Figure 1C, right) and mice appeared ill. Of note, some mice also developed biliary and gastric outlet obstruction during the course of disease progression (Figure 1D). By eight weeks, mortality was consistently 100% (data not shown).

In total, pancreatic tumors developed in 100% of mice following orthotopic implantation of LM-P cells (Table 1). Of these mice, 90% developed liver metastases while no mice developed peritoneal carcinomatosis or hemorrhagic ascites. No significant differences were noted in the frequencies of uptake and metastases with either technique. Lung metastases were noted in some mice, particularly those with more advanced disease; however, the frequency of metastasis to this organ was not systematically evaluated. Pancreatic tumors and liver metastases also developed with fewer than $10^6$ cells injected, but tumor volumes were initially smaller and disease progression occurred more slowly; in contrast, the use of cells passaged more frequently in vitro generated pancreatic tumors with more rapid disease progression (data not shown).

Pancreatic and metastatic tumor histology resembles human disease. Hematoxylin and eosin staining of representative LM-P pancreatic tumors demonstrated adenocarcinoma with high grade nuclear features, areas of necrosis and hemorrhage, and regions of desmoplasia (Figure 2A). Adjacent areas of poorly differentiated, solid sheets of cells were also noted, particularly in more advanced tumors. Cytokeratin-19, a marker expressed in most human pancreatic ductal adenocarcinomas, was expressed in tumors as demonstrated by immunostaining (Figure 2B). Glandular areas showed more consistent cytoplasmic CK-19 staining than poorly differentiated areas of the tumor. In many pancreatic tumors, perineural invasion was present (Figure 2C) and peri-pancreatic lymph nodes were often heavily infiltrated by tumor (Figure 2D). Histological examination of liver and lung metastases (Figures 2E and 2F, respectively) confirmed the presence of adenocarcinoma.
Immunocompetent Mouse Model of Pancreatic Cancer

Carcinomatosis and more aggressive disease progression occur when pancreatic tumors are generated from primary tumor cell lines. Orthotopic implantation of PDA1-1 and PDA3-5 cell lines, which were derived from primary pancreatic tumors (as opposed to liver metastases for LM-P) in the Kras^{G12D+};LSL-Trp53^{R172H+};Pdx-1-Cre mice, led to pancreatic tumor development in 100% of the mice (data not shown). Mice implanted with PDA1-1 cells had significantly worse survival compared to mice implanted with PDA3-5 or LM-P (Figure 1E). In contrast to LM-P implanted mice (Table), all of the PDA1-1 and PDA3-5 implanted mice developed extensive peritoneal carcinomatosis (Figure 1F) and only occasionally, small liver metastases. On histological analysis, PDA1-1 pancreatic tumors were poorly differentiated (Figure 2G) compared to PDA3-5 (Figure 2H) and LM-P (Figure 2A), which correlated with the survival data.

Comparative genomic hybridization on tumor cells from different sources. In order to characterize our tumor model at the genomic level, we performed array comparative genomic hybridization (aCGH) on in vitro propagated LM-P cells, a subcutaneous tumor derived from LM-P cells, and an orthotopic tumor derived from a transplanted subcutaneous tumor (Figure 3). The LM-P cells displayed several broad low level gains and losses along with one focal deletion on cytoband 4D1. It is possible that these genomic aberrations might cooperate with the Kras and p53 mutations to transform cells. The subcutaneous tumor mirrored the LM-P cells with the exception of modest gains on chromosome 5A1, 5G3, and 6A1. The orthotopic tumor genome shared some features with the LM-P cells and the subcutaneous tumor but also contained many new genomic aberrations, including further gains on the ends of chromosomes 5 and 6. Interestingly, among many other genes, Hepatocyte Growth Factor and its receptor, Met, reside in these regions on chromosome 5 and 6, respectively, which were further amplified with progression from in vitro cells to orthotopic tumors.

Immunophenotyping of tumor-infiltrating mononuclear cells reveals a complex immune response. Single cell suspensions obtained from processed LM-P pancreatic tumor and draining lymph nodes three weeks after orthotopic tumor implantation were analyzed by flow cytometry for surface marker expression. After gating in CD45^{+} hematopoietic cells and gating out lineage-positive (CD19, NK1.1,
TCR) cells, multiple expanded populations of CD11c- and CD11b-expressing cells were noted in pancreatic tumors compared to the pancreas of tumor-naïve normal mice (Figure 4A). Population 1 (CD11c-high, CD11b-negative) and population 2 (CD11c-high, CD11b-positive) were characterized as dendritic cells (DCs) as both populations also expressed high levels of MHC Class II (I-A<sup>B</sup>). A small proportion of cells in these two DC populations also co-expressed CD86, an activation marker and T cell co-stimulatory factor; however, the majority (80-90%) lacked CD86 expression. Population 3 (CD11c-negative/low, CD11b-positive) did not express either MHC Class II or CD86 (data not shown), but could be further subdivided into Gr-1 positive myeloid derived suppressor cells (MDSCs) and Gr-1 negative macrophages. Each of these three myeloid cell populations was also expanded, but to a lesser extent, in the adjacent tumor draining lymph nodes. In both the pancreatic tumors and draining lymph nodes, over two-thirds of the T cells (CD45+ CD3+) were CD4+ (Figure 4B). A proportion (>20%) of these cells expressed CD25 and intracellular FoxP3, the phenotype of regulatory T cells. A similar immune cell infiltrate consisting of DCs, MDSCs, macrophages, and regulatory T cells was noted in pancreatic tumor and draining lymph nodes as early as 1.5 weeks after orthotopic tumor implantation (data not shown). When compared to normal control tissues, no consistent changes in NK or B cell populations were found in either tumor or lymph nodes at 1.5 or 3 weeks (data not shown).

Impact of an adaptive immune response on survival. When LM-P tumor cells were orthotopically implanted into RAG-/- mice, which lack T and B cells, these mice demonstrated more rapid disease progression and, overall, significantly decreased survival compared to fully immunocompetent B6/129 mice (Figure 4C).

Effect of gemcitabine and TGF-β receptor-1 kinase inhibiton on LM-P tumor growth. To evaluate the sensitivity of LM-P tumors to an agent with known activity against human pancreatic cancer, we cultured these cells in the presence of gemcitabine. We also studied the effect of a TGF-β receptor-1 kinase inhibitor, LY364947, based on recent studies indicating a role for TGF-β in the growth of pancreatic tumors and development of metastases (12,13). In vitro, gemcitabine resulted in significant cytotoxicity.
Immunocompetent Mouse Model of Pancreatic Cancer

against LM-P cells at all concentrations tested. In contrast, some cytotoxicity was noted with LY364947 only at higher concentrations tested (Figure 5A). In vivo, gemcitabine alone significantly improved survival in mice with pancreatic tumors, an effect which was slightly enhanced by co-administration of LY364947 (Figure 5B).

DISCUSSION

The development of a genetically engineered mouse model of pancreatic cancer marked a significant turning point in preclinical model development in pancreatic cancer (14). Other groups have shown that mutant Kras in combination with other targeted genetic mutations, including Ink4a/Arf and MUC1, can also accelerate the development and progression of pancreatic cancer (15,16). These spontaneous mouse models of pancreatic cancer in immunocompetent mice have significant advantages over traditional, immunodeficient xenograft models, in which both the host environment and local tumor microenvironment bear little resemblance to the natural milieu in which tumors typically develop and metastasize in humans.

Although our orthotopic implantation model is not a spontaneous model, it has several advantages over genetically engineered models. The most obvious advantage is that the model is rapidly established without the need for time-consuming and expensive breeding. Furthermore, the disease develops rapidly and predictably. In LSL-Kras\(^{G12D/+}\);LSL-Trp53\(^{R172H/+}\);Pdx-1-Cre mice, disease latency is highly variable, ranging from weeks to almost one year of age (10). With either direct tumor cell injection or subcutaneous tumor fragment transplantation, all mice in our study developed palpable pancreatic tumors by two weeks that progressed to advanced disease by six to eight weeks (Figure 1). The rapidity and consistency of disease development in our model makes it possible to perform time-dependent and large-scale studies in a resource- and cost-effective manner.

Our orthotopic mouse model of pancreatic cancer is established by surgical implantation of tumor cells into the pancreas of an immunocompetent host. Although other cell line-based immunocompetent
mouse models of pancreatic cancer exist (17,18), the cells used in those models are toxin- and virally-induced. Close examination of reports using these models reveals that the tumor histology is predominantly sarcomatoid, with tumors mainly comprised of solid sheets of cells (19). In contrast, the cells used to establish our model were “induced” by mutations in Kras and p53, mutations found in over 90% and 60% of human pancreatic cancers, respectively (2). Histological examination of the tumors in our model demonstrated regions of clear glandular differentiation and some regions of desmoplasia (Figure 2A), which are characteristic of human pancreatic cancer. Moreover, pancreatic tumors in our model expressed cytokeratin-19 (Figure 2B), a marker expressed by most human pancreatic ductal adenocarcinomas. Recently, Olive et al. have also reported on the use of a mouse pancreatic cancer model generated by implantation of tumor cell lines derived from LSL-KrasG12D/+;LSL-Trp53R172H/+;Pdx-1-Cre mice (20); however, most mice had subcutaneous tumors and only human cell lines (e.g. MiaPaCa2) were used to establish orthotopic xenograft tumors.

With regard to initial disease development, when LSL-Kras$^{G12D/+;LSL-Trp53R172H/+;Pdx-1-Cre}$ mice develop tumors, they often occur throughout the pancreas, as all ductal epithelial cells within the organ harbor Kras and p53 mutations and are susceptible to malignant transformation (10). In contrast, in orthotopic implantation models such as ours, the host pancreas cells are wild-type and tumors eventually invade into this otherwise normal parenchyma, similar to what occurs in human pancreatic cancer. A disadvantage of orthotopic implantation, however, is that tumor development does not proceed through the typical stepwise progression of preinvasive stages (pancreatic intraepithelial neoplasia or PanIN); thus carcinogenesis may be better studied using the original LSL-Kras$^{G12D/+;LSL-Trp53R172H/+;Pdx-1-Cre}$ mice.

We chose cells derived from liver metastases found in the LSL-KrasG12D/++;LSL-Trp53R172H/++;Pdx-1-Cre mice based on the rationale that cells that have already metastasized to the liver have a higher propensity to metastasize again, as shown in other mouse models of cancer (21,22). Indeed, 90% of our mice implanted with LM-P cells developed liver metastases (Table). In contrast, we observed that mice with pancreatic tumors generated from PDA (primary tumor) cell lines had fewer liver
Immunocompetent Mouse Model of Pancreatic Cancer

metastases and uniformly developed carcinomatosis. Our findings suggest that depending on which cell line is used, different aspects of pancreatic cancer disease progression may be highlighted for closer study, with the advantage of more rapid and predictable kinetics compared to the original genetically engineered mice.

The genome of human pancreatic cancer exhibits substantial complexity, and CGH array analysis of the pancreatic tumors in our model indicates similar complexity. In addition to Kras and p53, it is well-accepted that many more genetic alterations are involved in pancreatic carcinogenesis (23). Additionally, these genomic abnormalities increase with disease progression (23). This progressive genomic instability is mimicked in our model as revealed by CGH data obtained from in vitro cultured cells, subcutaneous tumors, and invasive pancreatic tumors (Figure 3). The data point to interesting genomic regions that may harbor genes involved in disease progression; however, due to the complex nature of the genome of pancreatic cancer, analysis of additional samples together with mechanistic studies on candidate genes will be required to confirm the identities and roles of involved genes.

Characterization of the immune cells found within the tumors and draining lymph nodes in our model revealed that the immune response to invasive pancreatic cancer is highly complex and involves an infiltration of tumors by multiple immune cell types (Figure 4A, B). Some populations – myeloid derived suppressor cells (MDSCs), tumor-associated macrophages and regulatory T cells – are known to inhibit effector T cell and NK activity, allowing for tumor escape (5,24,25). In contrast, DCs, defined by their high expression of MHC Class II and activation markers/T cell co-stimulatory molecules (e.g. CD86), are the most potent antigen presenting cells and are capable of orchestrating an adaptive anti-tumor immune response (26,27). However, poorly activated “tolerogenic” DCs have been shown to induce T cell anergy, allowing for continued tumor growth and progression (28, 29). Immunophenotypic analysis of the tumor-infiltrating DCs in our model would suggest that, within the tumor microenvironment, the majority of DCs are tolerogenic; however, a small proportion of the DCs present may be appropriately activated, based on CD86 expression (Figure 4A).
Immunocompetent Mouse Model of Pancreatic Cancer

The results from the survival study (Figure 4C) suggest that, in the fully immunocompetent B6/129 mice, at least some degree of effective DC priming of T cells against tumor likely occurs, leading to improved survival compared to T lymphocyte-deficient RAG\(^{-/-}\) mice. This is supported by our finding of activated DCs in the tumor draining lymph nodes (Figure 4A and data not shown), which suggests that at least some of these cells successfully trafficked to sites where priming of naïve T cells can occur. However, in time, all mice succumbed to progressive disease, indicating that the adaptive immune response is ultimately ineffective, perhaps thwarted by the simultaneous presence of tolerogenic DCs, MDSCs, macrophages, and regulatory T cells. In fact, these particular cell types have been identified as key elements hindering the efficacy of vaccine-based immunotherapy for human pancreatic cancer (30).

Clark et al. analyzed the immune response in KrasG12D-expressing, p53 wild-type mice, which manifest precursor pancreatic intraepithelial neoplasia (PanIN) that occasionally progresses to invasive adenocarcinoma (31). They found, surprisingly, that MDSCs and regulatory T cells were already present in the early PanIN stages and continued to increase with disease progression (31). The DC response was not analyzed; however, the authors did note that about one-third of intra-tumor CD4+ T cells had an expression profile (CD45RB-low and CD44-high) which suggests evidence of DC priming and antigen experience (31). In another spontaneous, genetically engineered mouse model of pancreatic cancer (TGF-\(\alpha/p53\) mutant), tumor-specific CD8+ T cells were noted (32); however, the presence of activated DCs, which would likely contribute to this response, was also not assessed.

To further assess the similarity of our model to human pancreatic cancer, we evaluated the sensitivity of LM-P tumors to gemcitabine, a chemotherapeutic agent widely used in the treatment of human pancreatic cancer (2). We also evaluated the effect of LY364947, a novel TGF-\(\beta\) receptor-1 kinase inhibitor. TGF-\(\beta\) is one of several tumor-derived cytokines that are thought to induce extracellular matrix deposition and fibrosis, creating the desmoplastic stroma characteristic of pancreatic cancer (6). Also, inhibition of TGF-\(\beta\) signaling has been shown in a subcutaneous, xenograft (BxPC3) mouse model of pancreatic cancer to potentiate drug delivery by altering permeability at the tumor microenvironment.
Immunocompetent Mouse Model of Pancreatic Cancer

level (12). Whereas gemcitabine was highly cytotoxic to LM-P cells in vitro and extended survival of tumor-bearing mice in our orthotopic, immunocompetent model, LY364947 had little or no anti-tumor activity as a single agent in vitro but resulted in modest improvement in survival when used in combination with gemcitabine (Figure 5). These results are consistent with the concept that TGF-β inhibition likely acts on surrounding tumor stroma. It will be interesting to determine if optimizing the dose of this agent results in greater efficacy.

Our preliminary findings with gemcitabine and TGF-β inhibition, however, should be approached with some caution. Olive et. al. showed recently that due to differences in tumor-supporting stroma and microvasculature, transplant models of pancreatic cancer were much more sensitive to gemcitabine than the original genetically engineered mice (20). Although desmoplasia was noted in pancreatic tumors in our model (Figure 2A), the extent and pattern of fibrosis may not be as profound as in the genetically engineered mice or in humans. Our results with gemcitabine and TGF-β inhibition will likely need to be further validated in the original genetically engineered mice; nonetheless, we envision that our model can serve as a complementary resource- and cost-effective screening tool for preclinical testing of new therapeutic agents.

In conclusion, we have developed a rapid, predictable model of pancreatic cancer by orthotopic implantation of tumor cells into immunocompetent mice. Like the human disease, tumor cells have both Kras and p53 mutations, and the tumors that develop from these cells have a histological appearance and pattern of disease progression similar to the human disease. Genomic analysis suggests that this model may help to reveal new genes involved in disease progression. Immunophenotypic analysis highlights the complexity of the immune response to pancreatic cancer. Our hope is that this model will further the understanding of pancreatic cancer biology and prove useful for the preclinical evaluation of new therapeutic agents.
REFERENCES

Immunocompetent Mouse Model of Pancreatic Cancer


Immunocompetent Mouse Model of Pancreatic Cancer


**TABLE**

**Table 1.** Frequency of pancreatic tumor uptake, liver metastases and peritoneal carcinomatosis six to eight weeks following orthotopic implantation of LM-P cells.

<table>
<thead>
<tr>
<th></th>
<th>Pancreatic Tumor</th>
<th>Liver Metastases</th>
<th>Peritoneal Carcinomatosis / Hemorrhagic Ascites</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LM-P Cell Injection</strong></td>
<td>15 of 15</td>
<td>13 of 15</td>
<td>0 of 15</td>
</tr>
<tr>
<td><strong>Subcutaneous LM-P Tumor Fragment Transplantation</strong></td>
<td>15 of 15</td>
<td>14 of 15</td>
<td>0 of 15</td>
</tr>
<tr>
<td><strong>TOTAL (%)</strong></td>
<td>30 of 30 (100%)</td>
<td>27 of 30 (90%)</td>
<td>0 of 30 (0%)</td>
</tr>
</tbody>
</table>

Immunocompetent Mouse Model of Pancreatic Cancer
FIGURE LEGENDS

Figure 1. Early and late disease and in our orthotopic model. At 2 weeks, LM-P tumor uptake is noted with progressive growth at 4 weeks. Comparison of the two techniques for implantation (see Methods) reveals no difference in tumor appearance (A) or total tumor volume (B) at these early time points. By 6 to 8 weeks, mice develop extensive local growth into adjacent organs (dotted line = tumor invading into stomach) and liver metastases (open arrows) (C). In some mice, clinically relevant disease manifestations, such as biliary obstruction (arrowheads = dilated common bile duct) producing jaundice (swab tip of bilious peritoneal fluid), and gastric outlet obstruction (* = massively dilated stomach) are seen (D). In comparison to LM-P, orthotopic implantation of PDA cells results in more aggressive disease progression with decreased survival (for PDA1-1, E, *p<0.05) and development of carcinomatosis (for both PDA1-1 and PDA3-5, F).

Figure 2. Histology of pancreatic tumors and metastases. LM-P tumors form glands and regions of desmoplasia (A, arrows). Immunohistochemistry demonstrates that pancreatic tumors stain for cytokeratin-19, a ductal epithelial marker (B, CK19 in brown, hematoxylin counterstain in blue, original magnification, 200 X). Areas of perineural invasion (C, arrowheads = nerve, dotted line = tumor invasion) and peri-pancreatic lymph node infiltration (D) are also noted. Liver (E) and lung metastases (F) confirm adenocarcinoma. PDA pancreatic tumors show similar histology to LM-P but are predominantly poorly-(1-1, G) or well-differentiated (3-5, H) (H&E, original magnification, 100 X for A, and D-F; 200 X for G, H; 400 X for B, C; N = nerve; T = tumor, GC = germinal center).

Figure 3. Comparative genomic hybridization. Shown are genomic profiles of copy number alteration for LM-P cells (below, in green), a subcutaneous tumor (middle, in blue), and an orthotopic tumor (above, in red). Tumor/normal CGH log2 ratios (moving average 0.2 megabases) are plotted by chromosome position; gains and losses appear as peaks and valleys, respectively. Selected sites of gain and loss (see text) are indicated.
Immunocompetent Mouse Model of Pancreatic Cancer

**Figure 4.** LM-P tumors are infiltrated with distinct mononuclear cell types. Three expanded populations are seen in pancreatic tumors and, to a lesser extent, in draining lymph nodes, based on surface marker expression: 1) CD11c-high, CD11b-negative 2) CD11c-high, CD11b-positive and 3) CD11c-negative/low, CD11b-positive cells (A). The two CD11c-high populations (1 and 2) express MHC Class II, and a small proportion of the cells also express CD86. The CD11c-negative/low CD11b-positive cells (3) are comprised of Gr-1+ myeloid-derived suppressor cells and Gr-1- tumor-associated macrophages. The T cell response in pancreatic tumors and draining lymph nodes is shifted toward CD4+ cells (B). Tumor-infiltrating CD4+ T cells (+) include a distinct population of CD25+ FoxP3+ regulatory T cells. Numerical values represent CD4:CD8 ratios. Isotype controls (for CD86, FoxP3) are shown in gray. Lymphocyte-deficient RAG⁻/⁻ pancreatic tumor-bearing mice have decreased survival compared to fully immunocompetent mice (*p<0.05); however, both ultimately succumb to disease (C).

**Figure 5.** Treatment of LM-P tumors with gemcitabine and LY364947, a TGF-β receptor-1 kinase inhibitor (TGF-βR1 KI). In vitro, MTT assays demonstrate direct cytotoxicity of gemcitabine but not LY364947 against LM-P cells (A). In vivo, gemcitabine treatment results in improved survival, an effect slightly enhanced by addition of LY364947 (B, Gem = gemcitabine; *p<0.05 for Gem vs. PBS control **p<0.05 for Gem + TGF-βR1 KI vs. Gem alone).
Figure 1

A. t = 0, Week 2, Week 4

B. Tumor Volume (mm³) vs Time (weeks)

C.

D.

E. Percent survival vs Days

F.
Figure 2
Figure 3
Figure 4

A. Normal Pancreas vs. dLN

B. Tumor-bearing Pancreas vs. dLN

C. Survival rates of RAG and B6/129 mice
Figure 5
Clinical Cancer Research

Development of an Orthotopic Model of Invasive Pancreatic Cancer in an Immunocompetent Murine Host

William W Tseng, Daniel Winer, Justin A Kenkel, et al.

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