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An imageable highly metastatic orthotopic red fluorescent protein model of pancreatic cancer

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Abstract

In order to investigate the antitumor and antimetastatic efficacy of new chemotherapeutic agents, a novel, red-fluorescent, orthotopic model of pancreatic cancer was constructed in nude mice. MIA-PaCa-2 human pancreatic cancer cells were transduced with red fluorescent protein (RFP) and initially grown subcutaneously. Fluorescent tumor fragments were then transplanted onto the pancreas by surgical orthotopic implantation (SOI), facilitating high-resolution, real-time visualization of tumor and metastatic growth and dissemination *in vivo*. Tumor growth at the primary site was visible within the first postoperative week, while distant metastasis and the development of ascites became visible over the following week. This MIA-PaCa-2-RFP model produced extensive local disease and metastases to the retroperitoneum (100%), spleen (100%), intestinal and periportal lymph nodes (100%), liver (40%) and diaphragm (80%), and gave rise to malignant ascites and peritoneal carcinomatosis in 80% of cases. Growth and metastasis of tumor was more rapid and frequent than in previously described orthotopic pancreatic cancer models, leading to a median survival of only 21 days after tumor implantation. This unique, red fluorescent model rapidly and reliably simulates the highly aggressive course of human pancreatic cancer and can be easily non-invasively visualized in the live animal. The model can therefore be used for the discovery and evaluation of novel therapeutics for the treatment of this devastating disease.

Abbreviations: GFP – green fluorescent protein; RFP – red fluorescent protein; SOI – surgical orthotopic implantation

Pancreatic ductal adenocarcinoma is one of the most lethal of human malignancies, accounting for over 30,000 deaths yearly in the United States alone [1]. Only 10% to 15% of these cancers are typically found to be resectable at the time of diagnosis [2], due to the presence of locally advanced disease or distant metastases. Moreover, the frequent accumulation of residual local and metastatic disease after maximal therapy limits the survival of patients with this disease to less than 21 months [3–5] despite complete surgical resection and adjuvant chemotherapy and radiation.

Several animal models of pancreatic cancer have been developed in nude or SCID mice and Syrian hamsters to simulate the course of this disease and to facilitate studies of novel therapeutics. Subcutaneous xenograft models [6], in which human pancreatic tumors are grown in a pocket directly underneath the skin, are easily established and afford ready access to the tumor – features which facilitate both tumor measurement and intratumoral injection of drugs. These models are limited, however, because the subcutaneous implants are typically noninvasive, usually do not metastasize, and may not respond to chemotherapeutic agents in the same way as the human disease they have been designed to emulate [7].

Orthotopic pancreatic cancer models involve the injection of cultured pancreatic cancer cell suspensions [8] or the surgical implantation of tumor fragments [9] directly onto the pancreas. Orthotopic xenografts have been demonstrated to frequently give rise to spontaneous metastases, with surgically implanted fragments superior in this regard [10]. Importantly, in orthotopically implanted xenografts, metastases often maintain the same pattern of genetic alterations that is present in their primary tumors [11].

Recent advances in orthotopic cancer models have led to the ability to identify and characterize tumor growth and metastasis by engineering the tumors to express high levels of the *Aequorea victoria* jellyfish green fluorescent protein (GFP) [12, 13]. The tracking of cancer cells that inherit the GFP gene has been shown to facilitate *in vivo* identification of metastases as small as one cell in size, and permits real-time imaging of the growth and dissemination of tumor, in live animals, using simple, noninvasive equipment [16, 18].

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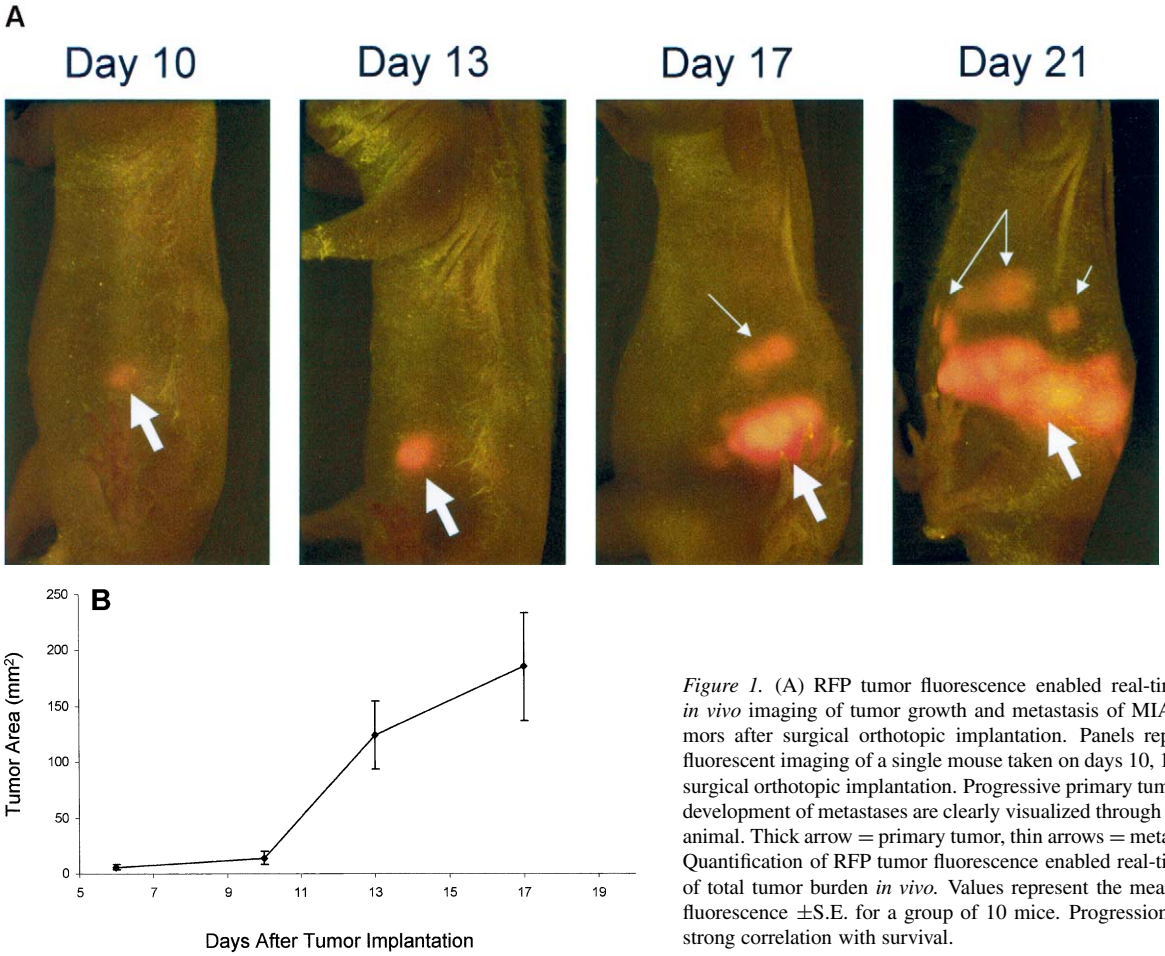

Figure 1. (A) RFP tumor fluorescence enabled real-time, non-invasive, *in vivo* imaging of tumor growth and metastasis of MIA-PaCa-2-RFP tumors after surgical orthotopic implantation. Panels represent sequential fluorescent imaging of a single mouse taken on days 10, 13, 17 and 21 after surgical orthotopic implantation. Progressive primary tumor growth and the development of metastases are clearly visualized through the skin in the live animal. Thick arrow = primary tumor, thin arrows = metastatic tumors. (B) Quantification of RFP tumor fluorescence enabled real-time determination of total tumor burden *in vivo.* Values represent the mean area of external fluorescence ±S.E. for a group of 10 mice. Progression of disease had a strong correlation with survival.

In this report, we describe a novel, highly metastatic model of pancreatic cancer that utilizes pancreatic cancer cells engineered to express very high levels of *Discosoma sp.* red fluorescent protein (RFP) [14]. This model clinically resembles human pancreatic cancer in its pattern of growth and metastasis. It rapidly and reliably produces distant metastatic disease, and frequently gives rise to malignant abdominal ascites and peritoneal carcinomatosis. Moreover, the enhanced fluorescence of this model enables real-time visualization and imaging of pancreatic tumor growth and metastasis in the live animal, and permits identification of both macro- and micrometastases. These features make the model an ideal system with which to study the effects of novel antineoplastic agents on tumor growth and metastasis.

Materials and methods

Cell line

The MIA-PaCa-2 pancreatic cancer cell line was obtained from the American Type Culture Collection (Rockville, Maryland). Cells were maintained in DMEM media supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin and streptomycin (Gibco-BRL, Life Technologies, Inc., Grand Island, New York). Cells were incubated at 37 °C in a 5% CO_2 incubator.

Animals

Male nude mice (NCr-nu) between 4–6 weeks of age were maintained in a barrier facility on HEPA-filtered racks. The animals were fed with autoclaved laboratory rodent diet (Teckland LM-485; Western Research Products, Orange, California). Animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH Publication Number 85-23) under NIH assurance number A3873-01.

RFP retroviral transduction and selection of MIA-PaCa-2-RFP pancreatic cancer cells

The *Discosoma* sp. pDsRed-2 vector (Clontech Laboratories Inc., Palo Alto, California) was used to engineer MIA-PaCa-2 clones stably expressing RFP. This vector expresses RFP and the neomycin resistance gene on the same bicistronic message. pDsRed-2 was produced in PT67 packaging cells. RFP transduction was initiated by incubating 20% confluent MIA-PaCa-2 cells with retroviral supernatants of the packaging cells and DMEM for 24 h. Fresh medium was replenished at this time and cells were allowed to grow in

the absence of retrovirus for 12 h. This procedure was repeated until high levels of RFP expression, as determined using fluorescence microscopy, were achieved. Cells were then harvested by trypsin/EDTA and subcultured into selective medium that contained 200 μg/ml of G418. The level of G418 (Geneticin, Invitrogen Corp., Carlsbad, California) was increased to 2 mg/ml stepwise. Clones expressing high levels of RFP were isolated and were amplified and transferred using conventional culture methods. High RFP-expression clones were isolated in the absence of G418 for 10 passages to select for stable expression of RFP *in vivo.*

Cell viability assay

MIA-PaCa-2 and MIA-PaCa-2-RFP cells were distributed into 96-well plates at a density of 2000 cells/well. The number of viable cells was subsequently determined using the CellTiter 96 Aqueous One Solution Cell Proliferation assay (Promega Corp., Madison, Wisconsin) at 24-, 48-, 72-, 96-, and 120-h time points. Briefly, at each time point, 20 μl of CellTiter 96 solution was added to each well. The plates were then incubated for one hour, after which the absorbance of each well was read at a wavelength of 490 nm. All assays were performed in quadruplicate and each assay was repeated at least twice.

Subcutaneous tumor growth

MIA-PaCa-2-RFP cells were harvested by trypsinization and washed three times with PBS. Approximately 5×10^6 cells were injected subcutaneously into nude mice in a total volume of 0.2 ml within 30 min of harvesting. The subcutaneous tumors were used as the source of tissue for surgical orthotopic implantation of tissue onto the pancreas, as detailed below.

Surgical orthotopic implantation of MIA-PaCa-2-RFP tumors

Orthotopic, red-fluorescent human pancreatic cancer xenografts were established in nude mice by surgical orthotopic implantation (SOI) [9]. Briefly, MIA-PaCa-2-RFP tumors in the exponential growth phase, grown subcutaneously in nude mice, were resected aseptically. Necrotic tissues were cut away, and the remaining healthy tumor tissues were cut with scissors and minced into 1 mm^3 pieces in RPMI 1640 medium. Mice were then anesthetized and their abdomens were sterilized with alcohol. An incision was created through the left upper abdominal pararectal line and peritoneum. The pancreas was carefully exposed and two tumor pieces were transplanted onto the middle of the gland using a single 8-0 surgical suture (Davis-Geck, Inc., Manati, Puerto-Rico). The pancreas was then returned into the peritoneal cavity, and the abdominal wall and the skin were closed in two layers using 6-0 surgical suture. All procedures were performed with a 7$\times$ microscope (Olympus) or standard surgical loupes.

External in vivo *whole body imaging*

Twice weekly, whole-body images of each mouse were obtained by placing the mouse in a fluorescent light box equipped with a fiberoptic light source of 470 nm (Lightools Research, Encinitas, California). Emitted fluorescence was collected through a long-pass filter GG475 (Chroma Technology, Battleboro, Vermont) on a Hamamatsu C5810 3-chip cooled color CCD camera (Hamamatsu Photonics Systems, Bridgewater, New Jersey). High resolution images of 1024 $\times$ 724 pixels were captured directly on an IBM PC and analyzed using Image Pro Plus 3.1 software (Media Cybernetics, Silver Spring, Maryland). Real-time determination of tumor burden was performed by quantifying fluorescent surface area, as described previously [13].

Internal imaging and analysis of metastasis

Mice were sacrificed and explored when they appeared premorbid. After euthanasia, each mouse underwent laparotomy and median sternotomy while selectively exciting RFP in the light box described above, facilitating identification of primary and metastatic pancreatic tumor by fluorescence visualization. After performing full-body, open images, the solid organs were removed and were thoroughly examined for any evidence of metastasis using a Leica fluorescence stereo microscope model LZ12 (Leica Microsystems, Inc., Bannockburn, Illinois) equipped with a mercury 50-W lamp power supply. Selective excitation of RFP was produced through a D425/60 band-pass filter and 470 DCXR dichroic mirror. Emitted fluorescence was collected on the Hamamatsu camera system described above.

Histological analysis

The primary orthotopic tumors were removed and saved for histological analysis with standard hematoxylin and eosin (H&E) staining.

Results and discussion

In vitro *MIA-PaCa-2 and MIA-PaCa-2-RFP isolation and growth*

Retroviral-vector transduced cells were able to grow *in vitro* at levels of G418 up to 2000 μg/ml. The selected G418-resistant pancreatic cancer cells had bright RFP fluorescence that remained stable in the absence of selective medium after numerous passages. Cell proliferation rates of the parental cells and the RFP transductants were found to be similar by cell viability assay (data not shown).

Real-time growth and metastasis of MIA-PaCa-2-RFP tumors

Internally growing MIA-PaCa-2-RFP primary tumors became visible through the skin in the live animal within the first week after implantation (Figure 1A). The progressive development of metastases was also clearly visible, even in

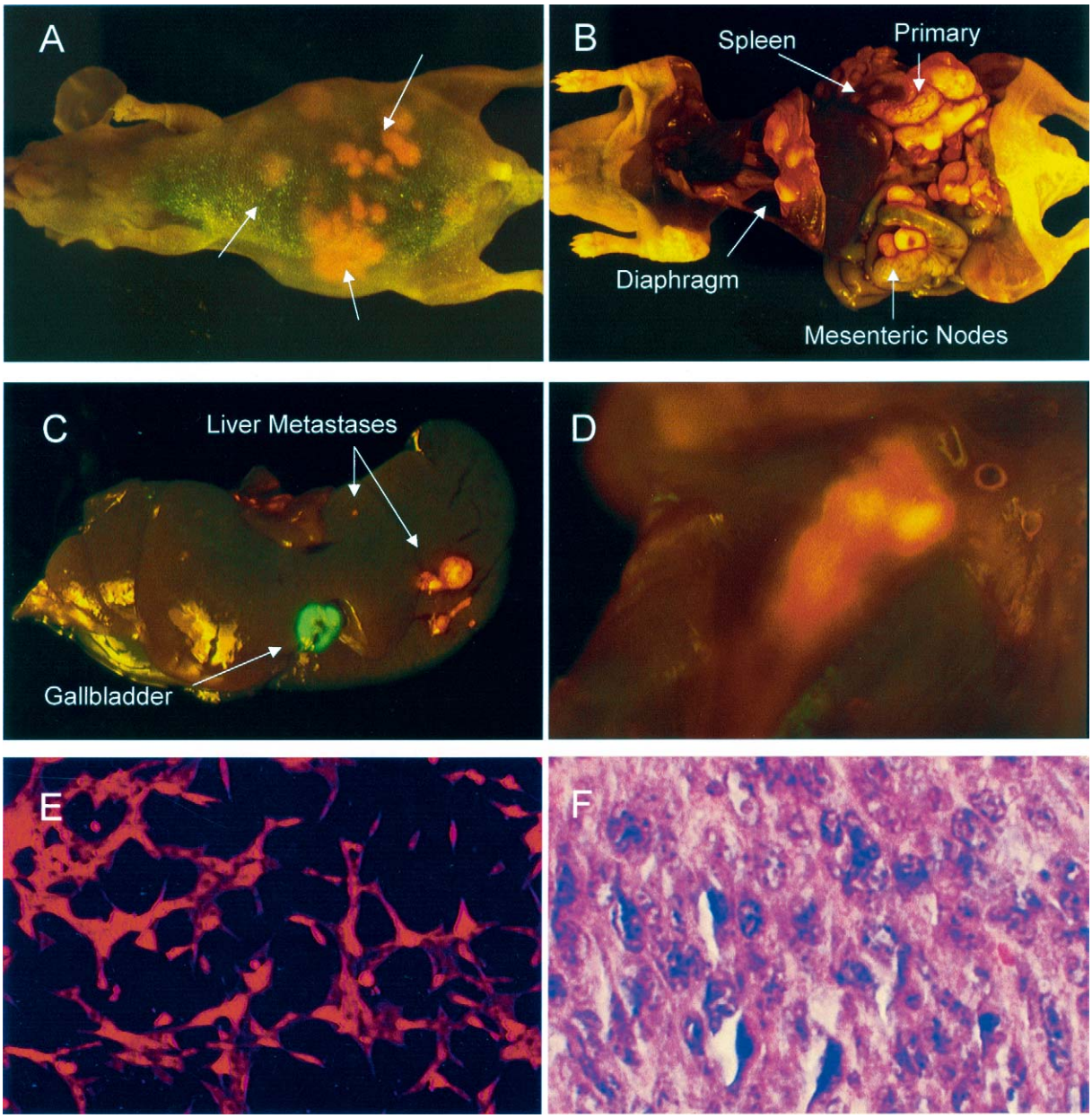

Figure 2 (A) Direct, fluorescence images taken upon autopsy demonstrated MIA-PaCa-2-RFP primary and metastatic tumors (arrows) that were externally visible through the skin. (B) Laparotomy/sternotomy of the same mouse in (A) visualized metastases to the lymphatics, peritoneum and solid organs. (C) Liver and other solid organs were removed, and gross metastases were visualized. (D) Fluorescence microscopy facilitated visualization of micrometastases to the liver and other organs (cross sectional view of solid liver micrometastasis, 40×). (E) Culture of ascites fluid from the SOI model two weeks after orthotopic tumor implantation. (F) H&E staining of primary MIA-PaCa-2-RFP tumors revealed histological characteristics of poorly differentiated pancreatic adenocarcinoma.

the presence of abdominal ascites, enabling direct, real-time evaluation and quantification of tumor growth and dissemination without the need for laparotomy or any invasive procedure (Figure 1B). The average tumor burden per mouse increased dramatically over the second week after surgical orthotopic implantation; by the end of the second week with distant metastases and abdominal ascites clearly visible by whole-body imaging (Figure 1A).

Patterns of growth and metastasis

Orthotopically transplanted MIA-PaCa-2-RFP tumors produced both extensive locoregional and disseminated disease. At the time of autopsy, all mice had metastatic disease in the periportal and intestinal lymph nodes, spleen and retroperitoneum (Figures 2A–C). Fluorescent microscopy enabled identification of micrometastases invisible to the naked eye (Figure 2D). Malignant ascites (80%), peritoneal carcinomatosis (80%), and metastasis to the diaphragm (80%) and liver (40%) were also common (Figure 3). Ascites fluid that was aspirated and cultured at the time of sacrifice formed colonies of brightly-fluorescent clones of MIA-PaCa-2-RFP cells (Figure 2E). Solid tumors were found to have histological features consistent with poorly differentiated pancreatic adenocarcinoma upon examination of hematoxylin and eosin stained tissue sections (Figure 2F).

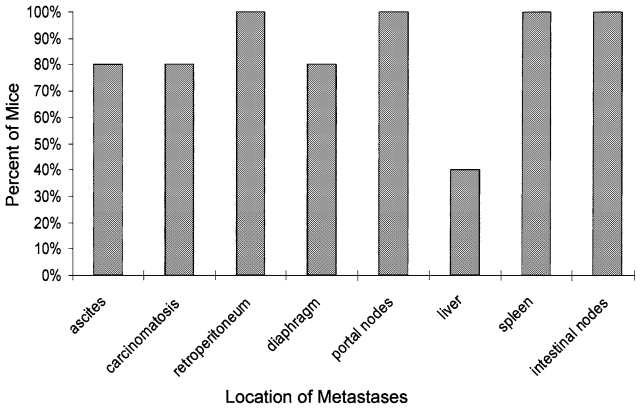

Figure 3 Frequency of specific organ metastases in the MIA-PaCa-2-RFP model after surgical orthotopic implantation. Bars represent the percentage of mice with metastatic tumor found in the specified location at autopsy.

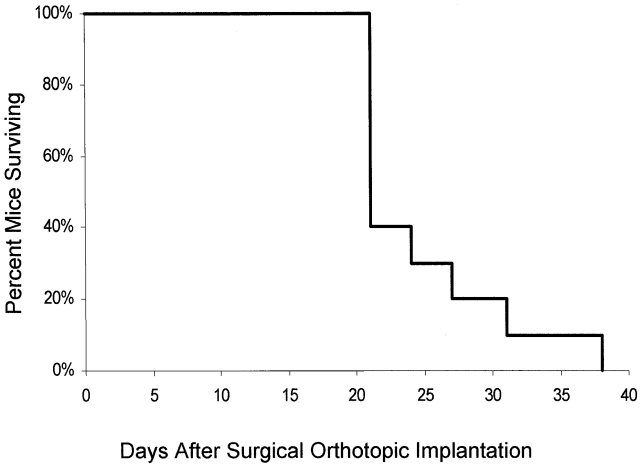

Figure 4 MIA-PaCa-2-RFP pancreatic cancer led to death in all mice within 40 days of implantation, with a median survival of only 21 days.

Survival

Death from disease was rapid in this model, with a median survival after implantation of only 21 days (Figure 4).

The novel orthotopic model of pancreatic cancer we have described in this report has several important features that make it an ideal system with which to study the antitumor and antimetastatic effects of novel therapeutics. This model facilitates qualitative and quantitative real-time optical imaging of progressive tumor growth and metastasis formation. The red fluorescent protein used in this model is particularly bright, and is selectively expressed by tumors and metastases that contain the RFP gene. High contrast images are therefore attainable through the skin without the need for laparotomy, contrast agents, or invasive procedures. We have previously demonstrated that the whole body, external images acquired over the time course of tumor progression using GFP fluorescent orthotopic models [15, 16], correlate well with direct intravital images performed under more invasive conditions [13].

In the present model, the brightness and reduced light scatter of RFP compared to GFP give it important advantages in whole-body imaging. Moreover, fluorescent-protein-based microscopy enables visualization of micrometastases as small as one cell in size – metastatic events that surely would be overlooked using non-fluorescent systems. The identification, retrieval and isolation of metastatic cells in ascites fluid are similarly facilitated by tumor cell fluorescence.

This MIA-PaCa-2-RFP model of pancreatic cancer reliably simulates the aggressive nature of human pancreatic ductal adenocarcinoma. Orthotopic implantation in this model yielded diffuse metastases to intestinal and periportal lymph nodes, retroperitoneum, and spleen in 100% of animals. Metastases to the diaphragm and liver, and the development of malignant ascites and peritoneal carcinomatosis, were also common. This malignant pattern of metastases occurs spontaneously, without intraperitoneal injection of cell suspensions, a procedure which may bypass important steps of the metastatic cascade [17].

The model we have described represents a clinically relevant, highly metastatic model of pancreatic cancer that is easily imaged over time. It should prove useful in the development and testing of novel treatment strategies of this lethal disease.

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