Color-Coded Imaging of Syngeneic Orthotopic Malignant Lymphoma Interacting with Host Stromal Cells During Metastasis

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Abstract. *Background: The EL4 cell line was previously derived from a lymphoma induced in a C57/BL6 mouse by 9,10-dimethyl-1,2-benzanthracene. In a previous study, EL4 lymphoma cells expressing red fluorescent protein (EL4-RFP) were established and injected into the tail vein of C57/BL6 green fluorescent protein (GFP) transgenic mice. Metastasis was observed at multiple sites which were also enriched with host GFP-expressing stromal cells. In the present study, our aim was to establish an orthotopic model of EL4-RFP. Materials and Methods: In the present study, EL4-RFP lymphoma cells were injected in the spleen of C57/BL6 GFP transgenic mice as an orthotopic model of lymphoma. Resultant primary tumor and metastases were imaged with the Olympus FV1000 scanning laser confocal microscope. Results: EL4-RFP metastasis was observed 21 days later. EL4-RFP tumors in the spleen (primary injection site), liver, supra-mediastinum lymph nodes, abdominal lymph nodes, bone marrow, and lung were visualized by color-coded imaging. EL4-RFP metastases in the liver, lymph nodes, and bone marrow in C57/BL6 GFP mice were rich in GFP stromal cells such as macrophages, fibroblasts, dendritic cells, and normal lymphocytes derived from the host animal. Small tumors were observed in the spleen, which were rich in host stromal cells. In the lung, no mass formation of lymphoma cells occurred, but lymphoma cells circulated in lung peripheral blood vessels. Phagocytosis of EL4-RFP lymphoma cells by macrophages, as well as dendritic cells and fibroblasts, were observed in culture. Conclusion: Color-coded imaging of the lymphoma microenvironment suggests an important role of stromal cells in lymphoma progression and metastasis.*

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With the use of multiple colored proteins, we have developed imaging of the tumor microenvironment (TME) by color-coding cancer and stromal cells (1-21). The TME is necessary for tumor growth and progression of disease (5). Color-coded imaging technology of the TME used green fluorescent protein (GFP) (2), red fluorescent protein (RFP) (7), or cyan fluorescent protein (CFP) (8) transgenic nude mice as hosts into which we transplanted cancer cells expressing a fluorescent protein not expressed by the host (1-21).

Tumors contain fibroblasts, lymphocytes, dendritic cells, macrophages and other myeloid cells in their microenvironment (22). Cancer-associated fibroblasts (CAFs) stimulate cancer cell growth, inflammation, angiogenesis, and invasion (22-26). As a tumor grows, it recruits CAFs and other host cells (27, 28). We reported the importance of CAFs in metastasis to the liver (9, 12). Non-colored HCT-116 human colon cancer cells were injected into the spleen of GFP-expressing nude mice which led to the formation of experimental liver metastases. 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 CAFs were recruited by the liver metastasis, and appeared to have a role in its progression (9).

A syngeneic color-coded imageable lymphoma model was previously developed to visualize recruitment of host stromal cells by malignant lymphoma during experimental

metastasis. The EL4 cell line was derived from a lymphoma induced in a C57/BL6 mouse by 9,10-dimethyl-1,2-benzanthracene. EL4 lymphoma cells expressing RFP (EL4-RFP) were established and injected into the tail vein of C57/BL6-GFP transgenic mice. EL4-RFP experimental metastasis was observed in the lymph nodes of the upper mediastinum and in the liver 28 days after cell injection. Large EL4-RFP liver metastases in C57/BL6-GFP mice contained GFP-expressing stromal cells derived from the host. In addition, EL4-RFP lymphoma metastasis was formed in peri-gastric lymph nodes, which were also enriched in host GFP-expressing cells. EL4-RFP lymphoma cells were also observed in the peripheral blood and bone marrow, where they were associated with GFP-expressing host cells (29).

The present study utilized color-coded fluorescent protein-based imaging to visualize the recruitment over time of host cells by lymph-node and liver metastases of the EL4-RFP lymphoma implanted in the spleen in syngeneic mice to establish an orthotopic model (29).

Materials and Methods

GFP transgenic mice. Transgenic C57/BL6-GFP mice (30) were obtained from the Research Institute for Microbial Diseases (Osaka University, Osaka, Japan). The C57/BL6-GFP mice expressed *Aequorea victoria* GFP under the control of the chicken β-actin promoter and cytomegalovirus enhancer (29).

Cell line and culture conditions. EL4, a mouse lymphoma cell line was established from a lymphoma induced in a C57/BL6 mouse by 9,10-dimethyl-1,2-benzanthracene (31). The cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin and streptomycin (Gibco-BRL, Grand Island, NY). The cells were cultured at 37˚C in a 5% CO_2 incubator (29).

RFP fluorescent protein transduction of lymphoma cells. Mouse EL4 lymphoma cells were labeled with RFP as previously reported using a retrovirus-based vector expressing RFP (4, 29, 32, 33).

Color-coded lymphoma-host cell model. Six-week-old GFP immunocompetent C57/BL6-GFP transgenic mice were used as the host for EL4-RFP lymphoma cells. EL4-RFP lymphoma cells growing *in vitro* were first harvested and washed three times with cold serum-free medium and then resuspended with serum-free RPMI-1640 medium. EL4-RFP lymphoma cells were injected in the spleen of C57/BL6-GFP transgenic mice (29).

Tumor imaging. SZX7 microscope, and FV1000 confocal microscope, all from Olympus Corp. (Tokyo, Japan), were used for imaging in this study (29).

Study approval. All experiments were conducted in accordance with the institutional guidelines of Gifu University and were approved by the Animal Research Committee and the Committee on Living Modified Organisms of Gifu University (approval number 26-37) (29).

Results and Discussion

EL4-RFP mouse malignant lymphoma cancer cells were injected in the spleen of GFP mice. EL4-RFP metastasis was observed in the liver (Figure 1A), in the lymph nodes of the upper mediastinum and abdomen (Figure 2C,E), and bone marrow (Figure 3F) after 21 days.

High-magnification confocal fluorescence microscopy showed extensive GFP fluorescence within and around RFP-expressing tumors indicating extensive recruitment of stromal cells. A large EL4-RFP liver metastases in a C57/BL6 GFP mouse had GFP stromal cells derived from the host animal around the edge of the metastasis (Figure 1B). The stromal cells included macrophages, dendric cells, lymphocytes, and fibroblasts.

In contrast to the liver and the lymph nodes, a small lymphoma was observed in the spleen at the primary injection site. However, the stroma cells in the spleen tumors were abundant (Figure 1C,D).

EL4-RFP lymphoma cells metastasis, formed in abdominal lymph nodes and mediastinal lymph nodes, also contained stromal cells which accumulated at the edge of the tumor. (Figure 2A-D).

EL4-RFP lymphoma cells also circulated in the peripheral blood in the lung and brain, although no metastasis occurred in these organs (Figure 1E,F and 2A,B).

Lymphoma cells from multiple metastases were cultured *in vitro* (Figure 3). Phagocytosis of lymphoma cells by macrophages, as well as fibroblasts and dendritic cells were observed in the cultures of the spleen and each metastatic lesion (at one and four days of culture). Interestingly, no metastasis formation was observed in the lungs and the brain where macrophages, fibroblasts, dendritic cells in addition to lymphoma cells did not infiltrate into the organ tissue.

Thus, we have demonstrated a color-coded imaging model in which the development of the TME during lymphatic and hematogenous metastasis can be visualized in a syngeneic orthotopic model of metastatic lymphoma. In GFP-expressing transgenic mice, only non-parenchymal cells of the liver have GFP fluorescence (29), which makes it a very high-contrast model for imaging stromal recruitment by liver metastases. The orthotopic model of malignant lymphoma can be used to study early tumor development, metastasis and the role of stroma, for the discovery and evaluation of novel therapeutics for targeting both the lymphoma and its host stromal cells.

Conflict of Interest

None of the Authors have any conflict of interest in regard to this study.

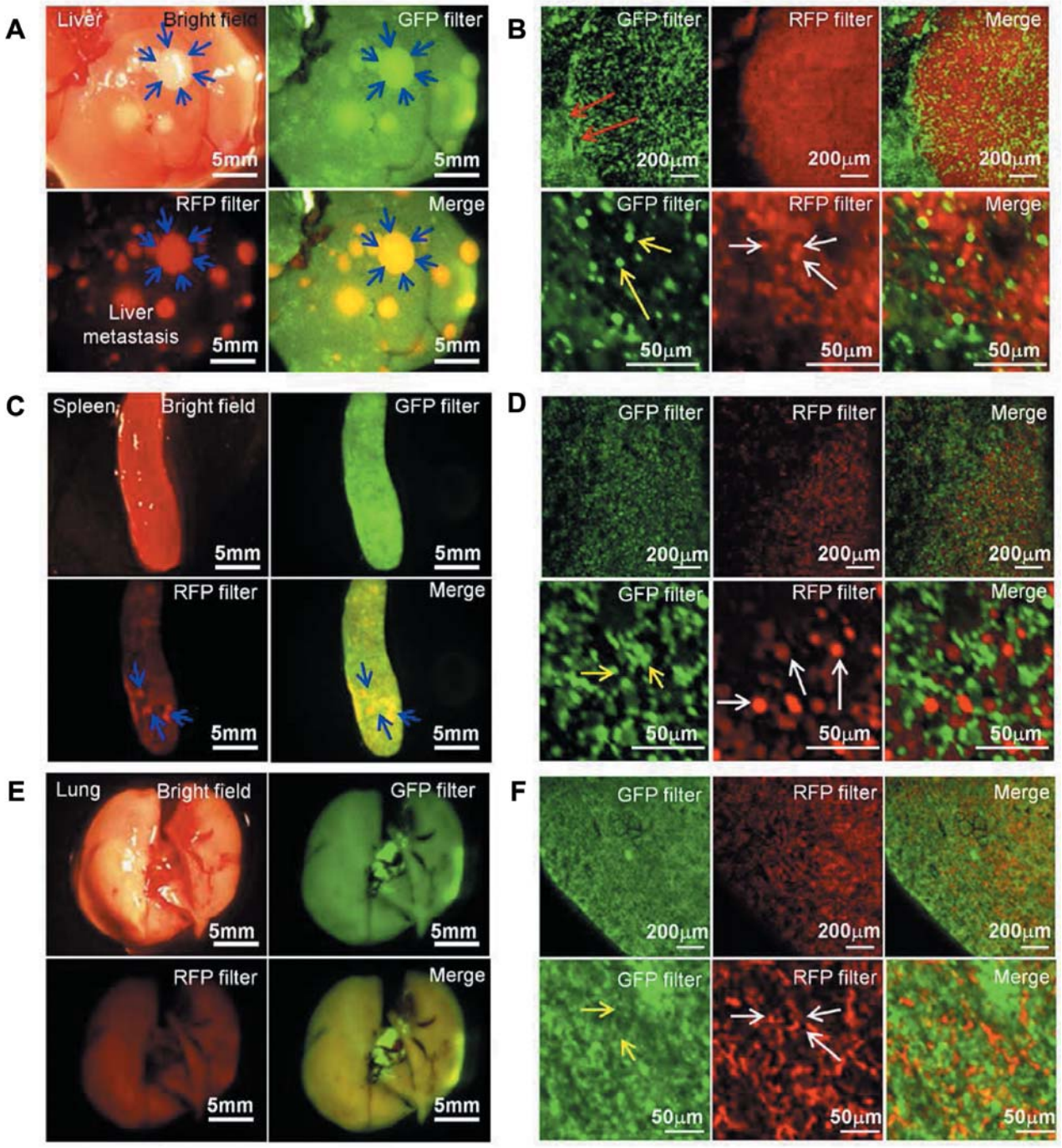

Figure 1. *(A) Bright-field and fluorescence images of EL4-RFP mouse lymphoma liver metastasis 21 days after splenic injection of cells. Blue arrows indicate liver metastasis. Images were captured with the Olympus SZX7 microscope (Bar=5 mm). (B) Images of liver metastasis (Upper panels: low magnification, Bar=200 μm; Lower panels: high magnification, Bar=50 μm): Yellow arrows indicate GFP-expressing normal lymphocytes and white arrows indicate EL4-RFP lymphoma cells. GFP-expressing stromal cells derived from the host animal accumulated around the edge of EL4-RFP lymphoma tumors (red arrows). Images were captured with the Olympus FV1000 confocal microscope. (C) Bright-field and fluorescence images of spleen (primary injected site) (Bar=5 mm): Blue arrows indicate the spleen tumor. The tumor in the spleen was smaller compared to the liver metastasis. (D) Images of spleen tumor (Upper panels: low magnification, Bar=200 μm; Lower panels: high magnification, Bar=50 μm): Yellow arrows indicate GFP-expressing normal lymphocytes and white arrows indicate EL4-RFP lymphoma cells. (E) Bright-field and fluorescence images of the lung: No lymphoma was observed in the lung. (F) Images of the lung (Upper panels: low magnification, Bar=200 μm; Lower panels: high magnification, Bar=50 μm): Yellow arrows indicate GFP-expressing lung white blood cells circulating in the peripheral blood. White arrows indicate EL4-RFP lymphoma cells circulating in the peripheral blood.*

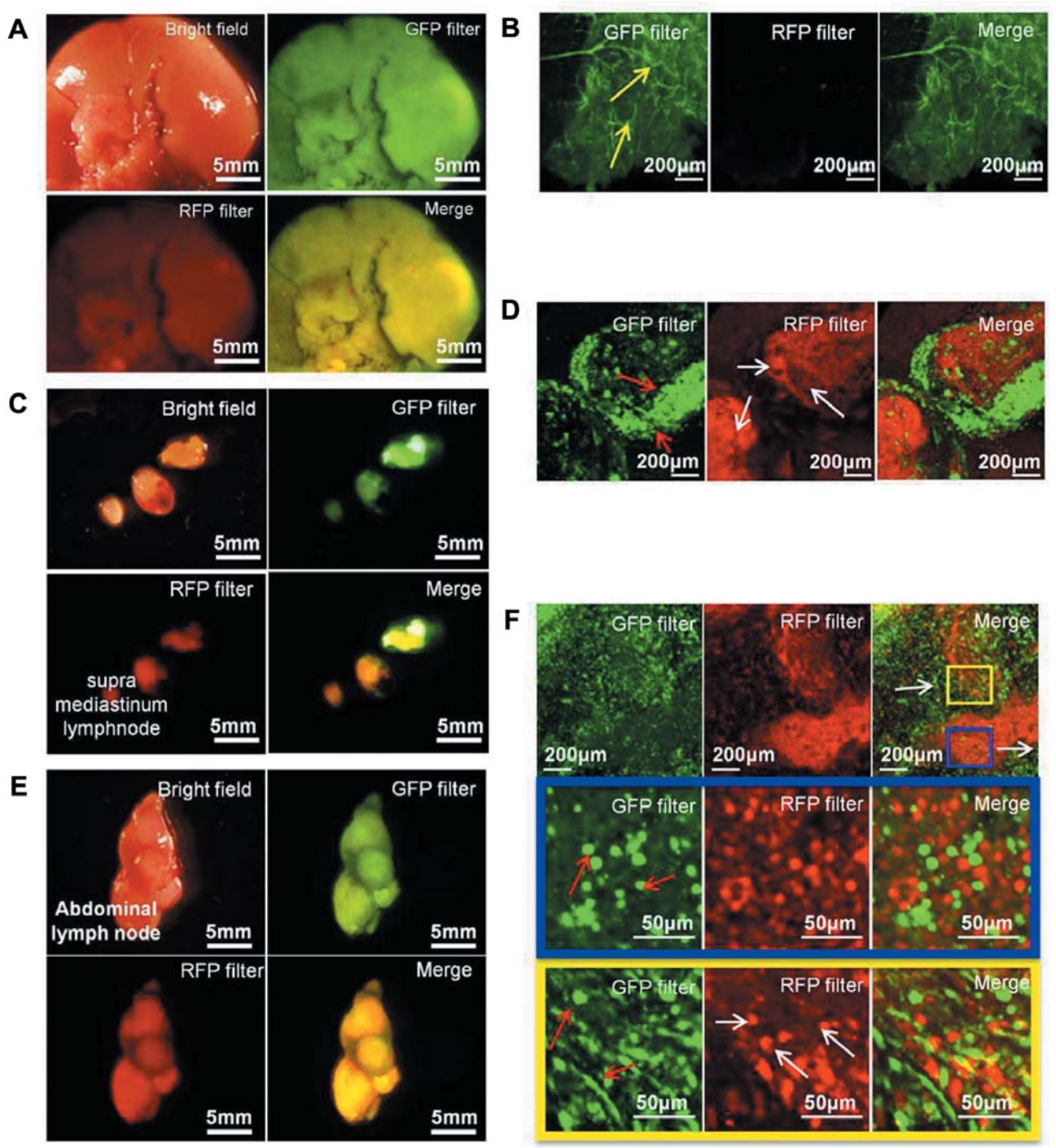

Figure 2. *(A) Bright-field and fluorescence images of brain 21 days after spleen injection of EL4-RFP lymphoma cells (Bar=5 mm). (B) Images of the cerebral cortex: GFP-expressing axon of a neuron was captured with the Olympus FV1000 confocal microscope (yellow arrows). No mass formation by lymphoma cells was observed in the brain (Bar=200 μm). (C) Bright-field and fluorescence image of supra-mediastinal lymph nodes: Lymph nodes swelled and RFP-expressing lymphoma mass formation was observed (Bar=5 mm). (D) Images of supra-mediastinal lymph nodes: Red arrows indicate GFP-expressing stromal cells derived from the host animal gathered around the edge of EL4-RFP lymphoma metastasis. White arrows indicate EL4-RFP lymphoma cells (Bar=200 μm). (E) Bright-field and fluorescence images of abdominal lymph nodes: Lymph nodes were swollen and RFP-expressing mass formation was observed. (F) Images of abdominal lymph nodes (Upper panels: low magnification, Bar=200 μm; Middle panels: high-magnification, Bar=50 μm; Lower panels: high magnification, Bar=50 μm). Red arrows indicate that GFP-expressing normal lymphocytes and fibroblast derived from the host animal gathered around the edge of EL4-RFP lymphoma cells. White arrows indicate EL4-RFP lymphoma cells.*

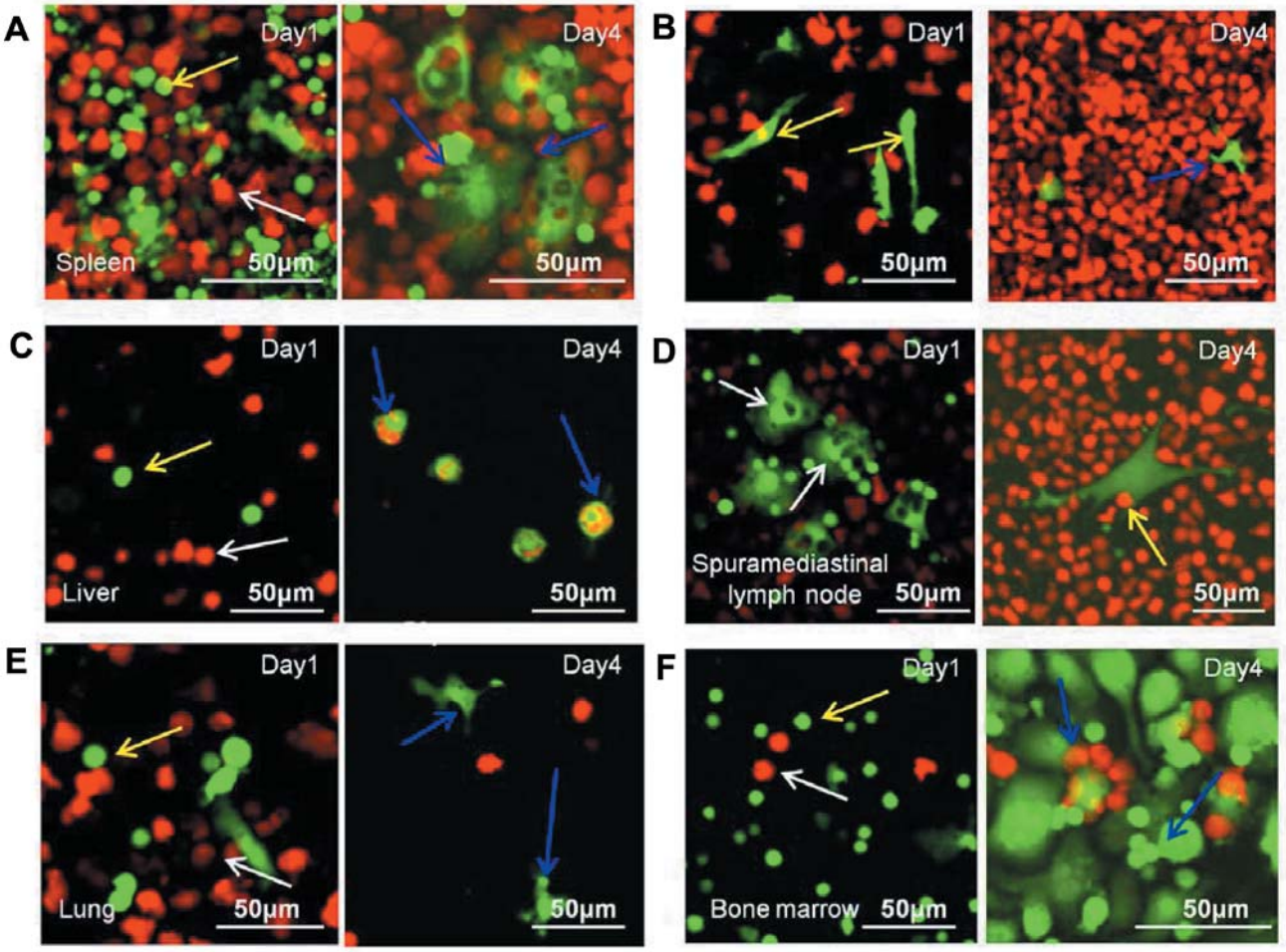

Figure 3. *High magnification images of cultured EL4-RFP tumors and metastasis which were collected from the mouse metastatic organs. Images were obtained with the Olympus FV 1000 confocal microscope. Left panel: Day 1; Right panel: Day 4. (A) Cultures of the spleen lymphoma (Bar=5 mm): yellow arrow indicates GFP-expressing normal lymphocyte and white arrow indicates EL4-RFP lymphoma cell. Blue arrows indicate GFP-expressing mouse macrophages. Phagocytosis of EL4-RFP expressing lymphoma cells was observed. (B) The spleen lymphoma contained GFP-expressing fibroblasts (yellow arrows) and dendritic cells (Blue arrow) among the EL4-RFP lymphoma cells. (C) Culture of liver lymphoma metastasis: yellow arrow indicates GFP-expressing normal lymphocyte and white arrow indicates RFP-expressing lymphoma cell (Bar=5 mm). Blue arrows indicate phagocytosis of RFP-expressing lymphoma cells by GFP-expressing macrophages. The liver lymphoma metastasis was rich in GFP-expressing stromal cells. (D) Cultures of metastatic lymphoma lymph nodes: lymph nodes were also rich in GFP-expressing stromal cells. White arrows indicate GFP-expressing macrophages and yellow arrow indicates EL4-RFP lymphoma cells. (E) Culture of lung tissues: yellow arrow indicates GFP-expressing normal lymphocyte. White arrow indicates RFP-expressing lymphoma cell and blue arrows indicate GFP-expressing dendritic cells. (F) Culture of bone marrow: yellow arrow indicates GFP-expressing normal lymphocyte. White arrow indicates RFP-expressing lymphoma cell. Blue arrows indicate GFP-expressing macrophages.*

References

1 Yang M, Li L, Jiang P, Moossa AR, Penman S and Hoffman RM: Dual-color fluorescence imaging distinguishes tumor cells from induced host angiogenic vessels and stromal cells. Proc Natl Acad Sci USA *100*: 14259-14262, 2003.

2 Yang M, Reynoso J, Jiang P, Li L, Moossa AR and Hoffman RM: Transgenic nude mouse with ubiquitous green fluorescent protein expression as a host for human tumors. Cancer Res *64*: 8651-8656, 2004.

3 Amoh Y, Yang M, Li L, Reynoso J, Bouvet M, Moossa AR, Katsuoka K and Hoffman RM: Nestin-linked green fluorescent protein transgenic nude mouse for imaging human tumor angiogenesis. Cancer Res *65*: 5352-5357, 2005.

4 Hoffman RM and Yang M: Color-coded fluorescence imaging of tumor–host interactions. Nat Protoc *1*: 928-935, 2006.

5 Bouvet M, Tsuji K, Yang M, Jiang P, Moossa AR and Hoffman RM: *In vivo* color-coded imaging of the interaction of colon cancer cells and splenocytes in the formation of liver metastases. Cancer Res *66*: 11293-11297, 2006.

6 Yang M, Jiang P and Hoffman RM: Whole-body subcellular multicolor imaging of tumor-host interaction and drug response in real time. Cancer Res *67*: 5195-5199, 2007.

7 Yang M, Reynoso J, Bouvet M and Hoffman RM: A transgenic red fluorescent protein-expressing nude mouse for color-coded imaging of the tumor microenvironment. J Cell Biochem *106*: 279-284, 2009.

8 Tran Cao HS, Reynoso J, Yang M, Kimura H, Kaushal S, Snyder CS, Hoffman RM and Bouvet M: Development of the transgenic cyan fluorescent protein (CFP)-expressing nude mouse for "Technicolor" cancer imaging. J Cell Biochem *107*: 328-334, 2009.

9 Suetsugu A, Osawa Y, Nagaki M, Saji S, Moriwaki H, Bouvet M and Hoffman RM: Imaging the recruitment of cancer-associated fibroblasts by liver-metastatic colon cancer. J Cell Biochem *112*: 949-953, 2011.

10 Suetsugu A, Hassanein MK, Reynoso J, Osawa Y, Nagaki M, Moriwaki H, Saji S, Bouvet M and Hoffman RM: The cyan fluorescent protein nude mouse as a host for multicolor-coded imaging models of primary and metastatic tumor microenvironments. Anticancer Res *32*: 31-38, 2012.

11 Yamauchi K, Tome Y, Yamamoto N, Hayashi K, Kimura H, Tsuchiya H, Tomita K, Bouvet M and Hoffman RM: Color-coded real-time subcellular fluorescence imaging of the interaction between cancer and host cells in live mice. Anticancer Res *32*: 39-43, 2012.

12 Suetsugu A, Katz M, Fleming J, Truty M, Thomas R, Saji S, Moriwaki H, Bouvet M and Hoffman RM: Imageable fluorescent metastasis resulting in transgenic GFP mice orthotopically implanted with human-patient primary pancreatic cancer specimens. Anticancer Res *32*: 1175-1180, 2012.

13 Suetsugu A, Katz M, Fleming J, Truty M, Thomas R, Moriwaki H, Bouvet M, Saji S and Hoffman RM: Multi-color palette of fluorescent proteins for imaging the tumor microenvironment of orthotopic tumorgraft mouse models of clinical pancreatic cancer specimens. J Cell Biochem *113*: 2290-2295, 2012.

14 Suetsugu A, Katz M, Fleming J, Truty M, Thomas R, Saji S, Moriwaki H, Bouvet M and Hoffman RM: Non-invasive fluorescent-protein imaging of orthotopic pancreatic-cancer-patient tumorgraft progression in nude mice. Anticancer Res *32*: 3063-3068, 2012.

15 Menen RS, Hassanein MK, Momiyama M, Suetsugu A, Moossa AR, Hoffman RM and Bouvet M: Tumor-educated macrophages promote tumor growth and peritoneal metastasis in an orthotopic nude mouse model of human pancreatic cancer. In Vivo *26*: 565-569, 2012.

16 Momiyama M, Kumamoto T, Suetsugu A, Kishimoto H, Chishima T, Tanaka K, Akiyama H, Ichikawa Y, Bouvet M, Endo I and Hoffman RM: Major liver resection stimulates stromal recruitment and metastasis compared with repeated minor resection. J Surg Res *178*: 280-287, 2012.

17 Uchugonova A, Zhao M, Weinigel M, Zhang Y, Bouvet M, Hoffman RM and Koenig K: Multiphoton tomography visualizes collagen fibers in the tumor microenvironment that maintain cancer-cell anchorage and shape. J Cell Biochem *114*: 99-102, 2013.

18 Snyder CS, Harrington AR, Kaushal S, Mose E, Lowy AM, Hoffman RM and Bouvet M: A dual-color genetically engineered mouse model for multispectral imaging of the pancreatic microenvironment. Pancreas *42*: 952-958, 2013.

19 Uehara F, Tome Y, Miwa S, Hiroshima Y, Yano S, Yamamoto M, Mii S, Maehara H, Bouvet M, Kanaya F and Hoffman RM: Osteosarcoma cells enhance angiogenesis visualized by color-coded imaging in the *in vivo* Gelfoam® assay. J Cell Biochem *115*: 1490-1494, 2014.

20 Hoffman RM: Transgenic nude mice ubiquitously-expressing fluorescent proteins for color-coded imaging of the tumor microenvironment. *In*: Mouse Genetics: Methods and Protocols. Chap. 20. Methods in Molecular Biology Shree Ram Singh (ed.) Vol. 1194: Springer Science+Business Media New York, pp. 353-365, 2014.

21 Hiroshima Y, Maawy A, Hassanein MK, Menen R, Momiyama M, Murakami T, Miwa S, Yamamoto M, Uehara F, Yano S, Mori R, Matsuyama R, Chishima T, Tanaka K, Ichikawa Y, Bouvet M, Endo I and Hoffman RM: The tumor-educated-macrophage increase of malignancy of human pancreatic cancer is prevented by zoledronic acid. PLoS One *9*: e103382, 2014.

22 Egeblad M, Ewald AJ, Askautrud HA ,Truitt ML, Welm BE, Bainbridge E, Peeters G, Krummel MF and Werb Z: Visualizing stromal cell dynamics in different tumor microenvironments by spinning disk confocal microscopy. Dis Model Mech *1*: 155-167, 2008.

23 Kalluri R and Zeisberg M: Fibroblasts in cancer. Nat Rev Cancer *6*: 392-401, 2006.

24 Gaggioli C, Hooper S, Hidalgo-Carcedo C, Grosse R, Marshall JF, Harrington K and Sahai E: Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. Nat Cell Biol *9*: 1392-1400, 2007.

25 Pietras K, Pahler J, Bergers G and Hanahan D: Functions of paracrine PDGF signaling in the proangiogenic tumor stroma revealed by pharmacological targeting. PLoS Med *5*: e19, 2008.

26 Erez N, Truitt M, Olson P Arron ST and Hanahan D: Cancer-associated fibroblasts are activated in incipient neoplasia to orchestrate tumor- promoting inflammation in an NF-kappaB-dependent manner. Cancer Cell *17*: 135-147, 2010.

27 Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL and Weinberg RA: Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell *121*: 335-348, 2005.

28 Egeblad M, Nakasone ES and Werb Z: Tumors as organs: Complex tissues that interface with the entire organism. Dev Cell 18: 884-901, 2010.

29 Matsumoto T, Suetsugu A, Shibata Y, Nakamura N, Aoki H, Kunisada T, Tsurumi H, Shimizu M and Hoffman RM: A color-coded imageable syngeneic mouse model of stromal-cell recruitment by metastatic lymphoma. Anticancer Res *35*: 4647-4654, 2015.

30 Okabe M, Ikawa M, Kominami K, Nakanishi T and Nishimune Y: 'Green mice' as a source of ubiquitous green cells. FEBS Lett 407: 313–319, 1997.

31 Zielinski MR, Muenchow M, Wallig MA, Horn PL and Woods JA: Exercise delays allogeneic tumor growth and reduces intratumoral inflammation and vascularization. J Appl Physiol *96*: 2249-2256, 2004.

32 Hoffman RM and Yang M: Subcellular imaging in the live mouse. Nat Protoc *1*: 775-782, 2006.

33 Hoffman RM and Yang M: Whole-body imaging with fluorescent proteins. Nat Protoc *1*: 1429-1438, 2006.

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