

Dual-color Imaging of Nascent Angiogenesis and its Inhibition in Liver Metastases of Pancreatic Cancer

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Abstract. *As previously shown, the stem cell marker nestin is expressed in nascent blood vessels in transgenic nestin-driven green fluorescent protein (ND-GFP) nude mice. This mouse model was recently utilized to evaluate angiogenesis in primary tumors in an orthotopic model of pancreatic cancer. In the present study, nascent angiogenesis of pancreatic cancer liver metastasis in the ND-GFP transgenic nude mice after splenic injection of low-passage xPA-1 human pancreatic cancer cells expressing red fluorescent protein (RFP) was visualized by dual-color fluorescence imaging. ND-GFP was highly expressed in proliferating endothelial cells and nascent blood vessels in the growing liver metastasis. Immunohistochemical staining showed that CD31 co-localized in ND-GFP-expressing nascent blood vessels. The density of nascent blood vessels in the tumor was readily quantitated. Gemcitabine significantly decreased the mean nascent blood vessel density in the pancreatic liver metastases. In conclusion, the dual-color model of the ND-GFP nude mouse with RFP-expressing pancreatic cancer liver metastases, enabled the simultaneous visualization and quantitation of nascent angiogenesis and its response to angiogenesis inhibitors. This model will be useful for understanding the mechanism of angiogenesis of pancreatic cancer liver metastasis and for the discovery of effective new inhibitors of this process.*

A novel transgenic nude mouse was recently developed for the imaging of human tumor angiogenesis. In this mouse

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model, the stem-cell marker nestin is expressed in nascent blood vessels. A regulatory element of nestin drives green fluorescent protein (ND-GFP) in this transgenic mouse enabling nascent blood vessels to be visualized by their GFP expression. Many human and rodent cancer cell lines expressing red fluorescent protein (RFP) were implanted in the ND-GFP nude mice and grew extensively. ND-GFP was highly expressed in proliferating endothelial cells and nascent blood vessels in the growing tumors, visualized by dual-color fluorescence imaging (1).

Doxorubicin inhibited the nascent tumor angiogenesis, as well as tumor growth in the ND-GFP mice transplanted with B16F10-RFP murine melanoma (2).

Primary tumor angiogenesis in the ND-GFP transgenic nude mice with orthotopically transplanted MIA PaCa-2 human pancreatic cancer expressing RFP was also visualized by dual-color imaging. Gemcitabine significantly decreased the mean nascent blood vessel density in the tumor, as well as decreased tumor volume. These results demonstrated for the first time that gemcitabine is an inhibitor of angiogenesis, as well as tumor growth in pancreatic cancer (3).

In the present study, angiogenesis of liver metastasis of the XPA-1-RFP human pancreatic cancer in the ND-GFP transgenic nude mice was visualized by dual-color fluorescence imaging. ND-GFP was highly expressed in proliferating endothelial cells and nascent blood vessels in the growing liver metastasis. The density of nascent blood vessels in the liver metastasis was readily quantitated by ND-GFP expression. Gemcitabine significantly decreased the mean nascent blood vessel density in the liver metastases.

Materials and Methods

ND-GFP transgenic nude mice (1, 4). ND-GFP transgenic C57/B6 mice carry GFP under the control of the nestin second-intron enhancer. In the present study, the ND-GFP gene was crossed into nude mice on the C57/B6 background to obtain ND-GFP nude mice.

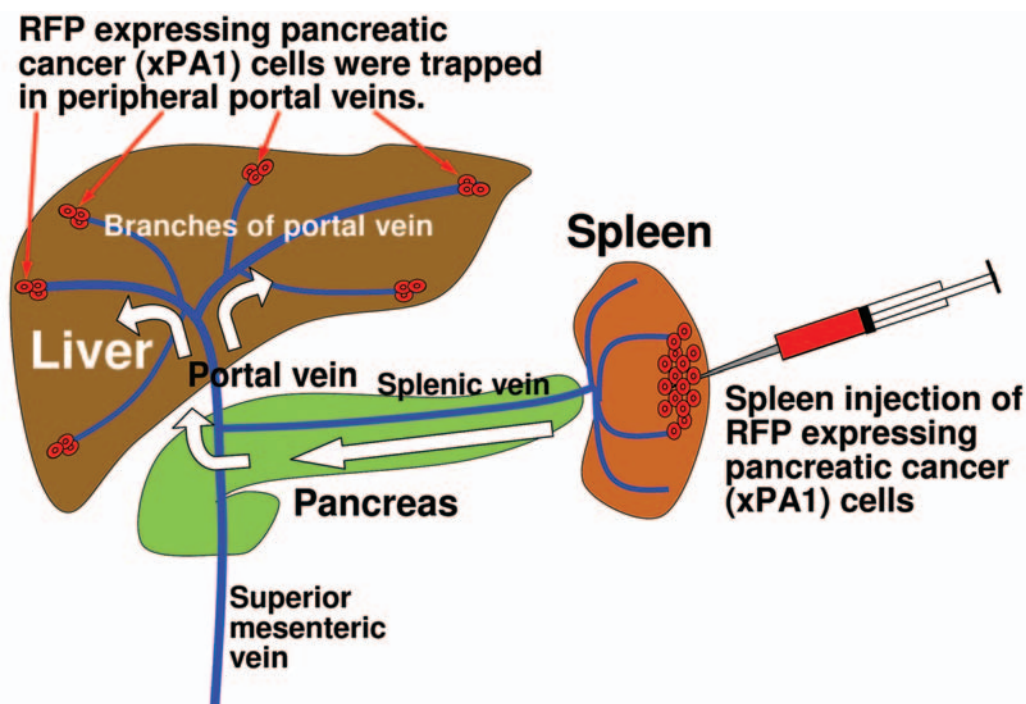


Figure 1. Experimental hepatic metastases of pancreatic cancer. RFP-expressing human pancreatic cancer (xPA1) cells (2×10^6 in $50 \mu\text{l}$) were injected in the spleen of ND-GFP transgenic mice.

Red fluorescent protein vector production (1). The RFP (DsRed-2) gene (Clontech, Palo Alto, CA, USA) was inserted in the retroviral-based mammalian expression vector pLNCX (Clontech) to form the pLNCX DsRed-2 vector. Production of retrovirus resulted from transfection of pLNCX DsRed-2 in PT67 packaging cells, which produced retroviral supernatants containing the DsRed-2 gene. Briefly, PT67 cells were grown as monolayers in DMEM supplemented with 10% FCS (Gemini Biological Products, Calabasas, CA, USA). Exponentially growing cells (in 10 cm dishes) were transfected with 10 μg expression vector using a Lipofectamine Plus (GIBCO-BRL, Grand Island, NY, USA) protocol. Transfected cells were replated 48 h after transfection in 100 $\mu\text{g}/\text{mL}$ G418. Two days later, the amount of G418 was increased to 200 $\mu\text{g}/\text{mL}$. After 25 days of drug selection, surviving colonies were visualized under fluorescence microscopy and RFP-positive colonies were isolated. Several clones were selected and expanded into cell lines after virus titering on the 3T3 cell line.

RFP gene transduction of tumor cell lines (1). For RFP gene transduction, 20% confluent xPA-1 human pancreatic cancer cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 cells and RPMI 1640 or other culture media (GIBCO) containing 10% fetal bovine serum (Gemini Biological Products) for 72 h. Fresh medium was replenished at this time. Tumor cells were harvested with trypsin/EDTA and subcultured at a ratio of 1:15 into selective medium, which contained 50 $\mu\text{g}/\text{mL}$ G418. To select brightly fluorescent cells, the level of G418 was increased to 800 $\mu\text{g}/\text{mL}$ in a stepwise manner. Clones expressing RFP were isolated with cloning cylinders (Bel-Art Products) with trypsin/EDTA and were amplified and transferred by conventional culture methods in the absence of selective agent.

Experimental model of liver metastases of pancreatic cancer. ND-GFP transgenic nude mice, 6-8 weeks old, were used. The mice were anesthetized with tribromoethanol. xPA1-RFP cells, grown in RPMI medium with 10% fetal bovine serum, were detached from the culture flask by a brief incubation with phosphate-buffered saline without calcium and magnesium (PBS) (Cellgro, Herndon, VA, USA) containing 2 mM EDTA. The cancer cells were suspended in RPMI 1640/10% FBS, washed and resuspended in PBS. Fifty microliters containing 2×10^6 xPA1-RFP cells per mouse were injected in the spleen with a 27G syringe (Becton Dickinson) (Figure 1).

Histology. Tumor samples were excised from the liver under anesthesia on days -21 and -35 after injection. The tumor samples were embedded in tissue-freezing embedding medium and frozen at -80°C overnight. Frozen sections 5- μm thick were cut with a CM1850 cryostat (Leica, Deerfield, IL, USA) and were air-dried.

Measurement of length and density of nestin-positive nascent blood vessels (1). Angiogenesis was quantified in the tumor tissue by measuring the length of ND-GFP nascent blood vessels in all fields under fluorescence microscopy. All fields at $\times 40$ or $\times 100$ magnification were measured to calculate the total length of ND-GFP-positive nascent blood vessels. The vessel density was calculated by total length of ND-GFP nascent blood vessels divided by tumor volume.

Immunohistochemical staining (1). Colocalization of ND-GFP fluorescence, the endothelial cell marker CD31, was visualized in frozen sections. Detection was with the anti-rat immunoglobulin horseradish peroxidase (HRP) detection kit (BD PharMingen, San Diego, CA, USA; CD31) following the instructions of the

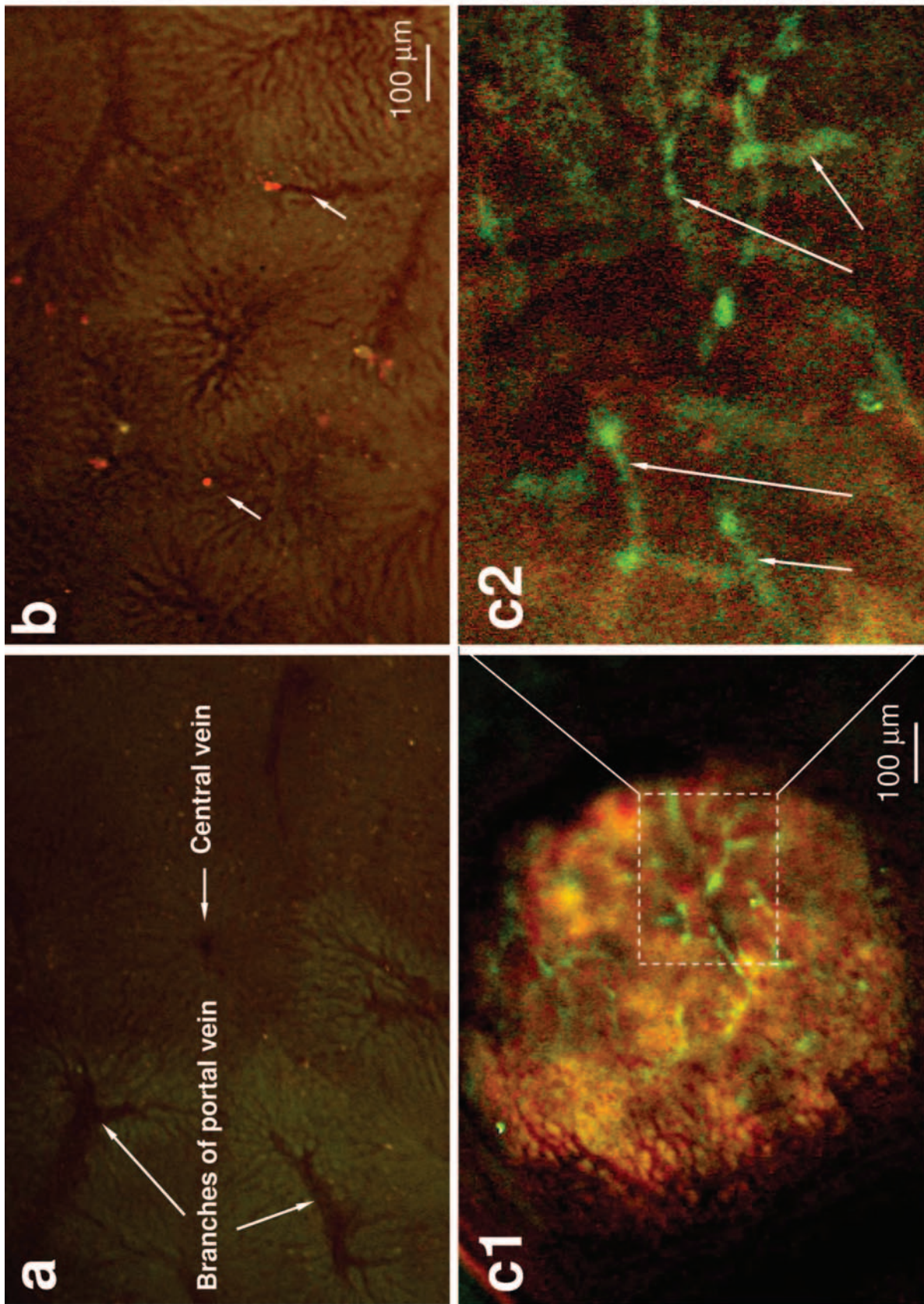


Figure 2. Dual-color imaging of nascent blood vessels in the hepatic metastases. (a) Normal liver of ND-GFP transgenic mice. (b) Human pancreatic cancer (xPA1) cells, expressing RFP, injected into the spleen of ND-GFP transgenic mice. The liver was observed directly in live mice with the Olympus OV100 Small Animal Imaging System. Right after splenic injection, RFP-expressing tumor cells were visualized in branches of the portal vein. (c1) At day-21, nascent blood vessels were seen to express ND-GFP around the growing RFP-expressing pancreatic tumor cells in the liver. (c2) This is a higher magnification image of c1.

manufacturer. The primary antibody used was CD31 monoclonal antibody (mAb; 1:50). Staining was with 3,3'-diaminobenzidine. Anti-CD31 mAb (CBL1337) was purchased from Chemicon (Temecula, CA, USA).

Dual-color intravital imaging (5). The Olympus OV100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan), containing an MT-20 light source (Olympus Biosystems, Planegg, Germany) and DP70 CCD camera (Olympus), was used for imaging in live mice. The optics of the OV100 fluorescence imaging system have been specially developed for macroimaging, as well as microimaging with high light-gathering capacity. The instrument incorporates a unique combination of high numerical aperture and long working distance. Five individually optimized objective lenses, parcentered and parfocal, provide a 10^5 -fold magnification range for seamless imaging of the entire body down to the subcellular level without disturbing the animal. The OV100 has the lenses mounted on an automated turret with a high magnification range of 1.6 to 16 and a field of view ranging from 6.9 to 0.69 mm. The optics and antireflective coatings ensure optimal imaging of multiplexed fluorescent reporters in small animals. High-resolution images were captured directly on a PC (Fujitsu Siemens, Munich, Germany). Images were processed for contrast and brightness and analyzed with the use of Paint Shop Pro 8 and Cell^R (Olympus Biosystems).

Fluorescence microscopy (1). Fluorescence microscopy was carried out using an Olympus IMT-2 inverted microscope equipped with a mercury lamp power supply. The microscope had a GFP filter set (Chroma Technology). Tissue samples were directly observed.

Treatment with gemcitabine (6-8). The mice were given intraperitoneal (*i.p.*) injections of 150 mg/kg of gemcitabine or phosphate-buffered saline (PBS) (Cellgro, Herndon, VA, USA) (vehicle controls) at days-24, -27, -30 and -33 after implantation of tumor cells. Tumor samples were excised under anesthesia at day-21 after implantation of tumor cells. At the end of the experiment, the mice were euthanized. Tumors visible with the naked eye were surgically removed. Tumors were measured in three dimensions with calipers. Tumor volume (mm^3) was calculated with the formula $V = 0.52 \times \text{length} \times \text{width} \times \text{height}$. Angiogenesis was quantified in the tumor mass by measuring the total length of ND-GFP-expressing nascent blood vessels under fluorescence microscopy. The vessel density at day-35 was calculated by the total length of nestin-positive nascent blood vessels divided by the tumor volume (mm^3). Each experimental group consisted of five mice.

Statistical analysis. The experimental data are expressed as the mean + SD. Statistical analysis was performed using the two-tailed Student's *t*-test.

Results

The RFP-expressing xPA-1 human pancreas cancer line was injected into the spleen of ND-GFP transgenic nude mice in order to establish experimental liver metastases. The metastatic tumors could then be directly imaged in the live mouse with the Olympus OV100 Small Animal Imaging System. Immediately after injection, RFP-expressing tumor cells could be visualized in the branches of the portal vein (Figure 2). ND-GFP-expressing nascent blood vessels were visualized to grow into

the RFP-expressing liver metastasis as early as day-21 following tumor cell injection. Immunohistochemical staining showed that CD31 and ND-GFP fluorescence were positive in the newly formed ND-GFP-expressing blood vessels growing into the RFP-expressing tumor metastasis (Figure 3).

ND-GFP mice with established xPA-1-RFP liver metastases were given daily *ip* injections of gemcitabine or PBS at days-24, -27, -30 and -33 after splenic injection. Control mice were given concomitant *i.p.* injections of PBS. The ND-GFP-expressing nascent blood vessels in the control mice had many branches and appeared to form a network (Figure 4). However, in the mice that were given gemcitabine, the ND-GFP-expressing nascent blood vessels were diminished and of smaller diameter (Figure 4). By day-35 after splenic injection, the number of nascent blood vessels in the gemcitabine-treated mice was significantly less than the PBS-injected mice ($p < 0.05$) (Figure 5).

Discussion

Angiogenesis is usually determined by immunohistochemical staining of tumor tissue using various antibodies specific for endothelial cells. In the present study, proliferating endothelial cells were visualized in the growing pancreatic cancer liver metastases by ND-GFP expression. Simultaneously, the metastatic tumor was visualized by RFP. The dual-color model provides a powerful and specific model to visualize nascent tumor angiogenesis simultaneously with the growth of the metastasis. These data suggest that nascent angiogenesis by the ND-GFP-expressing blood vessels is a critical target to prevent the growth of metastasis.

Hepatic metastases of primary tumours may be less reliant on traditional angiogenic pathways by co-opting pre-existing hepatic vasculature (9). Many anti-angiogenic agents are undergoing preclinical evaluation, with only a few entering clinical trials. However, early results suggest that anti-angiogenic therapy could be an important adjunct to conventional chemotherapy treatment of gastrointestinal neoplasia (9).

Gemcitabine is a nucleoside analogue that inhibits pancreatic tumor growth and has been first-line treatment for patients with metastatic pancreatic cancer since 1997 (10). In the present study, the growth-suppressing and antiangiogenic efficacy of gemcitabine on pancreatic cancer liver metastases was demonstrated. Similar findings on primary pancreatic tumors in an orthotopic model of pancreatic cancer (3) were previously observed. The concept of using chemotherapeutic agents as antiangiogenic agents, either alone or in combination with other known inhibitors of angiogenesis, has recently been proposed by several investigators (11-15). Therefore, our pre-clinical findings of the antiangiogenic effects of gemcitabine on liver metastasis of pancreatic cancer warrant further investigation. Furthermore, these results

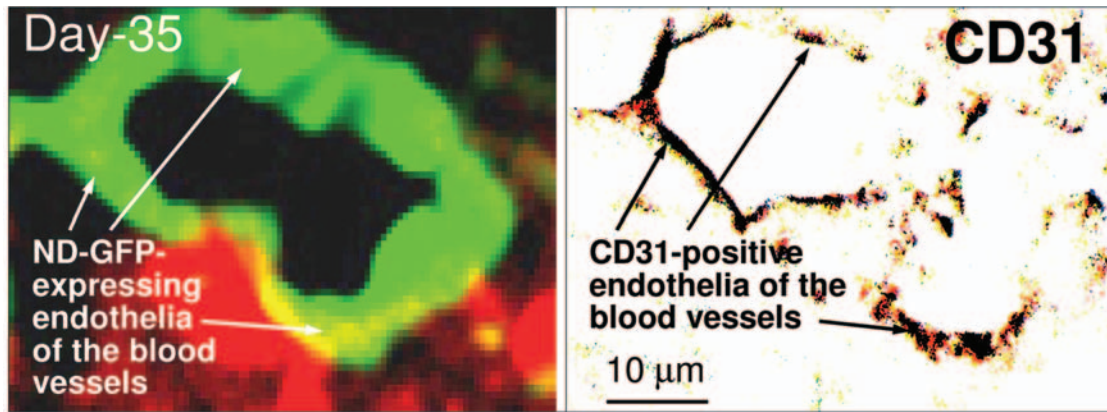


Figure 3. Expression of CD31 in ND-GFP blood vessels. Immunohistochemical staining showed that CD31 (black arrows, panel b), colocalized with ND-GFP fluorescence (white arrows, panel a) in ND-GFP-expressing blood vessels growing in the RFP-expressing liver metastasis of the xPA-1 pancreatic cancer.

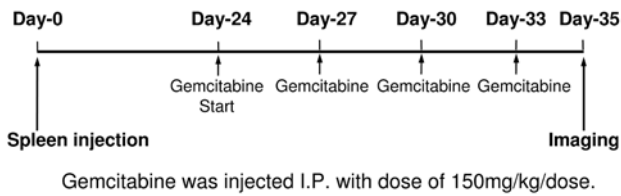


Figure 4. Gemcitabine treatment schedule. Mice with xPA-1 pancreatic cancer liver metastasis were given ip injections of gemcitabine or PBS (vehicle controls) at days -24, -27, -30 and -33 after injection of tumor cells. Liver tumors were observed at day-35 after injection.

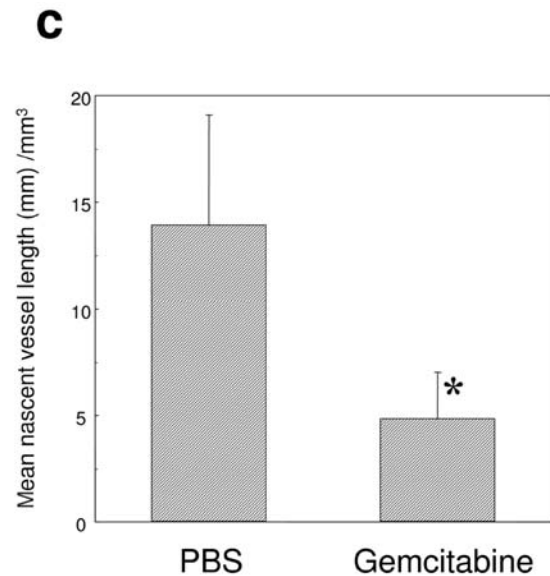
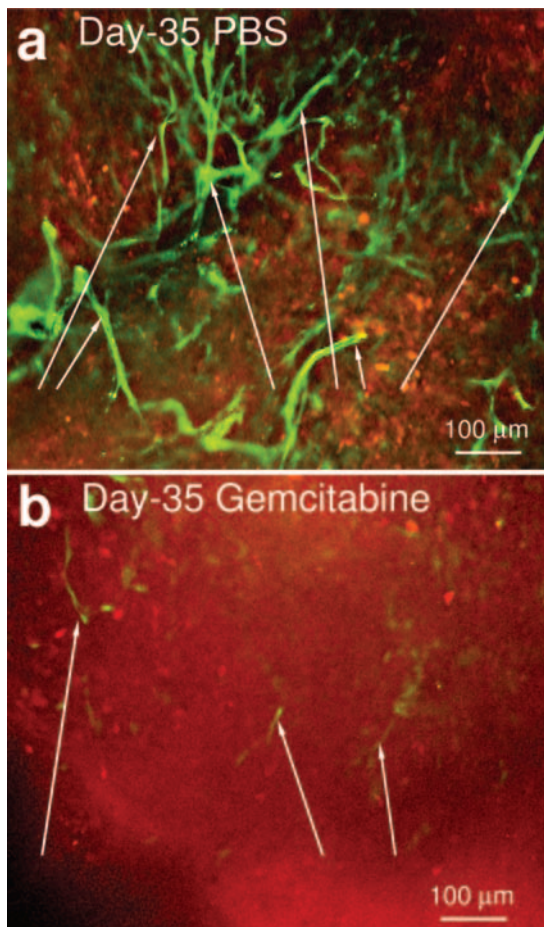


Figure 5. Anti-angiogenic effect of gemcitabine on xPA-1-RFP pancreatic liver metastases. (a) Control mice were given ip injections of PBS. In the control mice, the ND-GFP-expressing nascent blood vessels formed a network in the growing tumor mass. (b) Mice were given ip injections of gemcitabine. In the treated mice, the ND-GFP-expressing nascent blood vessels were diminished. (c) By day-35 after splenic injection, the mean nascent blood vessel density in the gemcitabine-treated mice was significantly less than the PBS-injected mice ($p < 0.05$).

suggest that the ND-GFP transgenic nude mouse model is uniquely useful for the visualization of nascent tumor angiogenesis in liver metastasis and evaluation of angiogenesis inhibitors against this critical target.

Conclusion

The dual-color model of the ND-GFP nude mouse with RFP-expressing liver metastases from pancreatic cancer enabled the simultaneous visualization and quantitation of angiogenesis and volume of the metastatic tumor. These results demonstrate for the first time that gemcitabine is an inhibitor of angiogenesis of liver metastasis, as well as metastatic growth in pancreatic cancer. These results have important implications for the clinical use of gemcitabine in this disease. Future studies will concentrate on the anti-angiogenesis and anti-metastatic efficacy of combination chemotherapy of pancreatic cancer in this and orthotopic (17) models. The present study further demonstrates the power of fluorescent-protein-based imaging to evaluate these powerful new therapies (16).

Acknowledgements

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