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Imaging the Recruitment of Cancer-Associated Fibroblasts by Liver-Metastatic Colon Cancer

Atsushi Suetsugu,[1,2,3] Yosuke Osawa,[3] Masahito Nagaki,[3] Shigetoyo Saji,[4] Hisataka Moriwaki,[3] Michael Bouvet,[1] and Robert M. Hoffman[1,2*]

[1] *AntiCancer, Inc., San Diego, California 92111*
[2] *Department of Surgery, University of California, San Diego, California 92103*
[3] *Department of Gastroenterology, Gifu University Graduate School of Medicine, Gifu, Japan*
[4] *Department of Surgical Oncology, Gifu University Graduate School of Medicine, Gifu, Japan*

ABSTRACT

The tumor microenvironment (TME) is critical for tumor growth and progression. However, the formation of the TME is largely unknown. This report demonstrates a color-coded imaging model in which the development of the TME can be visualized. In order to image the TME, a green fluorescent protein (GFP)-expressing mouse was used as the host which expresses GFP in all organs but not the parenchymal cells of the liver. 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 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 which have a role in tumor progression. The color-coded model of the TME enables its formation to be visualized at the cellular level in vivo, in real-time. This imaging model of the TME should lead to new visual targets in the TME. J. Cell. Biochem. 112: 949–953, 2011.

KEY WORDS: GFP AND RFP NUDE MICE; TUMOR MICROENVIRONMENT; STROMAL CELLS; CANCER-ASSOCIATED FIBROBLASTS; LIVER METASTASIS; COLOR-CODED IMAGING

The use of fluorescent proteins for imaging is revolutionizing in vivo biology [Hoffman, 2005, 2008]. Green fluorescent protein (GFP) and red fluorescent protein (RFP) have been shown to be able to be genetically linked with almost any protein providing a permanent and heritable label in live cells to study protein function and location. Many different colors of fluorescent proteins have now been produced in the laboratory or found in nature [Matz et al., 1999; Shaner et al., 2004]. With different color fluorescent proteins, many processes can be visualized simultaneously in cells. Thus, live cells can be multiply labeled for imaging processes that heretofore could be seen only on fixed and stained cells. The use of fluorescent proteins for imaging in vivo has been particularly useful to study tumor growth and progression [Hoffman, 2005].

With the use of multiple colored-proteins, we developed imaging of the tumor microenvironment (TME) by color-coding cancer and stromal cells. The TME is critical for tumor growth and progression. Indeed, cancer cells and stromal cells must replicate in parallel in order for the tumor to grow. Our original color-coded imaging technology of the TME used a GFP or RFP transgenic nude mouse as a host in which we transplanted cancer cells expressing a fluorescent protein not expressed by the host [Yang et al., 2003, 2004, 2009].

Recently, color-coded in vivo imaging has shown that stromal cells had higher motility in the microenvironment at the tumor periphery than within the tumor mass [Egeblad et al., 2008]. Solid tumors contain fibroblasts, lymphocytes, dendritic, macrophages, and other myeloid cells in their microenvironment [Egeblad et al., 2008].

Myofibroblasts and are a major population of carcinoma-associated fibroblasts (CAFs) [Kalluri and Zeisberg, 2006]. CAFs stimulate cancer cell growth, inflammation, angiogenesis, and invasion [Kalluri and Zeisberg, 2006; Gaggioli et al., 2007; Pietras

Abbreviations used: TME, tumor microenvironment; GFP, green fluorescent protein; RFP, red fluorescent protein; CAF, cancer-associated fibroblasts.

*Correspondence to: Robert M. Hoffman, PhD, AntiCancer, Inc., 7917 Ostrow Street, San Diego, CA 92111.
E-mail: all@anticancer.com

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et al., 2008; Erez et al., 2010]. As the tumor grows, it recruits CAFs [Orimo et al., 2005; Egeblad et al., 2010].

How the TME is formed in real time during cancer progression is not known. The present study utilizes color-coded fluorescent protein-based imaging to visualize the recruitment over time of stromal cells including CAFs by liver metastases of the colon cancer.

MATERIALS AND METHODS

CELL CULTURE

The HCT-116 human colon cancer cell line was originally obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in RPMI 1640 supplemented with 10% FCS. All media were supplemented with penicillin and streptomycin (Gibco BRL). The cell line was cultured at 37°C in a 5% incubator.

GFP TRANSGENIC MICE

Transgenic C57/B6-GFP mice [Okabe et al., 1997] were obtained from the Research Institute for Microbial Diseases (Osaka University, Osaka, Japan). The C57/B6-GFP mice expressed the *Aequorea victoria* GFP under the control of the chicken β-actin promoter and cytomegalovirus enhancer. The GFP gene was crossed into nude mice on the C57/B6 background [Yang et al., 2004]. Transgenic C57/B6-RFP mice were obtained from Jackson Labs (Bar Harbor, ME). C57/B6-RFP mice expressed the RFP (DsREDT3) under the control of a chicken β-actin promoter and cytomegalovirus enhancer [Vintersten et al., 2004]. The RFP gene was crossed into nude mice (Harlan, Indianapolis, IN) on the BALB/c and NCR background [Yang et al., 2009]. All animal studies were conducted in accordance with the principles of and procedures outlined in the NIH guide for the care and use of laboratory animals under assurance number A3873-1.

NON-COLORED COLON CANCER CELL (HCT-116)–GFP HOST MODEL

Six-week-old GFP nude mice were used as the host for non-colored HCT-116 human colon cancer cells. Non-colored HCT-116 cells were first harvested by trypsinization and washed three times with cold serum-free medium and then resuspended with serum-free RPMI 1640 medium. GFP nude mice were anesthetized with a ketamine mixture (10 μl ketamine HCl, 7.6 μl xylazine, 2.4 μl acepromazine maleate, and 10 μl H_2O) injected into the peritoneal cavity. Non-colored human HCT-116 colon cancer cells (2.0×10^6) were injected in the spleen of GFP nude mice during open laparotomy in order for experimental liver metastases to form.

IN VIVO IMAGING

For in vivo imaging based on fluorescent proteins [Hoffman, 2005; Hoffman and Yang, 2006a,b,c], 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), was 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. The instrument incorporates a unique combination of high numerical aperture and long working distance. Four 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) [Yamauchi et al., 2006].

HISTOLOGICAL ANALYSIS

The host livers were fixed with 10% buffered formalin, sectioned at a thickness of 4 μm, and stained with hematoxylin and eosin. The fluorescent cell area was measured using the National Institutes of Health (NIH) Image analysis software program (available at http://rsb.info.nih.gov/nih-image/). Desmin was stained with anti-desmin antibody (Lab Vision, Fremont, CA) using avidin–biotin–peroxidase complex technique (Vector, Burlingame, CA). Measurement of positive area was performed using the NIH Image.

STATISTICAL ANALYSIS

All data represent the mean of at least three independent experiments ± SD. For the determination of statistical significance, unpaired Student's *t*-tests were performed. *P*-values of less than 0.01 were considered statistically significant.

RESULTS AND DISCUSSION

FLUORESCENT ORGANS IN GFP AND RFP NUDE MICE

After sacrifice of the GFP and RFP transgenic nude mice, organs including lungs (Fig. 1A), kidney (Fig. 1B), esophagus (Fig. 1C), stomach (Fig. 1C), duodenum (Fig. 1C), small intestines (Fig. 1D), colon (Fig. 1D), the circulatory system, uterus and ovary, pancreas, brain, heart, and spleen (data not shown) were harvested, and imaged with the Olympus OV100 Small Animal Imaging System. All of the tissues from the RFP transgenic mouse, with the exception of erythrocytes, were red fluorescent under appropriate excitation light (Fig. 1). In contrast, although the other organs of the GFP-transgenic nude mice were brightly fluorescent, the liver of GFP nude mice fluorescence was weak except for the gallbladder (Fig. 1E).

IMAGING RECRUITMENT OF CANCER-ASSOCIATED FIBROBLASTS BY COLON CANCER LIVER METASTASIS

Human non-colored HCT-116-colon cancer cells were injected in the spleen of GFP nude mice. On day 28 after cancer-cell injection, GFP fluorescence was observed in the experimental liver metastatic colonies. High-magnification fluorescence microscopy showed extensive GFP fluorescence in the tumor. Liver sections were stained with hematoxylin–eosin, and GFP fluorescence of recruited GFP-expressing hosts cells in the liver metastasis was observed. There was a very large increase of GFP expression in the tumor compared to the non-tumor-bearing liver in nude mice. There was also significantly more GFP fluorescence in the liver metastasis

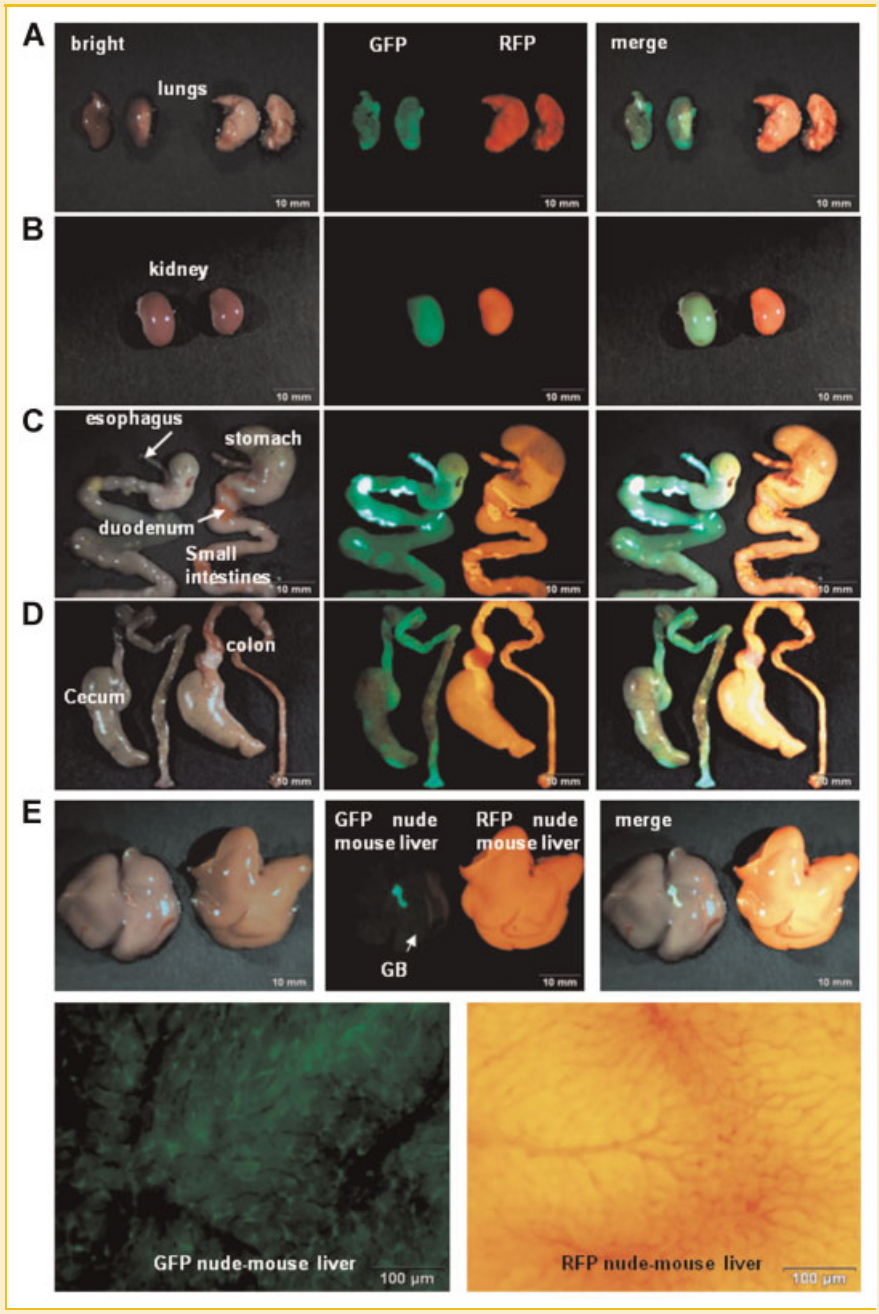

Fig. 1. Major organs of transgenic GFP and RFP nude mice. Fluorescence of organs after excitation with blue light. A: Lungs; (B) kidney; (C,D) digestive tract; (E) liver. Lower panel displayed high magnification (16×). The liver of GFP nude mice has very weak fluorescence.

compared to the non-tumor area of the liver ($P < 0.01$). Thus, the host GFP cells were imaged extensively accumulating in the liver metastasis. Expression of desmin was determined by immunohistochemistry with anti-desmin antibody and found to be positive in the tumor area. Thus, the liver metastasis contained GFP- and desmin-expressing cells, suggesting that cancer-associated fibroblasts (CAFs) were recruited and grew in the tumor (Fig. 2).

It is well known that CAFs have an important role in tumor progression [Erez et al., 2010]. CAFs have an increased rate of proliferation and differential expression of extracellular matrix (ECM) components and growth factors compared to normal fibroblasts [Bhowmick et al., 2004; Kalluri and Zeisberg, 2006; Erez et al., 2010]. CAFs promote tumor growth by inducing angiogenesis, recruiting bone marrow-derived endothelial progenitor cells, and remodeling the ECM [Olumi et al., 1999; Allinen et al., 2004; Orimo et al., 2005; Pietras et al., 2008; Erez et al., 2010]. CAFs can confer resistance to antiangiogenic therapy [Crawford et al., 2009; Erez et al., 2010]. CAFs also mediate tumor-enhancing inflammation mediated by NF-kappaB signaling [Erez et al., 2010].

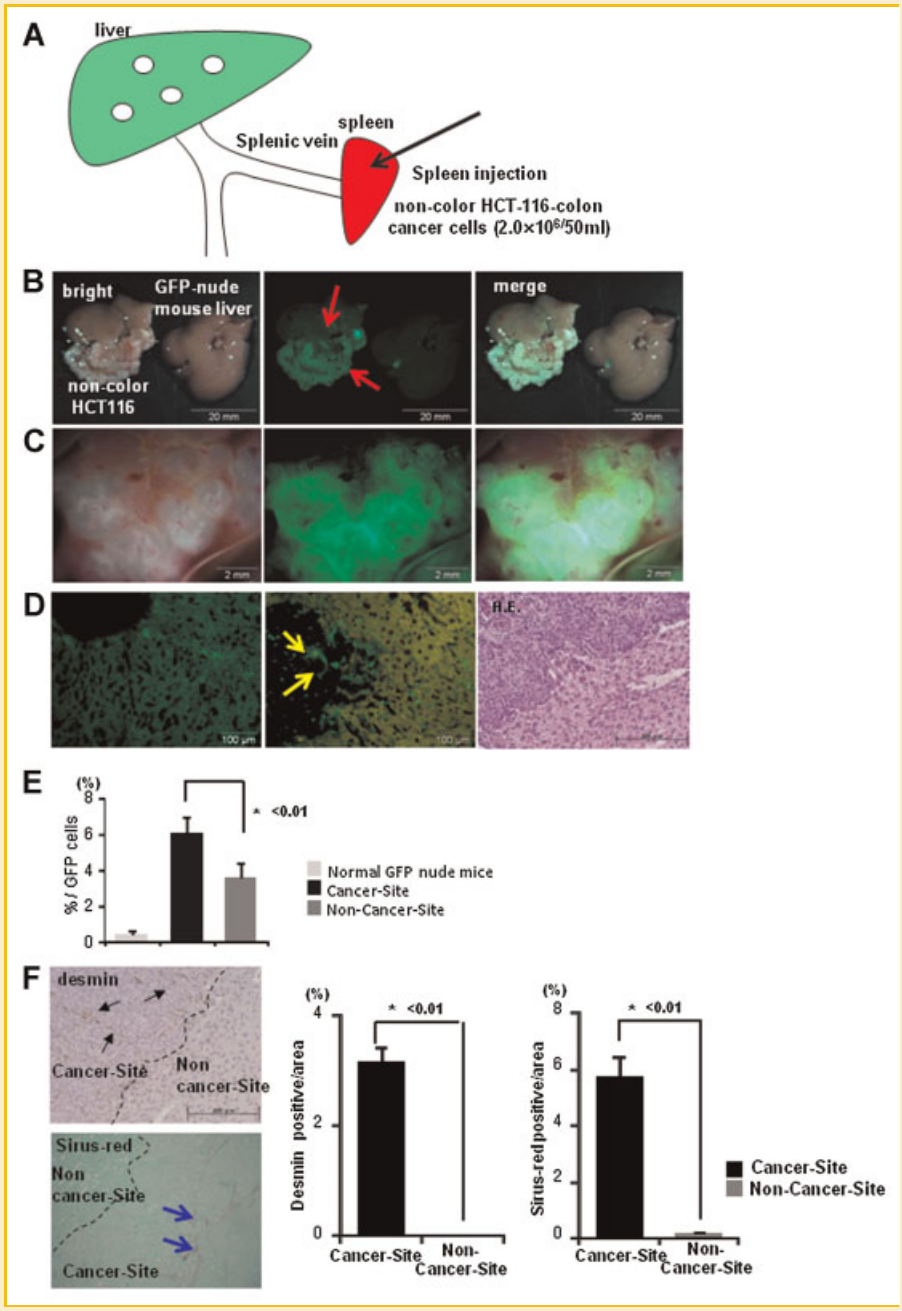

Fig. 2. Tumor–host interaction of non-colored human colon tumors in the liver of GFP nude mice. A: Schematic representation of experimental protocol. Human non-colored HCT-116-colon cancer cells (2.0×10^6/50 μl) were injected in the spleen of GFP nude mice during open laparotomy. B: On day 28 after cancer-cell injection, GFP fluorescence was observed in the liver metastasis (red arrows; left panel: bright field; central panel: fluorescence; right panel: merge; original magnification 0.14×). C: High magnification (left panel: bright-field; central panel: fluorescence; right panel: merge). D: Liver sections from GFP nude mice were stained with hematoxylin–eosin, and GFP fluorescence of non-parenchymal cells in the host GFP liver was observed as well (yellow arrows). E: There was significantly more GFP fluorescence in the metastasis than in the non-tumor part of the liver ($P < 0.01$). F: Expression of desmin was determined by immunohistochemistry with anti-desmin antibody (arrows; original magnification 100×). Measurement of the desmin-positive area was performed using the NIH Image analysis software program. Data are means ± SD from three independent experiments. Collagen deposition was assessed by Sirus red staining (blue arrows). The host GFP non-parenchymal cells were observed in the experimental liver metastasis. Non-parenchymal cells accumulated in tumors compared with normal liver. Liver metastasis contained GFP and desmin-expressing cells, suggesting that cancer-associated fibroblast cells have a role in metastasis.

The present report enables imaging of accumulation of CAFs of the tumor in real time.

The TME is critical for tumor growth and progression. However, the formation of the TME is largely unknown. This report demonstrates a color-coded imaging model in which the development of the TME can be visualized. In the GFP transgenic nude mice, only non-parenchymal cells of the liver have GFP fluorescence, which makes it a very useful model to image stromal development in the tumor, since GFP-expressing stromal cells were recruited by the metastatic tumors. A desmin-positive area increased around the

liver metastatic tumors over time, suggesting CAFs were recruited by the metastatic tumors and have an important role in tumor progression. Stromal cells essential for metastatic tumors to develop in the liver can be identified with the stromal imaging model described in this report.

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