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Functional Imaging of Angiogenesis in an Orthotopic Model of Pancreatic Cancer

Jason B. Fleming[1]* and Rolf A. Brekken[1,2]

[1]The Hamon Center for Therapeutic Oncology Research and the Department of Surgery, University of Texas, Southwestern Medical Center, Dallas, Texas 75390-8593
[2]Department of Pharmacology, University of Texas, Southwestern Medical Center, Dallas, Texas 75390-8593

Abstract Pancreatic cancer is a major unsolved health problem. The estimated overall 5-year survival rate of only 1–4% is due to aggressiveness of the disease and the lack of effective systemic therapies. Most pancreatic cancer-related deaths are due to the development of metastases, which represents the culmination of a complex interaction between the host organism and neoplastic cells within the primary tumor. Therefore, the study of tumor–host interaction in the context of the whole organism is necessary to evaluate the pathogenesis of tumor growth and metastasis so that effective therapies can be developed. Recent advances in functional imaging combined with animal models that faithfully recreate the biology of human tumors have elevated our ability to examine these complex interactions. In this review, we will use the example of orthotopic mouse models of pancreatic cancer as a tool to survey the challenges and possibilities of functional imaging of angiogenesis, a critical determinant of metastasis. J. Cell. Biochem. 90: 492–501, 2003.

Key words: pancreatic cancer; angiogenesis; non-invasive imaging; orthotopic models

ANGIOGENESIS AT THE INTERFACE OF TUMOR–HOST INTERACTION

Angiogenesis is a fundamental tumor–host interaction that represents the cornerstone for pancreatic tumor growth and metastasis. The oxygen and nutrient requirements supplied by the vasculature are crucial for cell function and survival, obligating virtually all cells in a tissue to reside within 100 μm of a capillary blood vessel [Tannock, 1970]. Malignant transformed cells within aberrant proliferative lesions initially lack angiogenic ability, which limits expansion and size. In order to advance to a larger size, neoplasias must develop their own vasculature [Hanahan and Weinberg, 2000]. The stimulus and maintenance of angiogenesis in tumors occurs by at least two mechanisms; (1) tumors secrete angiogenic growth factors, such as vascular endothelial growth factor (VEGF) that bind to specific receptors on endothelial cells and stimulate angiogenesis, (2) tumors co-opt existing mature blood vessels. Both types of vessels develop structural and physical abnormalities that result in increased vascular permeability and tumor blood flow that is sluggish and irregular [Carmeliet and Jain, 2000]. Despite efforts to elucidate the molecular determinants of angiogenesis, surprisingly little is known about the nature of the vascular bed in human tumors. It is clear that high microvessel density (MVD) counts within vascular hot spots of tumors correspond with a poor prognosis for the patient [Hlatky et al., 2002]. However, MVD studies using pan-endothelial cell markers do not give an indication of the angiogenic status of a tissue vascular bed. Alternative methods of quantitating angiogenesis in preclinical models of cancer are needed. These methods might encompass the use of activation-specific

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*Correspondence to: Jason B. Fleming, MD, Department of Surgery, UT-Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9155.
E-mail: jason.fleming@utsouthwestern.edu

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markers of angiogenesis [Brekken et al., 2002] and/or non-invasive methods of examining the function of tumor vasculature, such as blood flow and tissue perfusion measurements [Mason et al., 2002].

Most early studies of angiogenesis in pancreatic tumors were conducted with (ectopic) xenograft models in which human pancreatic cancer cells were implanted within the flank of immunocompromised mice. Although ectopic sites are easily accessible, increasing evidence demonstrates that orthotopic (tumor cells within organ of origin) and ectopic environments differentially influence tumor cell gene expression, tumor growth, invasiveness, angiogenesis, metastasis, drug delivery, and sensitivity to therapeutic agents [Killion et al., 1998; Fidler et al., 2002]. The microenvironment of the pancreas and the unique characteristics of the tumors that derive from it contribute to the aggressive growth of pancreatic cancer and its resistance to therapy. The importance of the microenvironment of the pancreas on the angiogenic process was highlighted by a recent study that demonstrated enhanced expression and activity of vascular endothelial growth factor (VEGF) in tumors grown in the pancreatic microenvironment compared to tumors grown subcutaneously [Tsuzuki et al., 2001]. Furthermore, increased VEGF levels were consistent with an observed increase in tumor growth in the orthotopic setting compared to the subcutaneous environment [Tsuzuki et al., 2001].

Given the importance of orthotopic models of pancreatic cancer, this laboratory and others have sought to develop animal model systems that will enable study of the growth, progression, metastasis, and therapy of pancreatic cancer [Capella et al., 1999; Bouvet et al., 2002] in the orthotopic setting. The use of orthotopic models, however, presents technical hurdles for accurate examination of complex biologic processes such as angiogenesis. The pancreas is a retroperitoneal organ that lies behind the stomach and is, therefore, difficult to examine. Tsuzuki et al. [2001] utilized abdominal wall window techniques to examine angiogenesis and microcirculation of orthotopic pancreatic tumors. While useful for this study, methods that are less invasive, more sensitive, and allow for whole animal evaluation are needed to further investigate tumor physiology related to angiogenesis.

FUNCTIONAL IMAGING OF ANGIOGENESIS

The broad imaging goals when measuring tumor angiogenesis in orthotopic models of pancreatic cancer include: (1) detection and accurate assessment of primary tumor development, growth, and metastasis; and (2) measurement of specific parameters related to tumor angiogenesis (sluggish blood flow, high capillary permeability, tissue hypoxia, and related metabolism). Technical advances now enable imaging of mice with ultrasound, magnetic resonance, fluorescence, and nuclear techniques. Because each of these modalities has inherent strengths and weaknesses, studies of angiogenesis in orthotopic animal models will likely require multiple approaches. We will examine how the functional imaging modalities available meet the stated goals of imaging angiogenesis in orthotopic models of pancreatic cancer.

Scanning tissue with ultrasound waves (2–10 MHz) is a quick, inexpensive, and portable procedure that is capable of generating structural and functional images of the target tissue [Turnbull et al., 1996]. Furthermore, because the images are obtained in real-time the animal does not need to be immobilized for an extended period of time or undergo anesthesia. A drawback to ultrasound imaging is the limited depth of penetration at the higher wave frequencies necessary to resolve small organs in mice. However, ultrasound examination with a 10 MHz transducer in B-mode (grayscale) has monitored successfully the development of carcinogen-induced hepatic tumors [Oh et al., 2002] as well as colorectal metastases in mice [Kruskal et al., 2000]. Ultrasound is an "operator-dependent" technique that requires practice. However, data from this laboratory and others demonstrate that this imaging modality does possess the ability to obtain structural information (size) about orthotopic pancreatic tumors (Fig. 1). Therefore, because of the efficiency with which it can be used, ultrasound is a highly functional method for following pancreatic tumor growth in mice.

Ultrasound methods can also be used to assess the function of the vasculature in the target organ. The Doppler effect created when sound returns after striking moving red blood cells enables ultrasound to provide real-time measurements of vascular flow in tumors. Tumor vasculature is tortuous, irregular, and inefficient and therefore, can be distinguished from

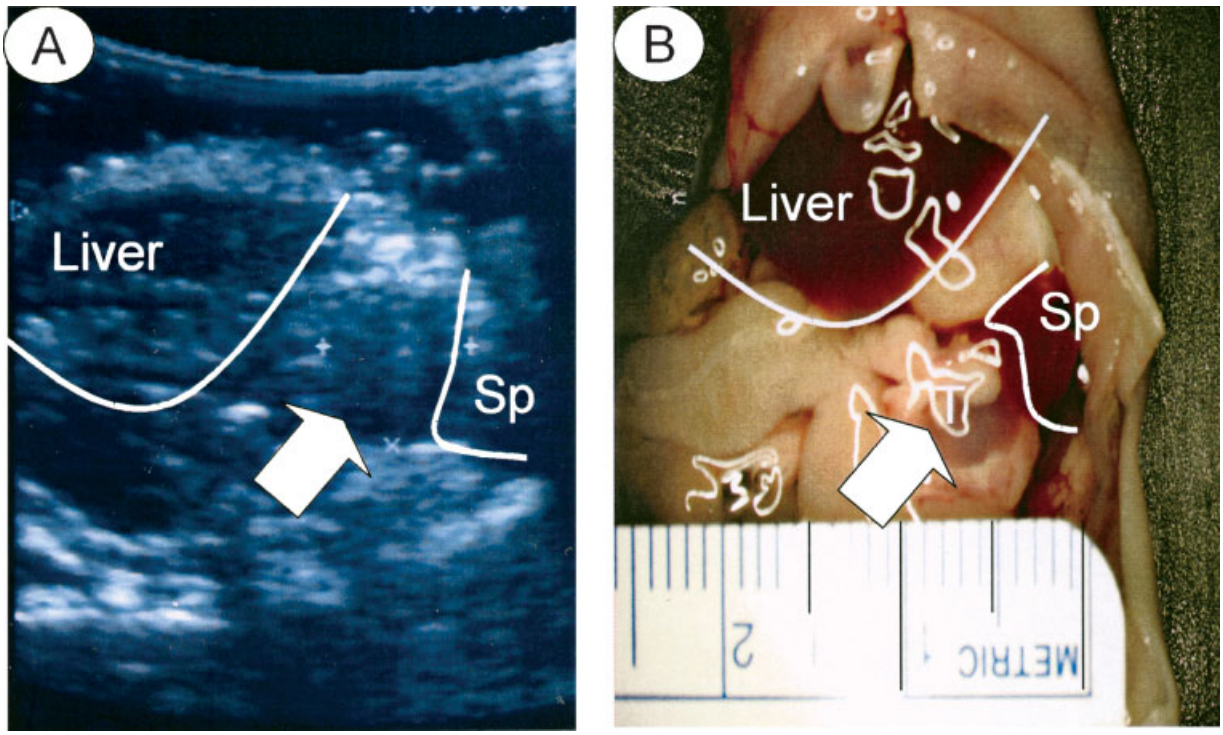

Fig. 1. **A**: Transabdominal ultrasound image obtained using 7.5 MHz probe of animal bearing orthotopic pancreatic tumor. **B**: Necropsy demonstrating same orthotopic tumor with the same diameter measurements (8 mm) found during ultrasound examination.

vasculature in normal tissue by color Doppler examination with direct, high-frequency ultrasound. The ease and speed of this technique elevates the likelihood that it will be used for imaging tumor vasculature in mouse orthotopic models of cancer, as evidenced by a recent study by Drevs et al. [2000], which used high-frequency ultrasound to demonstrate a reduction in blood flow in an orthotopic renal cell carcinoma after therapy with an anti-angiogenic agent. Contrast agents for ultrasound are a recent technological development; the most common approach is to use intravenous injection of small (<3 μm) air or gas bubbles (microbubbles). Microbubbles resonate at the high frequencies used for diagnostic imaging making them several thousand times more reflective than normal red blood cells. In this way they enhance both normal grayscale and blood flow mediated Doppler signals. Additionally, microbubbles can be engineered to facilitate drug delivery to the tumor vasculature, both by potentiating the production of transient cell membrane pores ("sonoporation") and by acting as drug delivery vehicles [Porter et al., 2001; Unger et al., 2001].

Radionuclides are attractive agents for selective nuclear imaging of tumors by chemical conjugation to tumor-specific antibodies and other biologically active molecules. Image resolution can be 1–2 mm with no depth limitation, an important fact given the deep location of many orthotopic tumors including those implanted into the pancreas. In single photon emission tomography (SPECT) imaging, a gamma-ray-emitting nuclide, such as ^{99}Tc, ^{111}In, ^{123}I, or ^{125}I, is used to track individual molecules such as monoclonal antibodies (mAb). We have used SPECT to track an ^{111}In-labeled anti-VEGF antibody that localizes to VEGF bound to stromal tissue in tumors (Fig. 2). This approach is effective for the localization of labeled mAbs to subcutaneous tumor xenografts; however, orthotopic pancreatic tumors have not yet been targeted in this manner. We anticipate that localization of the anti-VEGF mAb to tumor stroma will not only be an effective means of tumor identification in the orthotopic setting but that the strength of signal will correlate to the amount of VEGF-induced angiogenesis ongoing in the target tissue. In this way biodistribution of VEGF within the tumor can be

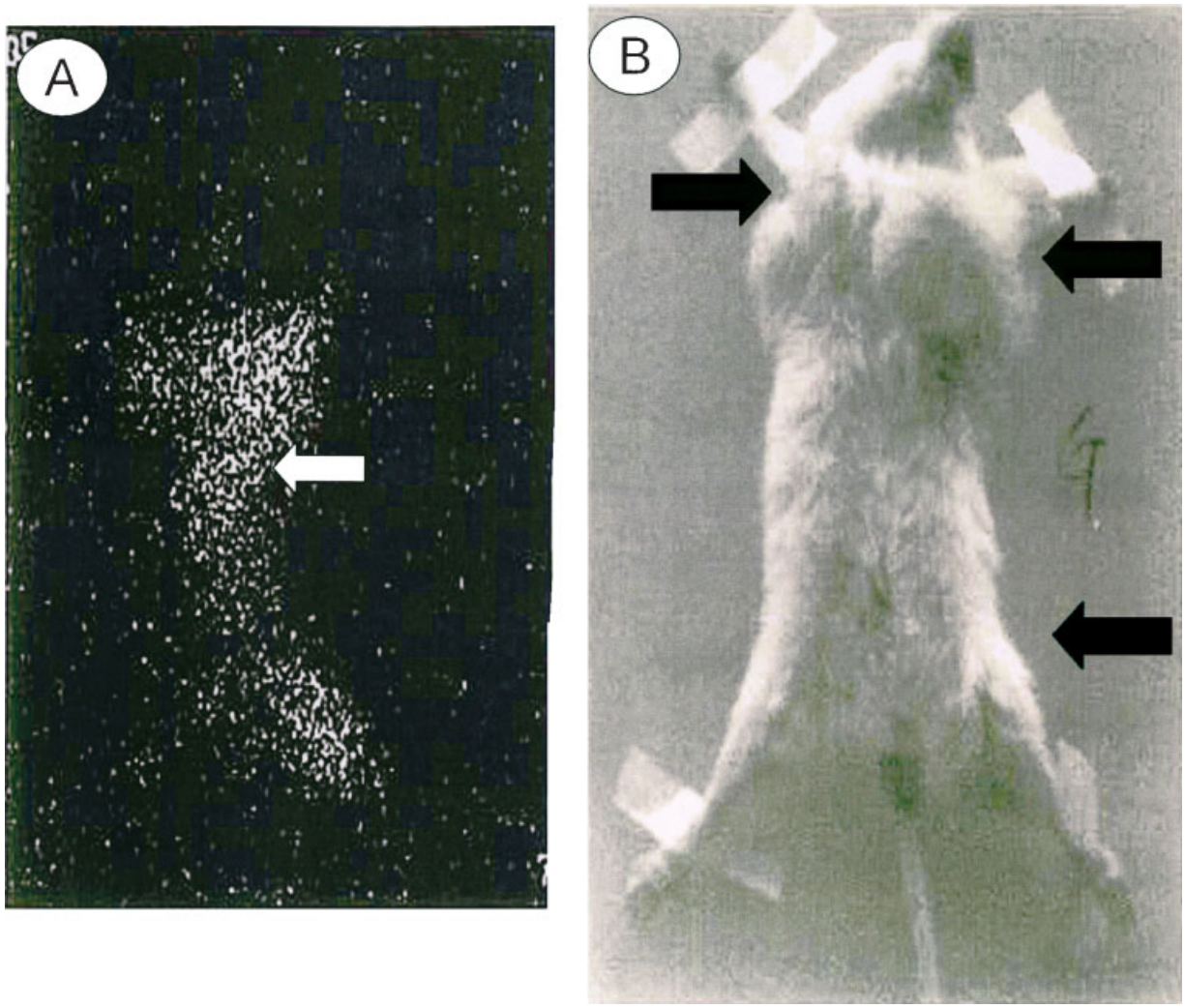

Fig. 2. **A**: Gamma camera images 120 h after intravenous delivery of ^{111}In-anti-VEGF mAb in SCID mice (**B**) bearing subcutaneous human tumor xenografts on three limbs (black arrows). Localization within the tumor is shown as well as some uptake in liver (white arrow).

mapped and compared to traditional ex vivo measurements of tumor angiogenesis (e.g., MVD and vascular area).

Positron emission tomography (PET) is an imaging technique used to detect decaying nuclides such as ^{15}O, ^{13}N, ^{11}C, ^{18}F, ^{124}I, and ^{99}Tc. PET generates tomographic images that provide both structural and functional information about the target tissue. The molecule ^{18}fluorodeoxyglucose (FDG) has a high positive predictive value in human patients diagnosed with pancreatic adenocarcinoma [Kalady et al., 2002], which is likely due to changes in the glycolytic pathway utilized by tumor cells within a hypoxic microenvironment [Bos et al., 2002]. PET studies using ^{18}FDG, which correlates with tissue metabolism and ^{18}fluoromisonidazole (FMISO), which correlates with tissue hypoxia [Rajendran et al., 2003] might be an ideal tool to study the relationship between glucose metabolism, hypoxia, and angiogenesis in pancreatic tumors. Initial studies of spontaneous orthotopic tumors arising in a transgenic mouse model of pancreatic cancer have been performed with FDG-PET [Seitz et al., 2001] with convincing results that this imaging modality can identify orthotopic pancreatic tumors. PET is also useful for imaging vasculature in tumors. For example, dermatan sulfate, a glycosaminoglycan (GAG) found on a variety of proteoglycans (e.g., decorin, biglycan, veriscan) [Trowbridge and Gallo, 2002] has been found to localize to tumor vasculature when delivered systemically in a tumor-bearing animal (Antich,

personal communication). Accumulation of dermatan sulfate conjugated to a positron-emitting agent (desferoxamine) can be followed with PET and used to image both primary and metastatic tumor tissue (Fig. 3). Additionally, a recent clinical study demonstrated that PET can be used to monitor changes in perfusion of tissues, including tumor tissue after adminstration of therapeutics designed to alter blood flow in the tumor [Anderson et al., 2003]. Disadvantages to PET are the expense associated with the assay and the need for a cyclotron and radiochemistry facility to use this technology.

Magnetic resonance imaging (MRI) is a non-invasive imaging technique that is being used with increasing frequency in conjunction with rodent tumor models. The fundamental principle underlying MRI is that unpaired nuclear spins (such as, hydrogen atoms in water and organic compounds) align themselves when placed into a magnetic field. A temporary radio-frequency pulse is then given to change the alignment of the spins, and their return to baseline is recorded as a change in electromagnetic flux [Weissleder, 2002]. Drawbacks to small animal MRI include motion artifacts induced by the rapid respirations and diaphragmatic excursion of the animal and establishing and maintaining intravenous access for injection of contrast materials. Once these hurdles are overcome monitoring tumor growth and response to therapy in vivo is, perhaps, the most straightforward application of MRI in the study of cancer. The non-invasive, non-destructive nature of MRI is particularly advantageous, as individual subjects can be serially monitored over an extended time period. These properties have prompted the use of MRI to monitor transgenic models for spontaneous cancer development [Messerli et al., 2002]. We have used MRI to obtain sequential measurements of orthotopic xenograft pancreatic tumors (Fig. 4) and are pursuing the use of MRI as a marker of vascular function in the face of different anti-angiogenic and anti-neoplastic strategies. Similar studies by other investigators have already shown that a differential response to therapy in a mouse orthotopic pancreatic tumor model can be monitored by MRI [He et al., 2000].

MRI techniques recently have been developed that yield physiologic information including levels of perfusion, diffusion, and oxygenation [Blood Oxygen Level Dependent MRI, BOLD] in addition to high-resolution anatomical detail

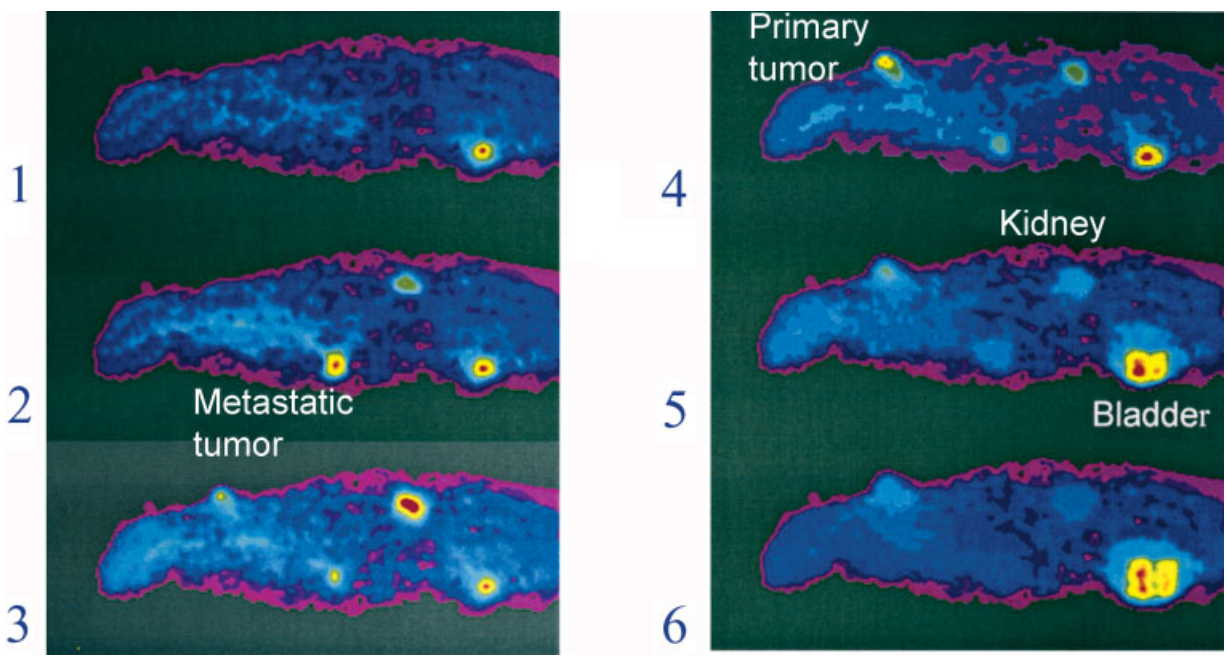

Fig. 3. Sagittal positron emission tomography (PET) images (six slices, 3 mm thickness, 6 mm interslice separation) of mammary adenocarcinoma (280 mg) after injection of tumor endothelial-specific molecule labeled with ^{68}Ga. Slices 2 through 4 demonstrate the primary tumor and metastatic focus within the chest. Uptake of radionuclide within the kidney and bladder is also visible. (Image courtesy of Dr. P. Antich, UT-Southwestern Medical Center.)

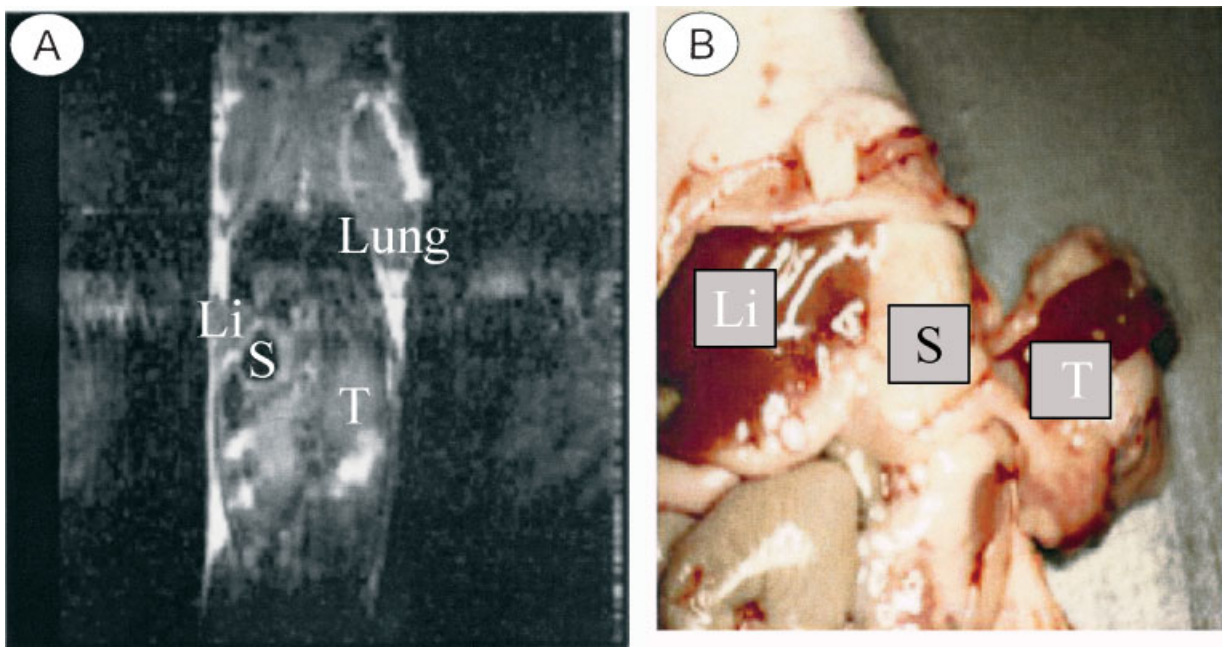

Fig. 4. **A**: T1 weighted coronal magnetic resonance image of a mouse bearing an orthotopic pancreatic tumor adjacent to stomach (S) and opposite side of the abdomen from the liver (Li). **B**: Necropsy of the same animal demonstrating orthotopic tumor within pancreas adjacent to spleen and stomach.

[Weissleder, 2002]. Fast injection of MR contrast agents into the blood circulation is followed by rapid distribution in the intravascular volume. Contrast medium, present in the capillaries and the extracellular extravascular space, provides signal enhancement during the first-pass, which then plateaus, and finally decreases. The enhancement rate and onset of enhancement in a given tissue depend upon: the number of vessels, perfusion (flow) through the vessels, vessel resistance, vessel wall permeability, composition of the extracellular space, and venous outflow. The kinetics of these parameters can be monitored in vivo yielding information on the perfusion and permeability of the target tissue [Degani et al., 1997]. Indeed, these techniques have been used to demonstrate changes of vascular permeability and vessel density after inhibition of VEGF in an animal model of gliobastoma multiforme [Gossmann et al., 2002].

The application of BOLD contrast MRI for non-invasive monitoring of vascular maturation depends on the ability to selectively detect the increased vasoreactivity to increased arterial CO_2 tension (hypercapnea) in mature vessels, while the entire functional vascular bed is detected by signal changes in response to hyperoxia. BOLD contrast mechanisms originate from the magnetic susceptibility of hemoglobin, which is dependent on its oxygen state. With this technique observed signal changes correlate with differing vascular densities and distributions in orthotopic tumor models [Robinson et al., 2003]. BOLD has also been used to map the mature vasculature in tumor xenografts. Importantly, these results were confirmed by the use of intravital microscopy, which co-registered mature vasculature as determined by BOLD analysis with vessels that have mural (smooth muscle or pericyte) cell support [Neeman et al., 2001]. Similar methods have been used to evaluate the response of human tumor xenografts in rodents to vascular targeting agents designed to occlude tumor blood vessels. Results from this study demonstrated a heterogeneous response within the tumor that corresponded with histological evidence of vascular occlusion and response of the tumor to therapy [Mason et al., 2002]. These studies highlight that functional MRI is capable of mapping heterogeneous regions of oxygenation and perfusion within the tumor and therefore, represents one of the most versatile imaging modalities for following angiogenesis in orthotopic models of pancreatic cancer.

In vivo fluorescence imaging is one of the most inexpensive and rapid ways to image particular

cells or targets in mice. The development of sensitive charge coupled device (CCD) cameras [Rice et al., 2001] combined with ability to label cells of interest with luciferase (usually of the North American firefly (*Photinus pyralis*)) or the *green fluorescent protein* (*GFP*) gene of the jellyfish *Aequorea victoria* has quickly made fluorescence imaging a useful method of tracking tumor growth and progression in vivo [Hoffman, 1998]. Cells expressing GFP will emit fluorescence after excitation with light of the appropriate wavelength (398 nm). A major advantage of fluorescence imaging of GFP in vivo is that imaging does not require any contrast agents, substrates, or light-tight boxes in order to achieve whole mouse imaging. For example, our laboratory has stably transfected enhanced GFP into multiple human and mouse pancreatic tumor cell lines and have obtained whole mouse images of orthotopic pancreatic tumors and metastases (Fig. 5). We have determined that with the use a highly sensitive CCD camera even limited disease can be detected in vivo, which is consistent with the results of other investigators who found that GFP positive metastatic nodules of 150 μm in diameter (about 1,000–10,000 cells) are detected easily in the liver and peritoneum of mice [Bouvet et al., 2002]. Problems that are typically associated with non-invasive imaging of GFP include autofluorescence of mouse skin and poor penetration of excitation and emission light through skin and surface organs (Fig. 5C). To overcome these difficulties the skin over tumor-bearing

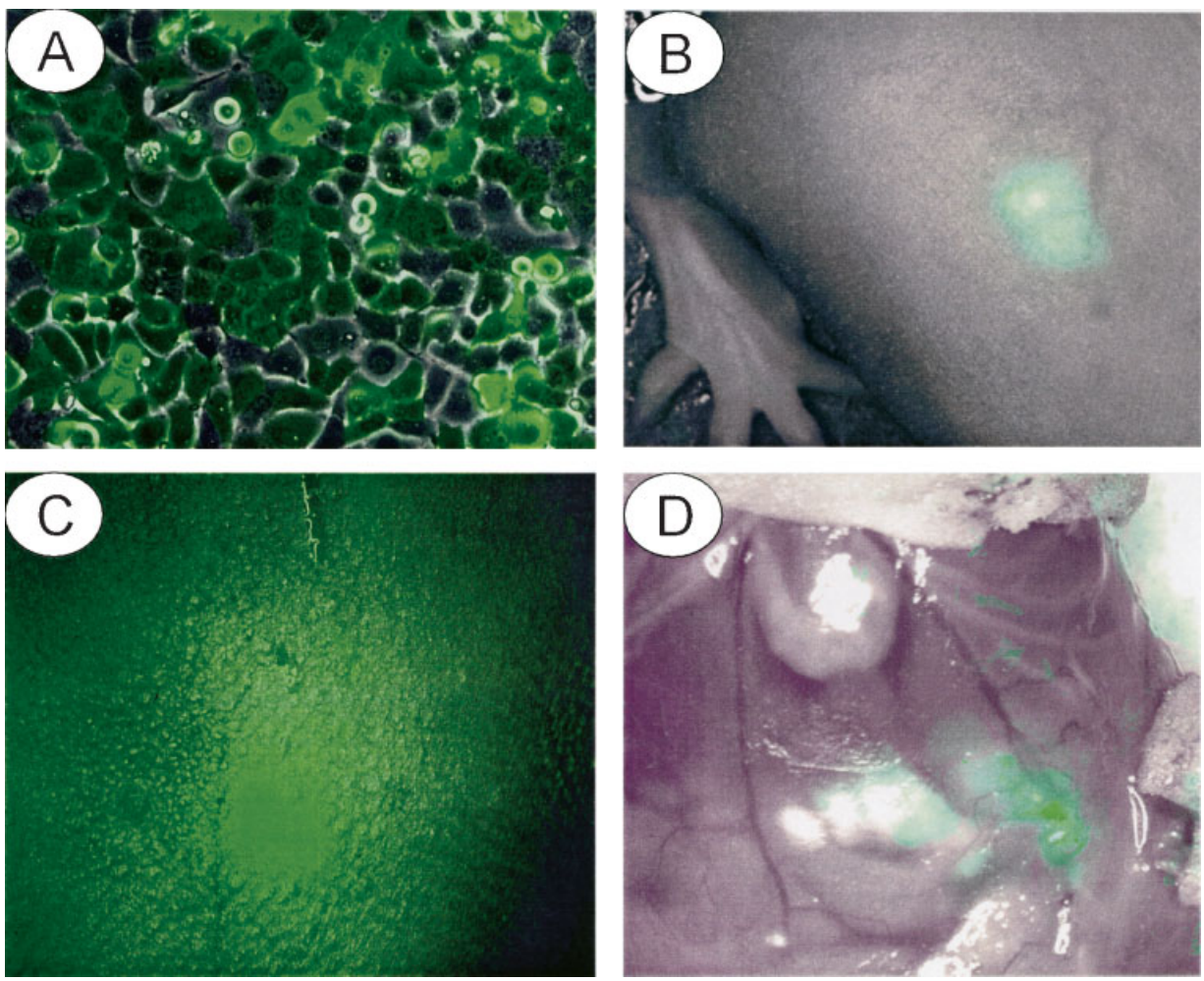

Fig. 5. Fluorescent images of (**A**) human pancreatic cancer cells stably transfected with enhanced GFP and (**B**) a subcutaneous tumor derived from these cells. **C**: Transabdominal image of orthotopic GFP-positive pancreatic tumor demonstrates autofluorescence of abdominal wall obscuring signal. **D**: After skin flap performed signal identified from tumor within mouse pancreas.

regions often must be exposed surgically (Fig. 5D). The skin flap method allowed Yang et al. [2002] to quantify tumor vascularity by measuring the number of tumor vessels per area during tumor growth. It is important to keep in mind that GFP is an exogenous gene product that might affect cell and tissue function. As the use of GFP-expressing cells has widened, several reports of GFP effects on cell function have surfaced. Murine models of leukemia and lymphoma using GFP-expressing tumor cells demonstrated a reduction in anticipated disease development after transplantation into immunocompetent mice suggesting that an immune response against GFP may interfere with results of animal cancer models [Stripecke et al., 1999]. Likewise, Zhang et al. [2003] demonstrated that endothelial cells engineered to express GFP showed increased levels of heat shock protein 70 (HSP70) and induction of cyclooxygenase-2 (COX-2) followed by increased prostaglandin E2 (PGE2) compared to control cells. Furthermore, adenoviral delivery of GFP into murine vasculature resulted in enhanced blood flow, suggesting that PGE2 produced as a result of GFP expression is sufficient to induce vasodilation [Zhang et al., 2003]. While fluorescent imaging through GFP is likely to increase in use, the potential affect of GFP on host immune and vascular function might compromise its utility in orthotopic models.

Bioluminescence imaging (BLI) is used to detect photons that emanate from cells that have been genetically engineered to express the luciferase gene. Luciferase, when exposed to the substrate luciferin, releases photons that can be detected and quantified using low light photon-counting cameras. BLI was initially used for gene expression studies in vitro [Engebrecht et al., 1985] but has also proven to be valuable for monitoring gene expression in vivo [Contag et al., 1995] and to non-invasively monitor tumor growth in mice [Edinger et al., 2002]. In contrast to fluorescent imaging, the target to be imaged does not need to be exposed to light of an excitation wavelength; but instead, the substrate, luciferin, must be injected into the animal and the substrate must interact with luciferase to produce a signal. However, like fluorescent imaging, tissue penetration is a concern such that deeper light sources are more difficult to reliably quantitate [Edinger et al., 2002]. A significant advantage of bioluminescent reporters is the low level of background noise and thus the high sensitivity that can be achieved, evidenced by the fact that as few as 100 luciferase positive tumor cells can be detected within the peritoneal cavity by BLI [Edinger et al., 1999]. Luciferase and its substrate, luciferin, have not been shown to be toxic to mammalian cells and no functional differences have been observed, thus far, between luciferase expressing clones and parental cells [Sweeney et al., 1999].

A logical direction for future functional imaging studies will be to use fluorescence reporters (luciferase and GFP) driven by promoters of a gene of interest so that temporal expression of genes related to tumor angiogenesis (hypoxia-induced genes) can be measured in vivo within an orthotopic tumor. Recently, real-time measurements of p53 transcriptional activity in colon cancer cells was detected non-invasively by BLI of subcutaneous tumors [Wang and El-Deiry, 2003]. In a similar study, 26S proteasome activity was measured in tumor xenografts by the use of a ubiquitin-luciferase reporter that was degraded rapidly under steady-state conditions and stabilized in a dose- and time-dependent manner in response to proteasome inhibitors [Luker et al., 2003]. Models in which cells to be implanted orthotopically are manipulated to allow monitoring of target gene expression will likely be a frequently used method of molecular imaging to monitor temporal changes during growth and metastasis or response to therapy.

The integration of BLI and fluorescent imaging into transgenic and knockout animals will allow examination of carcinogenesis at the earliest time points since spatial and temporal information about gene expression can be repeatedly examined in real-time. An example is the recent examination of a GFP transgenic mouse in with the mouse mammary virus promoter (MMTV) was used to drive GFP expression; when used in combination with a Cre-responsive promoter, strong mammary-specific expression was achieved. Highly metastastic GFP-expressing tumors within the mammary tissue developed from precurser lesions that could also be imaged [Ahmed et al., 2002]. In addition to imaging the tumor compartment transgenic models will allow for investigation of the host response. Fukumura et al. [1998] established a line of transgenic mice expressing GFP under the control of the promoter for VEGF. Implantation of solid tumors in the transgenic

mice lead to an accumulation of green fluorescence resulting from tumor induction of VEGF promoter activity within host cells (mainly fibroblasts) invading the tumor. Additionally, spontaneous mammary tumors induced by oncogene expression in the VEGF-GFP mouse show strong stromal, but not tumor, expression of GFP [Fukumura et al., 1998].

CONCLUDING REMARKS

In the future multifunctional reporter genes that link two or more modalities will likely be used, in fact fusion genes for bioluminescent and GFP imaging are already available [Day et al., 1998]. Similarly, dual reporters for combining nuclear imaging techniques and fluorescence for gene expression have been developed [Doubrovin et al., 2001] and used successfully to image ectopic xenograft tumors in mice with PET and BLI [Ray et al., 2003]. As these technologies mature and become broadly accessible, new reporter combinations will be explored to optimize the strategies for following tumor growth and response to therapy in orthotopic settings. With these advancements our ability to dissect the complex tumor–host interactions that contribute to angiogenesis in pancreatic cancer will also improve.

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