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Chronologically-specific metastatic targeting of human pancreatic tumors in orthotopic models

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Abstract

Pancreatic cancer is a highly metastatic disease that responds poorly to currently-available treatment. In order to better visualize and understand the chronology and specificity of metastatic targeting of pancreatic cancer, two human pancreatic cancer cell lines, expressing green fluorescent protein (GFP), were studied in orthotopic models. MIA-PaCa2-GFP and BxPC-3-GFP tumor fragments were transplanted by surgical orthotopic implantation (SOI) to the nude mouse pancreas for fluorescence visualization of the chronology of pancreatic tumor growth and metastatic targeting. BxPC-3-GFP tumors developed rapidly in the pancreas and spread regionally to the spleen and retroperitoneum as early as six weeks. Distant metastases in BxPC-3-GFP were rare. In contrast, MIA-PaCa-2-GFP grew more slowly in the pancreas but rapidly metastasized to distant sites including liver and portal lymph nodes. Regional metastases in MIA-PaCa-2-GFP were rare. These studies demonstrate that pancreatic cancers have highly specific and individual 'seed-soil' interactions governing the chronology and sites of metastatic targeting.

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

Introduction

A greater understanding of the metastatic process of pancreatic cancer is needed [1]. Transgenic rodent models of pancreatic cancer [2] as well as chemically induced pancreatic cancer in rodents have been useful to study this disease [3]. Metastases of human pancreatic cancer after s.c. transplantation in nude mice have only occasionally been reported, however [4, 5]. Vezeridis et al. [6] reported a metastatic model using splenic injection of a fast-growing variant of human pancreatic cancer. Although this was a valuable model for the study of certain steps of the metastatic process, it bypasses invasion from the primary tumor and in essence generates colonization rather than metastases.

It is important, however, to study clinically relevant metastases and their treatment. Tan and Chu [7], Marincola et al. [8, 9], and Bruns et al. [10] reported metastatic models of human pancreatic cancer using orthotopic implantation of tumor-cell suspensions, which resulted in invasive local tumor growth and subsequent metastases. Vezeridis et al. [11] used tumor tissue for orthotopic transplantation, resulting in extensive local growth and metastases to liver, lung, and lymph nodes. We have used histologically intact specimens of pancreatic cancer, including those directly removed from patients, for surgical orthotopic implantation (SOI) to nude mice to construct highly metastatic nude-mouse models of human pancreatic cancer [12–15]. The models using intact tissue for orthotopic implantation have been shown to have considerably higher metastatic rates than orthotopic models using cell suspensions [7–10].

The present report utilized nude mouse models of SOI human pancreatic tumors genetically engineered with green fluorescent protein (GFP) [8–9]. These models allow, for the first time, early visualization of the chronology of tumor growth, progression, and metastases of pancreatic tumors to specific target organs. Our hypothesis was that these models would demonstrate chronologically tumor-specific metastatic targeting. The results demonstrated that pancreatic tumors are highly specific with regard to metastatic sites and chronology of metastatic development.

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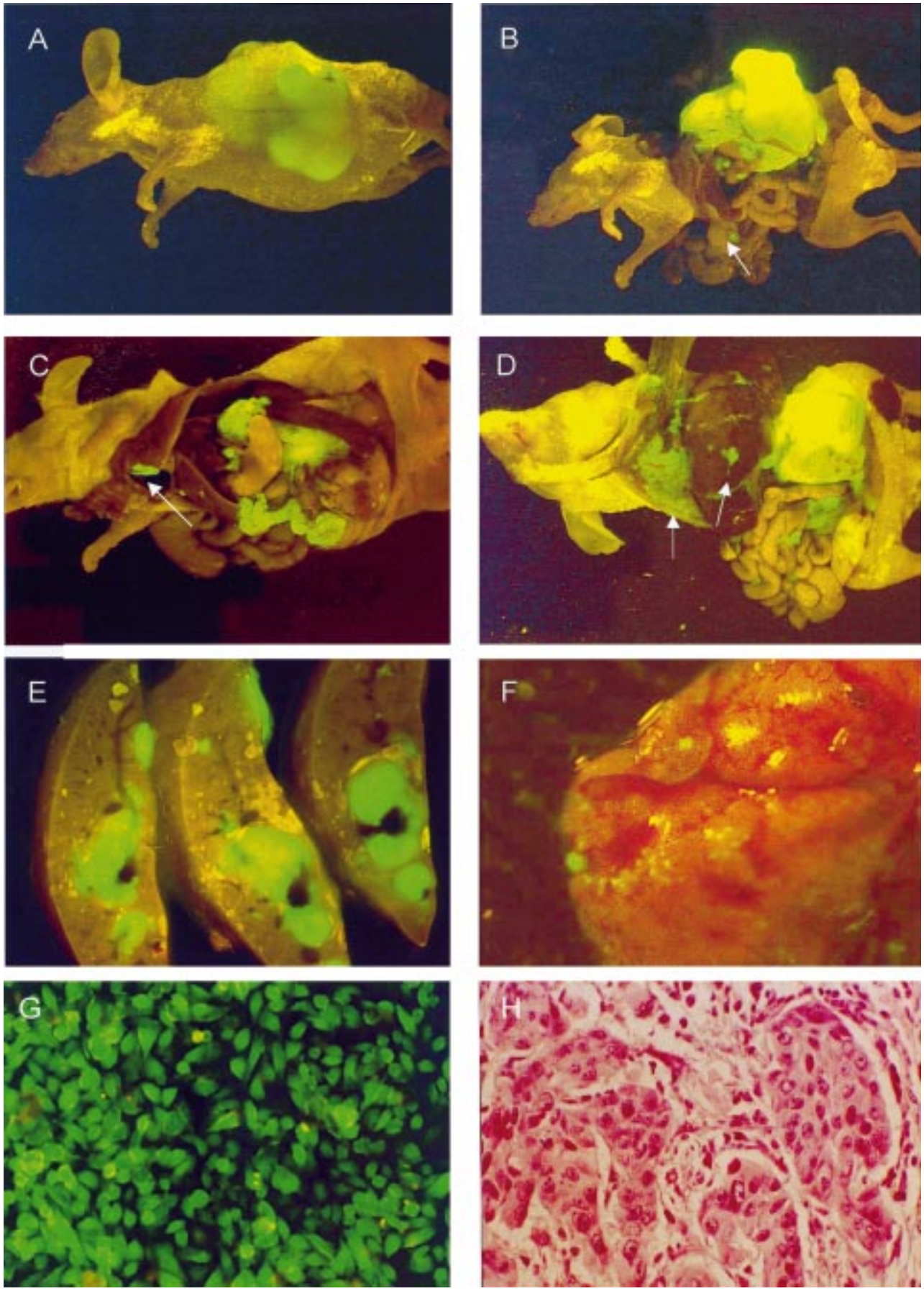
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Figure 1. (A) The orthotopic BxPC-3-GFP pancreatic tumor is externally visualized with fluorescence through the skin of the nude mouse. (B) Laparotomy of the same mouse in (A) showing locally advanced BxPC-3-GFP tumor with portal lymph node metastases. This pattern of metastases was typical of BxPC-3-GFP. (C) Three 1-mm^3 fragments of s.c.-grown MIA-PaCa-2-GFP tumor were implanted to the pancreas of nude mice by SOI. The primary tumor formed in the pancreas at 12 weeks is visualized under bright-field microscopy. Numerous metastases and micrometastases, typical of MIA-PaCa-2-GFP, can be visualized by GFP under fluorescence microscopy in the stomach, spleen, periportal nodes (arrow), liver, and mediastinum (arrow). (D) MIA-PaCa-2-GFP tumor at week-10 post-SOI. Left arrow shows diaphragm metastases and right arrow shows liver metastases. (E) Bi-lobar high expressing MIA-PaCa-2 GFP liver metastases are noted under fluorescence microscopy. (F) MIA-PaCa-2 GFP-expressing lung metastases are noted. (G) Stable high-level expression GFP transductant *in vitro*. The human pancreatic cancer cell line MIA-PaCa-2 was transduced with the RetroXpress vector pLEIN that expresses enhanced GFP and the neomycin resistance gene on the same bicistronic message. The stable high expression clone was selected in 800 μg/ml of G418. (H) H&E section of MIA-PaCa-2-GFP tumor.

Materials and methods

Pancreatic cancer cell lines

The MIA-PaCa-2 and BxPC-3 human pancreatic cancer cell lines were obtained from the American Type Culture Collection (Rockville, Maryland). The cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 μ/ml penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B (Gibco-BRL, Life Technologies, Inc., Grand Island, New York). Both cell lines were incubated at 37 °C in a 5% CO_2 incubator.

GFP DNA expression vector

The RetroXpress vector pLEIN was purchased from Clontech Laboratories, Inc. (Palo Alto, California). The pLEIN vector expresses enhanced GFP and the neomycin resistance gene on the same bicistronic message, which contains an IRES site.

GFP-retrovirus vector production [20]

PT67, an NIH3T3-derived packaging cell line expressing the 10 A1 viral envelope, was purchased from Clontech Laboratories, Inc. PT67 cells were cultured in DMEM (Irvine Scientific, Santa Ana, California) supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bioproducts, Calabases, California). For GFP-retrovirus production, packaging cells (PT67), at 70% confluence, were incubated with a precipitated mixture of DOTAP reagent (Boehringer Mannheim) and saturating amounts of pLEIN plasmid for 18 h. Fresh medium was replenished at this time. The cells were examined by fluorescence microscopy after 48 h. For selection of packaging cells producing high levels of GFP retrovirus, the cells were cultured in stepwise increasing amounts of 500–2000 μg/ml G418 (Life Technologies, Inc., Grand Island, New York) for 7 days.

GFP-retroviral transduction and selection of high GFP-expression MIA-PaCa-2 and BxPC-3 pancreatic cancer cells

For GFP gene transduction [16, 17], 20% confluent MIA-PaCa-2 or BxPC-3 cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of the PT67 packaging cells and RPMI 1640 (Life Technologies, Inc.) for 72 h. Fresh medium was replenished at this time. MIA-PaCa-2 or BxPC-3 cells were harvested by trypsin/EDTA 72 h after infection and subcultured at a ratio of 1:15 into selective medium that contained 200 μg/ml of G418. The level of G418 was increased to 800 μg/ml stepwise. MIA-PaCa-2 and BxPC-3 clones expressing GFP (MIA-PaCa-2-GFP or BxPC-3-GFP) were isolated with cloning cylinders (Bel-Art Products, Pequannock, New Jersey) by trypsin/EDTA and were amplified and transferred by conventional culture methods. High GFP-expression clones were then isolated in the absence of G418 for more than 10 passages to select for stable expression of GFP.

Subcutaneous tumor growth

BALB/c nu/nu female mice, 6 weeks of age, were injected subcutaneously with a single dose of 5×10^6 MIA-PaCa-2-GFP or BxPC-3-GFP cells. Cells were first harvested by trypsinization and washed three times with cold serum-free medium and then injected in a total volume of 0.2 ml within 40 min of harvesting.

Surgical orthotopic implantation (SOI) [12–15]

Pancreatic tumors at 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 approximately $1 \times 1 \times 1$ mm pieces in Hanks' balanced salt solution containing 100 units/ml penicillin and 100 μ/ml streptomycin. Each piece was weighed and adjusted with scissors to be 50 mg. Mice were anesthetized by isoflurane inhalation. The abdomen was sterilized with alcohol. An incision was then made through the left upper abdominal pararectal line and peritoneum. The pancreas was carefully exposed and three tumor pieces were transplanted on the middle of the pancreas with a 6-0 Dexon (Davis-Geck, Inc., Manati, Puerto-Rico) surgical suture. The pancreas was then returned into the peritoneal cavity, and the abdominal wall and the skin were closed with 6-0 Dexon sutures. Animals were kept in a sterile environment. All procedures of the operation described above were performed with a 7× microscope (Olympus). A total of 44 mice were used for the BxPC3-GFP model and 26 for the MIA-PaCa-2 model.

Fluorescence microscopy [20]

A Leica stereo microscope MZ 12 equipped with a mercury bulb as a light source was used for the imaging experiments. Selective excitation of GFP was produced through a D425/60 band-pass filter and a 470 DCXR dichroic mirror. Fluorescence was emitted through a GG475 long-pass

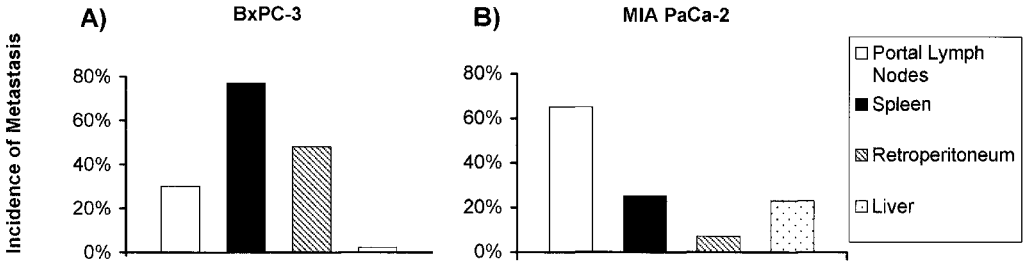

Figure 2. Site-specific metastases in human pancreatic cancer orthotopic models. A) BxPC-3-GFP. B) MIA-PaCa-2-GFP. Forty-four and twenty-six mice were used for the BxPC-3-GFP and MIA-PaCa-2 models, respectively. The y-axis represents cumulative percentage of mice with metastasis.

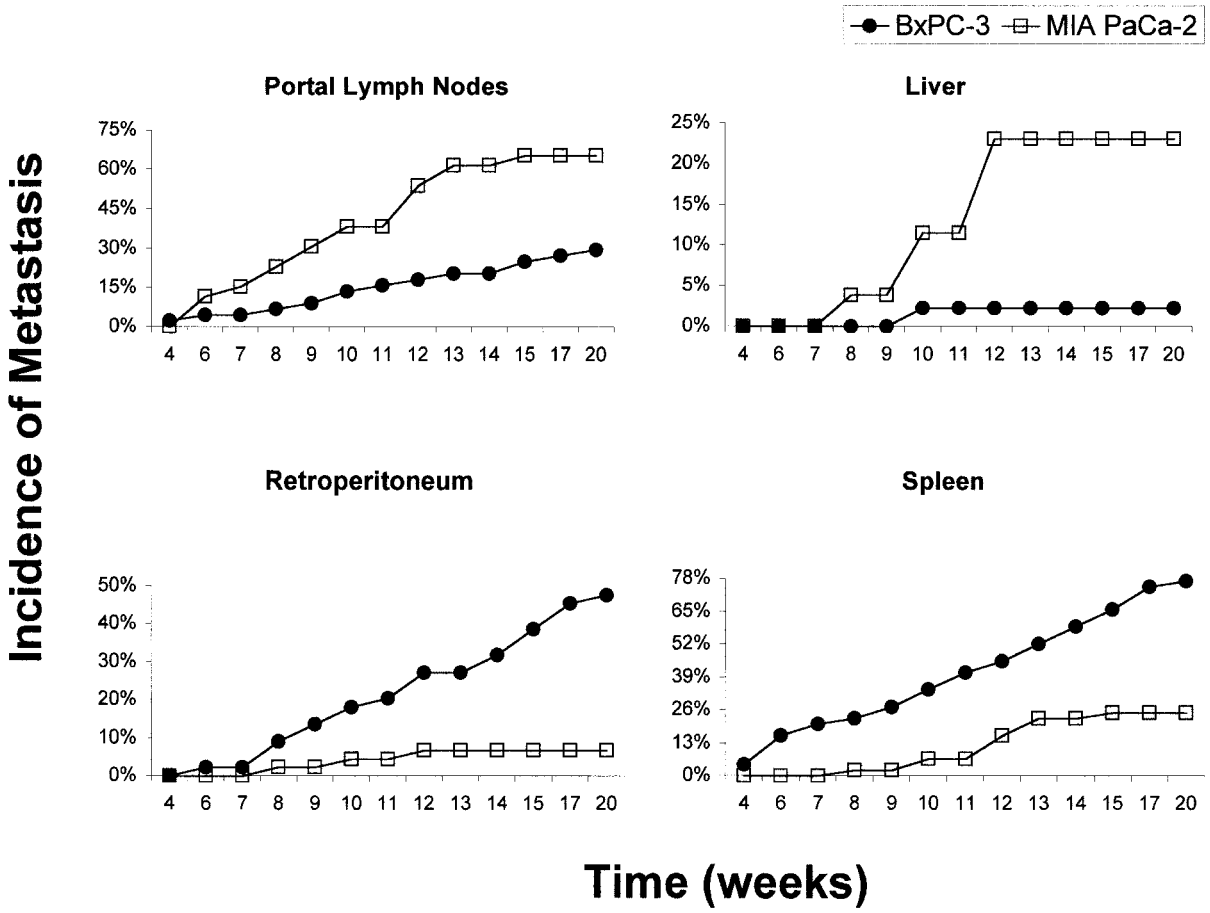

Figure 3. Chronology of site-specific metastasis. See Figure 2 for details of experimental conditions.

filter (Chroma Technology, Brattleboro, Vermont) and collected by a Hamamatsu Color Cooled CCD Video Camera HM C5810. High-resolution images were captured and processed with a Pro-Series Frame-Grabber and acquired by a Pentium-IV PC with Image Pro Plus 3.1 software (Media Cybernetics, Silver Spring, Maryland). Whole-body images were obtained by placing the mice in a fluorescent light box equipped with a fiberoptic light source of 490 nm (Lightools Research, Encinitas, CA) utilizing the CCD camera and filters mentioned above as previously described by Yang et al. [20].

Histological analysis

The primary orthotopic tumor was removed, weighed, and saved for histologic analysis carried out with standard hematoxylin and eosin (H&E) staining.

Analysis of metastasis

At indicated time points following SOI, mice were sacrificed and explored. The orthotopic tumor and all major organs were observed directly under fluorescence microscopy, images captured, and location and number of metastases were recorded for each mouse. At least one metastatic lesion per organ was needed to be present for an organ to be considered positive for metastasis. The sensitivity of the system was a single fluorescent tumor cell. The cumulative incidence

for each site of metastasis was determined by sacrificing approximately three mice per week.

Results and discussion

Isolation of stable high-level expression GFP transductants of MIA-PaCa-2 and BxPC-3 cells

The retroviral-vector transduced cells were able to grow *in vitro* at levels of G418 up to 800 μ/ml. The selected G418-resistant pancreatic cells had bright GFP fluorescence (Figure 1G). There was no difference in the cell proliferation rates of parental cells and the GFP transductants as determined by comparing their doubling times *in vitro* (data not shown).

Growth kinetics of primary pancreatic tumors

Both the MIA-PaCa-2-GFP and BxPC-3-GFP tumors grew extensively in the pancreas after orthotopic implantation. By 3–4 weeks after SOI, brightly fluorescent tumors were noted in the pancreata. MIA-PaCa-2-GFP tumors grew in a logarithmic manner, reaching a maximum weight of 3.8 grams by week-14 after SOI. BxPC-3-GFP tumors also grew logarithmically and reached a maximum weight of 10.4 grams by 15 weeks post-SOI. Both tumors showed pathologic features typical of human pancreatic adenocarcinoma on H&E staining (Figure 1H).

Tumor selective metastatic organ targeting

Figure 2 represents the incidence of metastasis in each model at week-20 post-SOI. The BxPC-3-GFP cell line produced locally-advanced, invasive tumors that metastasized regionally selectively to the spleen and the retroperitoneum with distant liver metastases very rare (Figure 2A). In contrast, metastases in the MIA-PaCa-2 model were selective to distant sites in the portal lymph nodes and liver with regional retroperitoneal lymph node metastasis very rare (Figure 2B).

Tumor selective metastatic chronology

Metastatic chronology markedly differed between the two pancreatic cancer models (Figure 3). With GFP expression, the metastatic targeting of each pancreatic tumor was readily detectable even early after transplantation (Figure 1). Rapid targeting of portal lymph nodes and liver was seen in MIA-PaCa-2 with metastases visible by 6 weeks and 8 weeks, respectively (Figure 3). At later time points, metastases were also seen in the lung (Figure 1F). In contrast, even after 20 weeks, only one mouse had retroperitoneal metastasis (Figure 3). In contrast, BxPC-3 preferentially targeted the retroperitoneum and spleen with metastasis visible by 8 and 6 weeks, respectively (Figure 3). Even at 20 weeks after SOI, only one animal with the BxPC-3 tumor had liver metastasis (Figure 3). Little data is available in the literature about the metastatic potential of the MIA-PaCa-2 and BxPC-3 cell lines [9]. Marincola previously observed liver metastasis following injection of MIA-PaCa-2 cell suspension into the pancreas of nude mice [9]. In our model, using the GFP and SOI techniques, numerous metastases at other sites in addition to the liver were visualized (Figures 1–3).

In the present study, we have utilized SOI and GFP in order to visualize the chronological specificity of pancreatic metastatic tageting for two human pancreatic cancer lines. We observed distinct chronological organ-specific metastasis for the two cancers. Both BxPC-3 and MIA-PaCa-2 have mutant p53 genes which therefore does not account for their distinct metastatic behavior [24]. BxPC-3 has wild type *ras* genes but MIA-PaCa-2 has a mutant k-*ras* [18]. Metastatic models such as described in the present report should be very useful to determine the role of such mutant oncogenes.

These two different metastatic models also provide new insights into clinical pancreatic cancer. For instance, approximately 25% of patients with pancreatic cancer present with locally-advanced unresectable tumors [19] similar to the metastatic pattern seen in the BxPC-3-GFP model. In contrast, 50% of patients have distant metastases to the liver and regional lymph nodes as seen in the MIA-PaCa-2-GFP model. Treatment strategies and prognosis differ for these two groups of patients and therefore clinically relevant models for these two major groups of pancreatic cancer patients such as we have described can be used to test new treatment modalities.

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