Imaging the Interaction of Pancreatic Cancer and Stellate Cells in the Tumor Microenvironment during Metastasis

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Abstract. *Background/Aim: Pancreatic stellate cells are involved in fibrosis of pancreatic cancer termed desmoplasia, which may contribute to both pancreatic cancer growth and metastasis, as well as to drug resistance. A better understanding of pancreatic cancer-cell interactions with stellate cells is therefore critical to our ability to develop effective anti-metastatic therapeutics for pancreatic cancer. Materials and Methods: The human pancreatic cancer cell line XPA-1 was engineered to express green fluorescent protein (GFP) in the nucleus and red fluorescent protein (RFP) in the cytoplasm. Pancreatic stellate cells were engineered to express RFP. The pancreatic cancer cells and stellate cells were co-cultured and their interaction was imaged in vitro. The pancreatic cancer cells and stellate cells were then co-injected in the spleen of transgenic cyan fluorescent protein (CFP) nude mice and imaged in liver, lung and diaphragm metastasis. Results: The interaction of the pancreatic cancer cells expressing GFP in the nucleus and RFP in the cytoplasm and stellate cells expressing RFP was first imaged in vitro. The intimate relationship between the two cell types could be seen. Three hours after splenic co-injection, dual-color pancreatic cancer cells and pancreatic stellate cells were found distributed in the host liver. By 28 days after splenic co-injection of the pancreatic cancer and stellate cells, liver metastases were observed in host CFP nude mice. Metastases were also observed in the lung and diaphragm. Stellate cells were observed along with the pancreatic cancer cells at all metastatic sites suggesting that stellate cells may be necessary for metastasis. With high-resolution intravital imaging afforded by the Olympus FV1000 confocal microscope, the interaction of the dual-colored pancreatic cancer cells and the RFP-expressing pancreatic stellate cells could be clearly imaged in the liver and other metastases, further suggesting that stellate cells participate in metastasis formation. Conclusion: Pancreatic cancer cells and stellate stem cells form a very close relationship and accompany each other to distant metastatic sties. Our hypothesis is that pancreatic stellate cells form a niche for metastasis of pancreatic cancer.*

A breakthrough occurred in the study of the tumor microenvironment (TME) with the use of fluorescent proteins to color-code the cellular elements of the TME for multispectral imaging (1-6).

The TME is critical for tumor progression (7). Solid tumors contain fibroblasts, lymphocytes, dendritic, macrophages and other myeloid cells in the TME (3). Color-coded *in vivo* imaging has shown that stromal cells had higher motility at the tumor periphery than within the tumor mass (3). Angiogenesis and lymphangiogenesis occur in the TME and are regulated by a variety of molecules released by cancer cells, as well as host stromal cells (8, 9). The cancer-associated fibroblast (CAF) is the most prominent cell type within the stroma of the TME (10-13). CAFs promote cancer cell growth and increase angiogenesis, invasion and metastasis (14-16). Macrophages within the tumor stroma are called tumor-associated macrophages (TAMs). Although TAMs may have antitumor activity (17), they also promote tumor progression and invasion (18-20), including intravasation (21). Stromal cells accompanying cancer cells are necessary for metastasis (7, 22, 23).

We have previously developed six different color-coded TME nude mouse models using the cyan fluorescent protein (CFP) mouse as a host. (i) Red fluorescent protein (RFP)- or green fluorescent protein (GFP)-expressing HCT-116 human

This paper is dedicated to the memory of A. R. Moossa, MD.

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Key Words: Pancreatic cancer, dual color cells, GFP, RFP, stellate cells, interaction, CFP nude mouse, color-coded fluorescence imaging.

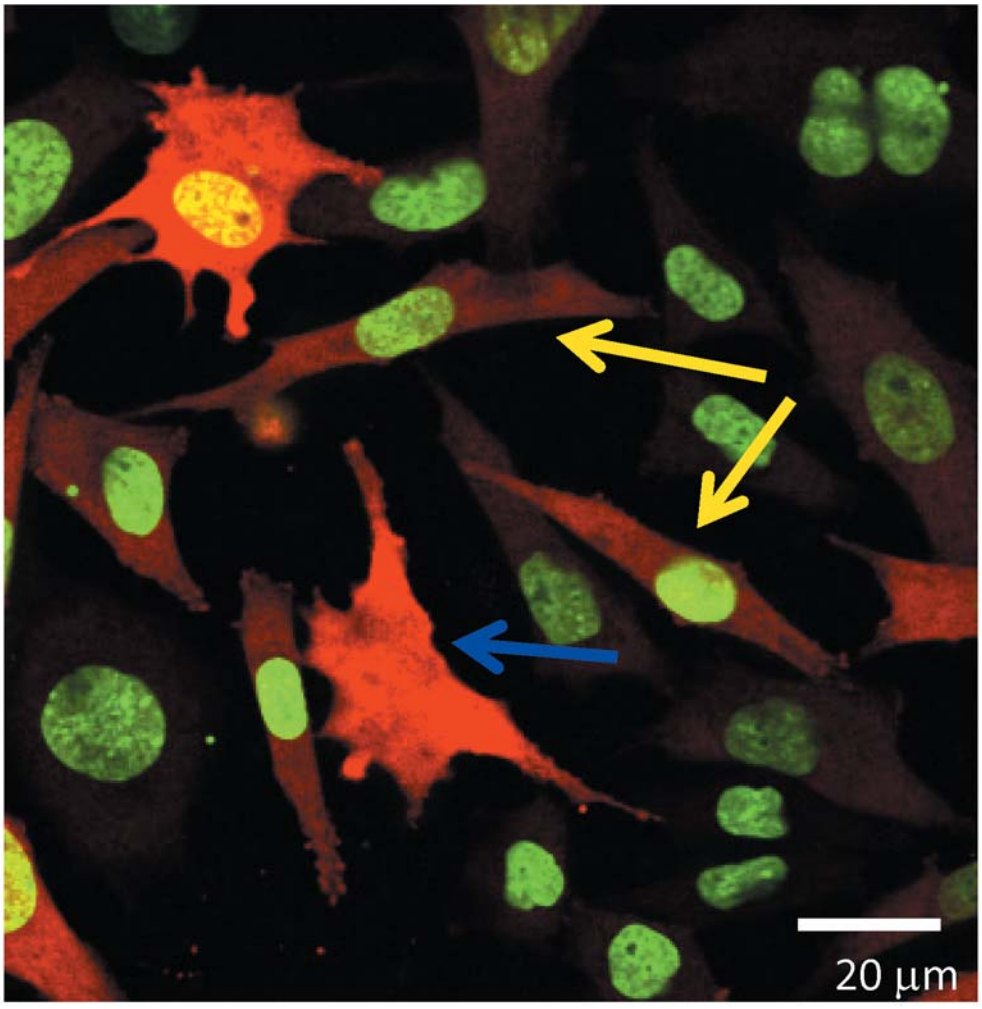

Figure 1. *Color-coded imaging of the interaction of dual-color pancreatic cancer cells and RFP-pancreas stellate cells in vitro. XPA-1-GFP-RFP cells and RFP pancreas stellate cells were co-cultured on plastic with RPMI 1640 and fetal calf serum (FCS). Blue arrow indicates RFP stellate cell. Yellow arrows indicate dual-color XPA-1 cells. (Bar=20 µm).*

colon cancer cells were implanted subcutaneously in the CFP-expressing nude mice. CFP stromal elements from the subcutaneous TME were recruited and interacted with the RFP- or GFP-expressing tumors. (ii) RFP-expressing HCT-116 cells were transplanted into the spleen of CFP nude mice. Experimental metastases were subsequently formed in the liver. CFP stromal elements from the liver were recruited and interacted with the RFP-expressing tumor. (iii) RFP-expressing HCT-116 cancer cells were transplanted in the tail vein of CFP-expressing nude mice, forming experimental metastases in the lung. CFP stromal elements from the lung were subsequently recruited and interacted with the RFP-expressing tumor. (iv) GFP-expressing and RFP-expressing HCT-116 cancer cells were co-implanted subcutaneously in CFP-expressing nude mice. A 3-color TME was formed subcutaneously in the CFP mouse. CFP stromal elements were recruited and interacted with the RFP or GFP-expressing tumors. (v) GFP-expressing HCT-116 cells were initially injected subcutaneously in RFP-expressing nude mice. The resulting tumor consisted of GFP cancer cells and recruited RFP stromal cells derived from the RFP nude mouse. This 2-color tumor was transplanted into the CFP nude mouse. CFP stromal cells were recruited by the growing transplanted tumor containing GFP cancer cells and RFP stroma. (vi) Mouse mammary tumor (MMT 060562) cells expressing GFP in the nucleus and RFP in the cytoplasm were implanted in the spleen of a CFP nude mouse. The dual-color MMT cells formed experimental metastasis in the liver, where CFP hepatocytes, as well as CFP non-parenchymal cells of the liver, interacted intimately (23).

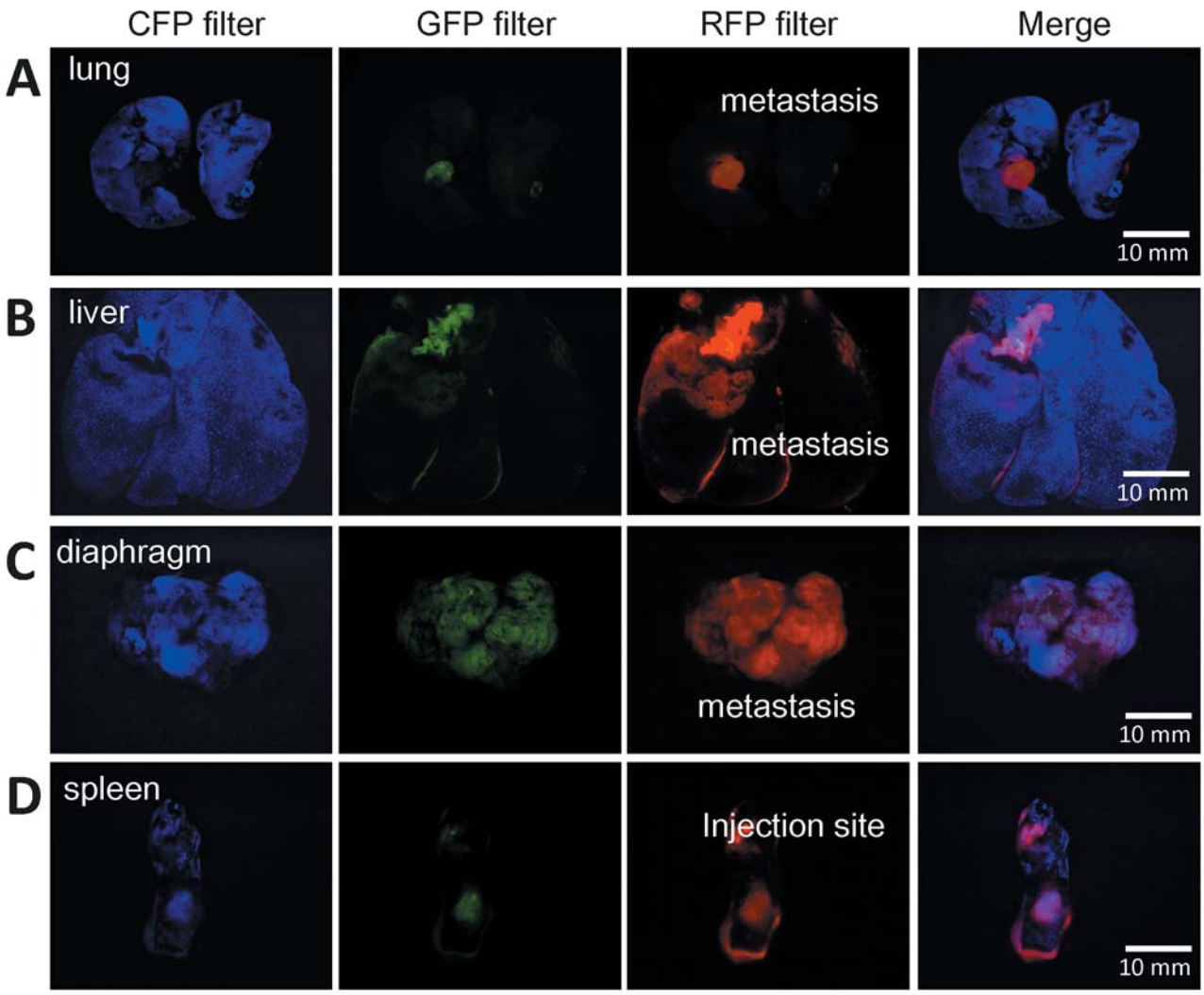

Figure 2. *Low magnification imaging the presence of RFP-expressing stellate cells in distant pancreatic cancer metastasis of XPA-I-GFP-RFP cells in the CFP mouse. A. Imaging of stellate cells and pancreatic cancer lung metastasis. B. Imaging of whole liver. C. Imaging of diaphragm. D. Imaging of spleen. Imaging was performed with the MVX10 microscope (Olympus).*

Another TME model was developed in our laboratory in which non-colored HCT-116 human colon cancer cells were injected in the spleen of GFP nude mice. HCT-116 cells subsequently formed experimental metastatic colonies in the liver by 28 days after transplantation to the spleen. GFP-expressing host cells were recruited by the metastasis and increased around and within the metastasis over time, indicating that CAFs were, thereby, recruited by the liver metastasis and probably increased their growth (24).

Pancreatic stellate cells (PSCs) are the fibrogenic cells in the pancreas (25, 26). In the normal pancreas, they are quiescent with low extracellular-matrix synthetic capacity (ECM) (27, 28). In cancer, PSCs can transform to a myofibroblast-like phenotype and have a high mitotic index and an increasing capacity to produce ECM proteins, cytokines and growth factors (29).

Pancreatic cancer cells can stimulate the motility, proliferation and matrix synthesis of PSCs with transforming growth factor-β1 (TGF-β1), fibroblast growth factor-2 and platelet-derived growth factor (PDGF) all involved in the process. PSCs may accelerate cancer cell proliferation and invasion (26, 30, 31). Co-injection of cancer cells with PSCs in orthotopic mouse models resulted in increased primary tumor growth and metastasis in previous experiments (32). PSCs have been shown to accompany cancer cells to metastatic sites and stimulate angiogenesis (29, 32).

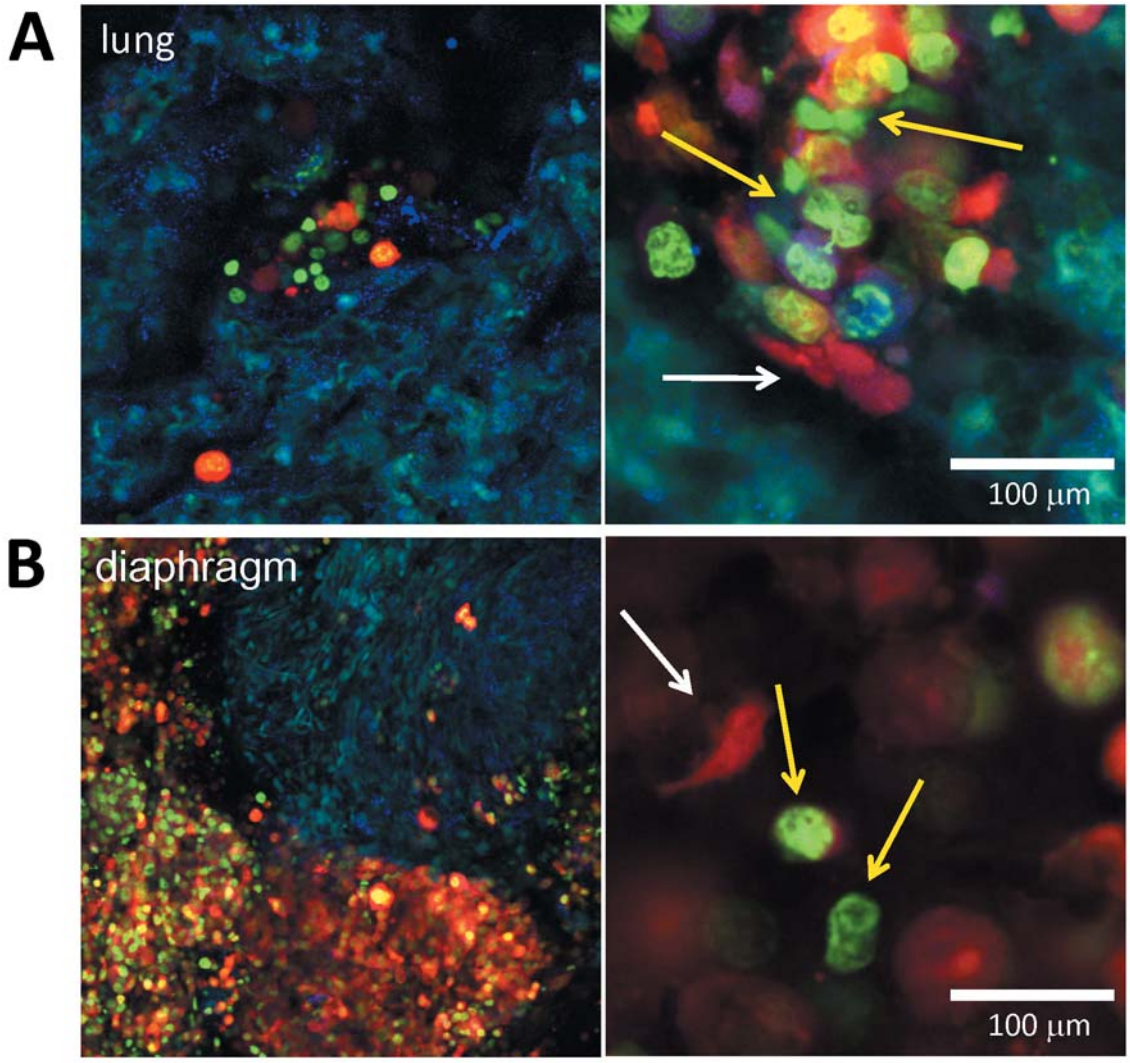

Figure 3. *High magnification imaging of the interaction of pancreatic cancer cells and stellate cells in organ metastasis. A. Interaction between XPA-I-GFP-RFP pancreatic cancer cells and RFP stellate cells in lung metastasis. B. Interaction of XPA-I-GFP-RFP pancreatic cancer cells and RFP stellate cells in diaphragm metastasis. (Left panels, low magnification; right panels, high magnification). Metastasis in the CFP mouse occured after co-injection of RFP pancreatic stellate cells and XPA-I-GFP-RFP pancreatic cancer cells in the spleen. Imaging was performed with the FV1000 confocal microscope (Olympus). (White arrows, RFP pancreatic stellate cells; yellow arrows, XPA-I-GFP-RFP pancreatic cancer cells).*

In the present report, using color-coded imaging we demonstrate the interaction of PSCs with pancreatic cancer cells *in vitro* and during metastasis to the liver, lung and diaphragm.

Materials and Methods

Cell culture. The human pancreatic cancer cell line XPA-1 was engineered to express GFP linked to histone H2B in the nucleus and RFP in the cytoplasm (33-34). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). All media were supplemented with penicillin and streptomycin (Gibco BRL, Grand Island, NY). The cell line was cultured in a 37˚ C incubator with 5% CO_2, 95% air.

Isolation of pancreatic stellate cells. PSCs were isolated from human pancreatic carcinoma tissue obtained during subtotal pancreatectomy. Patient tissue was obtained with informed consent according to Institutional Review Board-approved human subject protocols at the University of California, San Diego. Tissue samples used for cell isolation were submitted for histological examination to confirm the presence of carcinoma within the dense fibrotic stroma. PSCs were isolated using outgrowth techniques according to previously published methods (26, 28). Briefly, tissues were minced and small tissue fragments (1-2 mm^3) plated in Bachem medium (Dulbecco's modified Eagle's medium [DMEM]: Ham's F12 medium at 1:1) supplemented with 10-20% fetal bovine serum, L-glutamine, penicillin and streptomycin. After several days, PSCs that grew out from the tissue fragments were harvested and maintained in culture at 37°C with 5% CO_2.

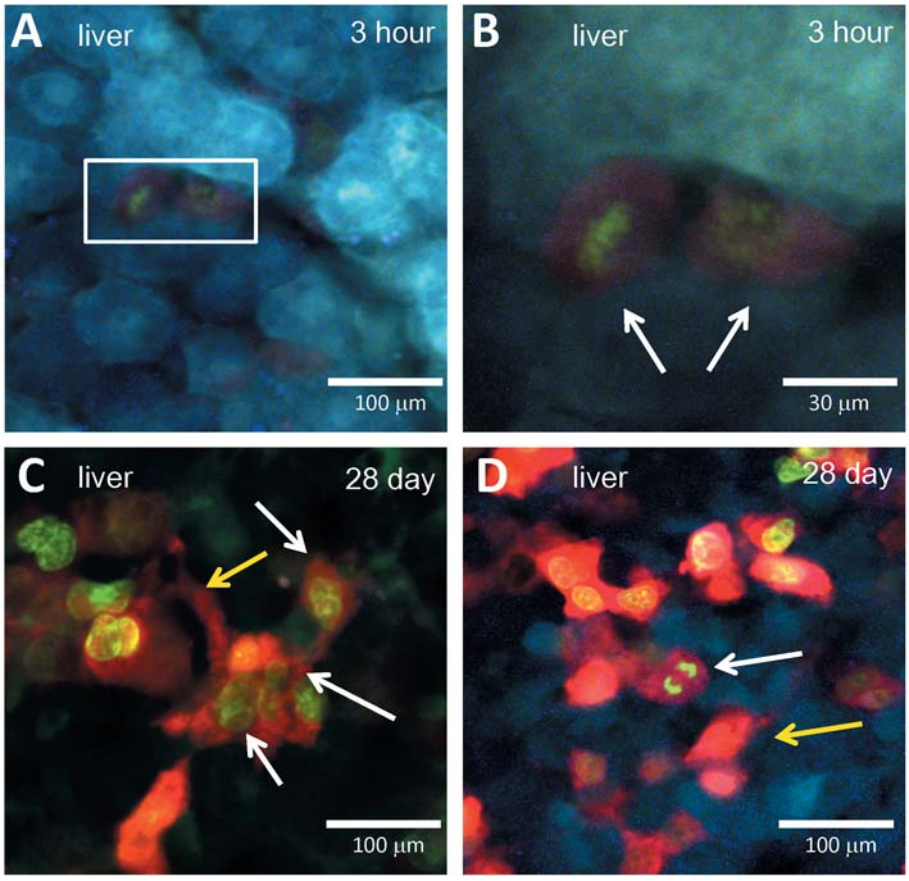

Figure 4. *High magnification imaging of pancreatic cancer cells and stellate cells in the liver of CFP nude mice. A. B. XPA-I-GFP-RFP pancreatic cancer cells in the host liver three hours after splenic injection. White arrows indicate XPA-1 pancreatic cancer cells. C.D. 28 days after co-injection of XPA-I-GFP-RFP pancreatic cancer cells and RFP stellate cells, liver metastases were observed in the host CFP nude mice. White arrows indicate XPA-I-GFP-RFP pancreatic cancer cells. Yellow arrows indicate RFP stellate cells. White arrow in the lower right panel shows dividing cancer cells. Imaging was performed with the FV1000 confocal microscope (Olympus).*

RFP transduction of pancreatic cancer cells. PSCs were incubated with a 1:1 mixture of retroviral supernatants of PT67-RFP packaging cells (33-35) and RPMI 1640 medium (Irvine Scientific, Irvine, CA, USA) containing 10% FBS for 72 h. Fresh medium was replenished at this time. Cells were harvested with trypsin/EDTA 72 h post-transduction and sub-cultured at a ratio of 1:15 into selective medium, which contained 200 μg/ml G418 (Invitrogen, Carlsbad, CA, USA). The level of G418 was increased stepwise up to 800 μg/ml in order to select for high expression of RFP (33-36).

Animal care. Transgenic nude mice, expressing CFP under the control of chicken β-actin promoter coupled with the cytomegalovirus (CMV) immediate early enhancer, were used (AntiCancer Inc., San Diego, CA, USA) (5, 23). Transgenic nude mice expressing CFP were bred and maintained in a high efficiency particulate arrestance (HEPA)-filtered environment with cages, food and bedding sterilized by autoclaving. The animal diets were obtained from Harlan Teklad (Madison, WI, USA). Ampicillin (5.0%, w/v; Sigma, St. Louis, MO, USA) was added to the autoclaved drinking water. All surgical procedures and imaging were performed with the mice anesthetized by intramuscular injection of a solution of 50% ketamine, 38% xylazine and 12% acepromazine maleate (0.02 ml). All animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals under PHS Assurance number A3873-01 (23).

Transplantation of pancreatic cancer and stellate cells in the spleen. Six-week-old CFP transgenic nude mice were used as the host. Pancreatic cancer cells and stellate cells were harvested by trypsinization and washed three times with cold serum-free medium, then re-suspended with serum-free RPMI medium 1640. CFP nude mice were anesthetized as described above. PSCs, engineered to express RFP, were co-injected with XPA-1 pancreatic cancer cells expressing H2B GFP and RFP into the spleen of transgenic CFP nude mice. The cancer cells subsequently formed liver metastases, as well as lung and diaphragm metastases (23).

In vivo imaging. Imaging was performed using the long-working-distance MVX10 *in vivo* fluorescence microcope with high numerical

aperture objectives for variable magnification imaging in live mice from macro- to subcellular (Olympus Corp., Tokyo, Japan) (37) or an FV1000 confocal fluorescence microscope (Olympus Corp.) (38).

Results and Discussion

Imaging of the interaction of dual-color GFP-RFP pancreatic cancer cells and RFP-pancreas stellate cells in vitro. Dual-color XPA-1-GFP-RFP cells and RFP PSCs were found to be closely associated during culture (Figure 1).

Stellate cells accompany pancreatic cancer cells to distant metastatic sites. RFP PSCs and XPA-1-GFP-RFP pancreatic cancer were imaged at low magnification in lung, diaphragm and liver metastases along with pancreatic cancer cells (Figure 2). The stellate cells accompanied the pancreatic cancer cells to all distant metastatic sites.

Imaging the interaction of dual-color pancreatic cancer cells and pancreas stellate cells in distant metastasis. With high-resolution confocal imaging, XPA-I-GFP-RFP pancreatic cancer cells and RFP PSCs could be visualized closely interacting in lung, liver and diaphragm metastasis (Figure 3).

High resolution imaging of stellate and pancreatic cancer cells in liver metastasis. High-resolution, high-magnification confocal microscopy imaging demonstrates the interaction of the XPA-I-GFP-RFP pancreatic cancer cells and the RFP-expressing PSCs in liver metastasis. Dividing cancer cells were observed juxtaposed to stellate cells (Figure 4).

Conclusion

Our results suggest that PSCs and pancreatic cancer cells interact very closely with each other and accompany each other to distant metastatic sites. The stellate cells may form a niche for the pancreatic cancer cells, thereby enabling metastasis. The presence of PSCs may also enhance pancreatic cancer to form primary tumors and metastasis (39).

Acknowledgements

This study was supported in part by National Cancer Institute grant CA132971.

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Received February 5, 2015
Revised February 17, 2015
Accepted February 18, 2015