

Recruitment of Cancer-Associated Fibroblasts and Blood Vessels by Orthotopic Liver Tumors Imaged in Red Fluorescent Protein (RFP) Transgenic Nude Mice

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Abstract. *Background/Aim:* The tumor microenvironment (TME) is critical for tumor growth and progression. We report here an imageable model of the TME of orthotopic liver cancer. *Materials and Methods:* The transgenic red fluorescent protein (RFP)-expressing nude mouse was used as the host. The RFP nude mouse expresses RFP in all organs. Non-colored Huh-7 human hepatoma cells were injected in the spleen of RFP nude mice to establish an orthotopic liver cancer model. TME formation resulting from the orthotopic liver tumor was observed using the Olympus OV100 small animal fluorescence imaging system. *Results:* Non-colored liver cancer cells formed tumor colonies in the liver 28 days after cell transplantation to the spleen. RFP-expressing host cells and blood vessels were recruited by the liver tumors as visualized by fluorescence imaging. A desmin- and sirius-red-positive area increased around and within the liver tumor over time. *Conclusion:* These results indicate cancer-associated fibroblasts (CAFs) were recruited by the liver tumors suggesting that CAFs, along with the angiogenic tumor blood vessels, were necessary for liver-tumor growth and could serve as visible therapeutic targets.

Our laboratory previously developed a color-coded model of the tumor microenvironment (TME) using red fluorescent protein (RFP), cyan fluorescent protein (CFP), and green fluorescent protein (GFP) transgenic nude mice as hosts for cancer cells expressing a different color fluorescent protein (1-11).

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Our color-coded TME imaging technology was used by Egeblad *et al.* (12) to show that stromal cells had higher motility in the periphery of the tumor. Our color-coded model was also used by Egeblad to demonstrate macrophages, fibroblasts, dendritic cells, and lymphocytes in the TME (12).

We have also demonstrated, using color-coded imaging, that tumors acquired fluorescent protein-expressing stroma over time, during growth in fluorescent protein-expressing nude mice, including during passage. The acquired stroma grew along with the tumor. The bright stroma enabled non-invasive imaging of a pancreatic-cancer patient-derived orthotopic xenograft (PDOX) mouse model (7-9). We previously showed that CAFs are recruited by metastasis, which stimulate their growth (6, 8, 11). We also discovered that stromal cells are necessary for metastasis (13).

We previously used a GFP-expressing transgenic nude mouse as the host which expresses GFP in all organs, but only dimly in the parenchymal cells of the liver, to study the role of the TME in liver metastasis of colon cancer. Non-colored HCT-116 human colon cancer cells were injected in the spleen of GFP nude mice, which led to the formation of experimental liver metastasis. TME formation resulting from the liver metastasis was observed using the Olympus OV100 small animal fluorescence imaging system. HCT-116 cells formed tumor colonies in the liver by 28 days after cell transplantation to the spleen. GFP-expressing host cells were recruited by the metastatic tumors as visualized by fluorescence imaging. A desmin-positive area increased around and within the liver metastasis over time, suggesting cancer-associated fibroblasts (CAFs) were recruited by the liver metastasis (6).

The present study images the recruitment of blood vessels and cancer-associated fibroblasts by orthotopic liver tumors growing in transgenic RFP nude mice.

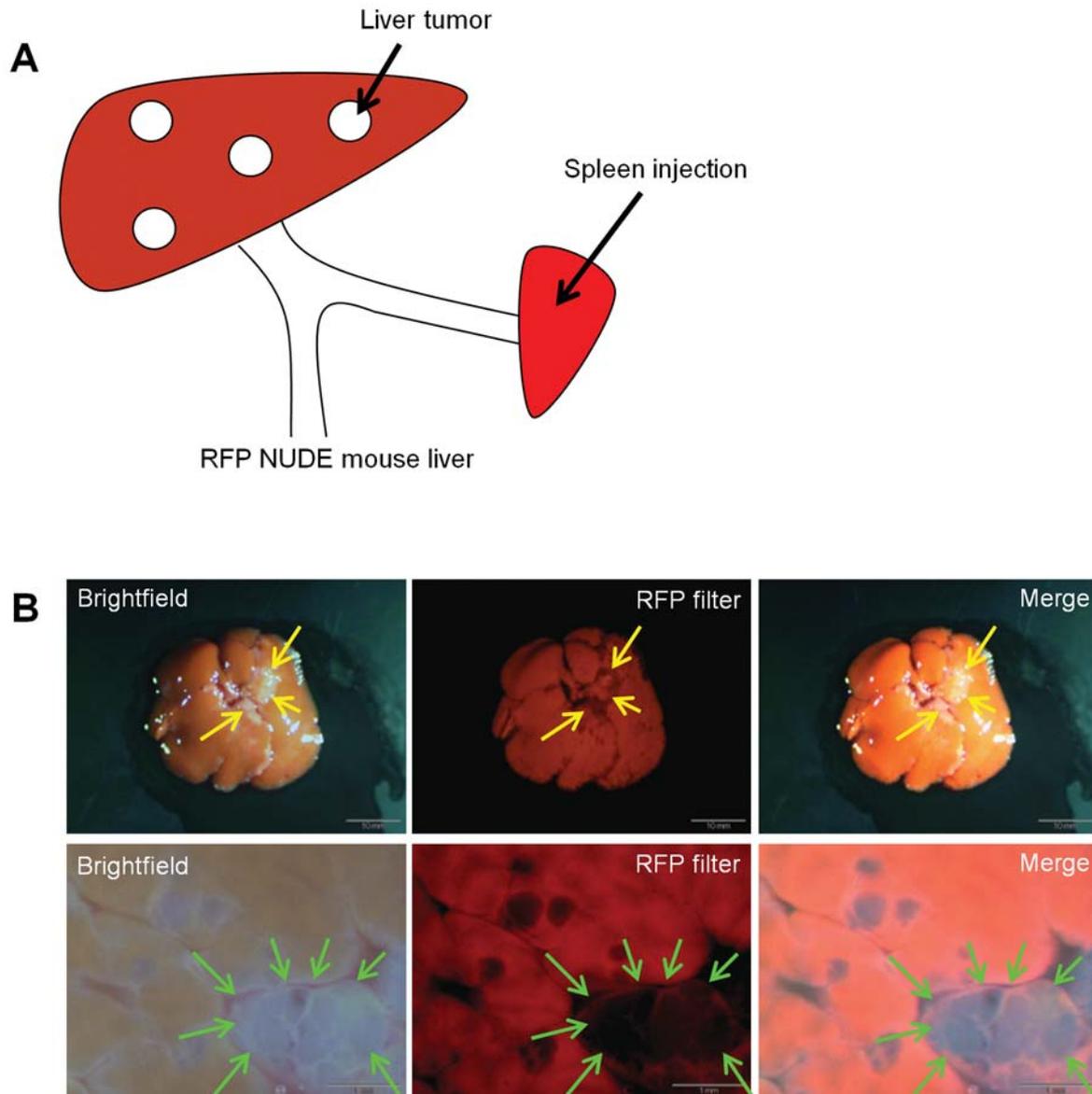


Figure 1. A: Schematic representation of experimental protocol. Non-colored Huh-7 human hepatoma cells ($2.0 \times 10^6/50 \mu\text{l}$) were injected in the spleen of RFP nude mice to establish an orthotopic liver tumor model. B: Imaging of an orthotopic liver tumor on day 28 after cancer-cell injection. RFP fluorescence was observed in the orthotopic liver tumor. The liver tumor was observed using the Olympus OV100 small animal fluorescence imaging system. Upper panels are low magnification (left panel: bright-field; central panel: fluorescence; right panel: merge). Yellow arrows indicate non-colored tumor (Bar=10 mm). Lower panels are high magnification (left panel: bright-field; central panel: fluorescence; right panel: merge). Green arrows indicate non-color tumor (Bar=1 mm).

Materials and Methods

Cell culture. Huh-7 human liver cancer cells were maintained in RPMI 1640 medium supplemented with 10% FCS (14) as well as with penicillin and streptomycin (Gibco BRL, Grand Island, NY, USA). The Huh-7 cell line was cultured at 37°C in a 5% incubator.

Transgenic red fluorescent protein-expressing nude mice. Transgenic red fluorescent protein (RFP) nude mice (AntiCancer, Inc., San

Diego, CA, USA), expressing RFP (DsRed.T3) under the control of a chicken β -actin promoter and cytomegalovirus enhancer, were used in this study. All tissues from this transgenic line, with the exception of erythrocytes, were red under blue excitation light.

Orthotopic liver-cancer model. Non-colored Huh-7 cells were first harvested by trypsinization and washed three times with cold serum-free medium and then resuspended with serum-free RPMI 1640 medium. RFP nude mice were anesthetized with a ketamine mixture (0.02 ml solution of 20 mg/kg ketamine, 15.2 mg/kg xylazine, and

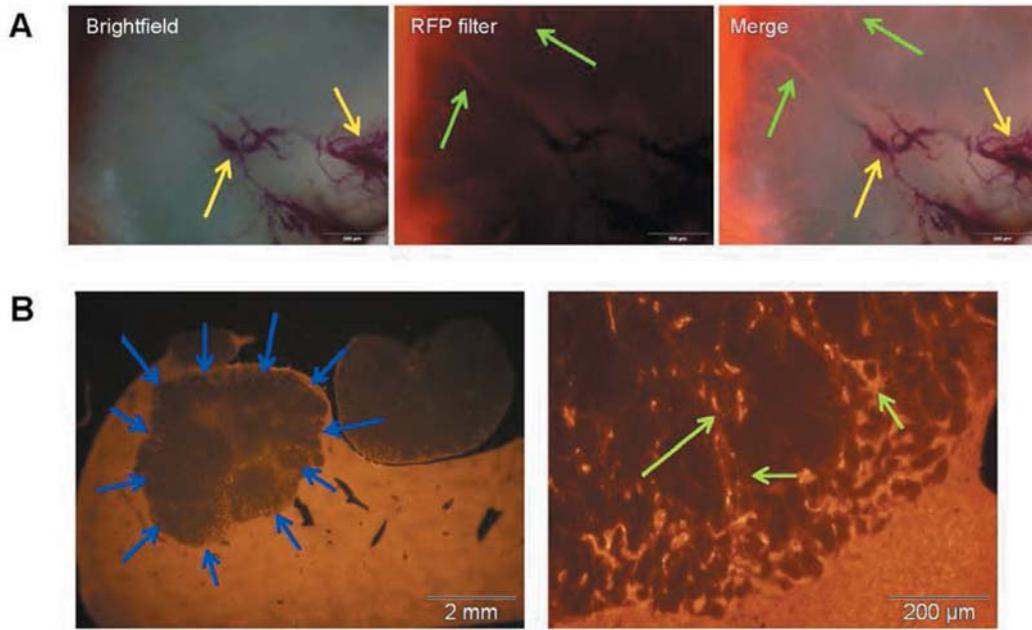


Figure 2. Fluorescence imaging of liver sections. A: High magnification image of the orthotopic Huh-7 liver tumors. The orthotopic liver tumor was observed using the Olympus OV100 small animal fluorescence imaging system. Yellow arrows indicate tumor vessels. Green arrows indicate RFP stroma. (Bar=500 μ m). B: Image of Huh-7 liver tumor section. Blue arrows indicate Huh-7 tumor. Green arrows indicate RFP stroma recruited by the liver tumor. (left panel: Bar=2 mm); (right panel: Bar=200 μ m).

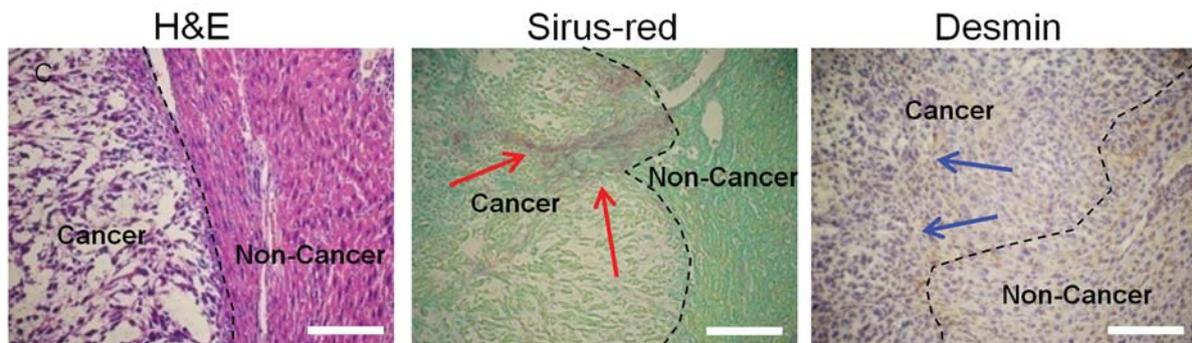


Figure 3. Hematoxylin and eosin, sirius-red and desmin staining. Liver sections from RFP nude mice with orthotopic Huh-7 liver tumors were stained with hematoxylin and eosin (H&E), sirius-red and desmin. There were significantly more sirius-red and desmin positive areas in the tumor than in the non-tumor part of the liver. The liver tumor contained RFP-, and desmin-expressing cells, sirius-red-stained cells as well as suggesting that cancer-associated fibroblast (CAFs) have a role in liver tumor development. Left panel: H&E stain; central panel: sirius-red stain; right panel: desmin stain. Non-colored Huh-7 liver tumor sections (original magnification, $\times 200$). Red arrows indicate sirius red- positive area. Blue arrows indicate desmin-positive area. (Bar=100 μ m).

0.48 mg/kg acepromazine maleate) injected into the peritoneal cavity. Non-colored Huh-7 cancer cells (2.0×10^6) were injected in the spleen of RFP nude mice during open laparotomy in order for tumors in the liver to form (Figure 1).

In vivo imaging. For *in vivo* imaging based on fluorescent proteins (16-19), the Olympus OV100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan) was used. The Olympus OV100, which contains

an MT-20 light source (Olympus Biosystems, Planegg, Germany) and DP70 CCD camera (Olympus), were used for cellular imaging in live mice. The optics of the OV100 fluorescence imaging system have been specially developed for macro-imaging as well as micro-imaging with high light-gathering capacity. 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 CellR (Olympus Biosystems) (20).

Histological and immunohistochemical analysis. The host livers were fixed with 10% buffered formalin, sectioned at a thickness of 4 μm , and stained with hematoxylin and eosin. Desmin was stained with anti-desmin antibody (Lab Vision, Fremont, CA, USA) using an avidin–biotin–peroxidase complex technique (Vector, Burlingame, CA). Collagen deposition was assessed by Sirius-red staining.

Results and Discussion

Imaging recruitment of blood vessels and cancer-associated fibroblasts by a non-colored orthotopic liver tumor. Huh-7 human liver cancer cells were injected in the spleen of RFP nude mice. Low-magnification imaging showed the contrast between the non-colored liver tumor and RFP-expressing liver (Figure 1). High-magnification fluorescence imaging showed extensive RFP fluorescence in the tumor due to recruitment of RFP-expressing blood vessels and fibroblast-like cells (Figure 2). Thus, host RFP cells were imaged extensively accumulating in the liver tumor.

Immunohistochemical experiments showed that expression of desmin was prominent in the liver tumor, but not in the host liver, suggesting that cancer-associated fibroblasts (CAFs) were recruited and grew in the tumor (Figure 3). The tumor also was stained with sirius-red, which marks collagen, possibly produced by the CAFs. CAFs have an important role in tumor progression (21). CAFs have a high rate of proliferation and differential expression of extracellular matrix (ECM) components, such as collagen, and growth factors compared to normal fibroblasts (21-23). CAFs promote tumor growth by inducing angiogenesis, recruiting bone marrow-derived endothelial-progenitor cells, and remodeling the ECM (24-28). CAFs can confer resistance to anti-angiogenic therapy (21, 29). CAFs also mediate tumor-enhancing inflammation mediated by NF- κ B (21).

However, the formation of the TME is still largely unknown. The present report demonstrates a color-coded imaging model in which the development of the TME can be visualized in an orthotopic liver cancer model. RFP transgenic nude mice are useful to image stromal development in the tumor, since bright RFP-expressing stromal cells are recruited by the liver tumors. A desmin- and sirius-red-positive area increased around the liver tumors over time, suggesting CAFs were recruited by the liver tumors and have an important role in tumor progression. Stromal cells essential for the liver tumors to develop can be imaged as visual therapeutic targets. RFP-expressing blood vessels are also recruited by the liver tumor, enabling visual therapeutic targeting of angiogenesis as well.

Dedication

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

Acknowledgements

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