Imaging tumor angiogenesis with fluorescent proteins

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We have developed three unique mouse models to image angiogenesis with fluorescent proteins, which are described in this review. First, we have adapted the surgical orthotopic implantation (SOI) model to image angiogenesis of human tumors labeled with green fluorescent protein (GFP) transplanted in nude mice. The nonluminous induced capillaries are clearly visible by contrast against the very bright tumor fluorescence examined either intravitaly or by whole-body imaging in real time. Intravital images of an SOI model of human pancreatic tumors expressing GFP visualized angiogenic capillaries at both primary and metastatic sites. Whole-body optical imaging showed that blood vessel density increased linearly over a 20-week period in an SOI model of human breast cancer expressing GFP. Opening a reversible skin-flap in the light path markedly reduces signal attenuation, increasing detection sensitivity many-fold and enabling vessels to be externally visualized in GFP-expressing tumors growing on internal organs. The second model utilizes dual-color fluorescence imaging, effected by using red fluorescent protein (RFP)-expressing tumors growing in GFP-expressing transgenic mice that express GFP in all cells. This dual-color model visualizes with great clarity the details of the tumor-stroma interaction, especially tumor-induced angiogenesis. The GFP-expressing tumor vasculature, both nascent and mature, are readily distinguished interacting with the RFP-expressing tumor cells. Using a spectral imaging system based on liquid crystal tunable filters, we were able to separate individual spectral species on a pixel-by-pixel basis. Such techniques non-invasively visualized the presence of host GFP-expressing vessels within an RFP-labeled orthotopic human breast tumor by real-time whole-body imaging. The third model involves a transgenic mouse in which the regulatory elements of the stem cell marker nestin drive GFP. The nestin-GFP mouse expresses GFP in areas of the brain, hair follicle stem cells, and in a network of blood vessels in the skin interconnecting hair follicles. RFP-expressing tumors transplanted to nestin-GFP mice enable specific visualization of nascent vessels in skin-growing tumors such as melanoma. Thus, fluorescent proteins expressed in vivo offer very high resolution and sensitivity for real-time imaging of angiogenesis.

Key words: Green fluorescent protein; red fluorescent protein; imaging; multi-color; real-time; in vivo.

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INTRODUCTION

Previous models used to visualize angiogenesis

The discovery and evaluation of angiogenic substances initially relied on methods such as the chorioallantoic membrane assay (1, 2), the monkey iris neovascularization model (3), the disk angiogenesis assay (4), and various models that use the cornea to assess blood ves-

Invited Review.
Tumor xenografts are not representative models of human disease.

Tumors transplanted in the cornea of the rodents (11–13) and rodent skin-fold window chambers have also been used for angiogenesis studies (14–20). The cornea and skin-fold chamber models provide a means for studying tumor angiogenesis in living animals. However, quantification requires specialized procedures, and the sites do not represent natural environments for tumor growth. The cornea and skin-fold window chamber tumor models do not allow metastasis and angiogenesis to occur, which may involve mechanisms of angiogenesis (21) that are qualitatively different from those occurring in ectopic models.

**FLUORESCENT ANGIOGENESIS MODELS**

*Fluorescent proteins to image angiogenesis*

Fluorescent proteins have been shown to be very useful for imaging in tumors including the formation of nascent vessels. We have developed three unique mouse models to image tumor angiogenesis with fluorescent proteins, which are described in this review.

*Orthotopic tumor models expressing fluorescent proteins to visualize tumor angiogenesis (Model #1)*

For realistic and real-time tumor angiogenesis models, we have developed surgical orthotopic implantation (SOI) metastatic models of human cancer (22). These models place tumors in natural microenvironments and replicate clinical tumor behavior more closely than do ectopic implantation models (22). The orthotopically-growing tumors, in contrast to most other models, give rise to spontaneous metastases that resemble, both in target tissues and in frequency of occurrence, the clinical behavior of the original human tumor (23). The tumors implanted in the orthotopic model have been transduced and selected to strongly express green fluorescent protein (GFP) in vivo (22). Orthotopically-implanted GFP-labeled tumors enable the visualization of the role of angiogenesis in metastasis. As Li et al. (18) point out, angiogenesis initiation in metastatic tumors may be very different from that of primary tumors and require different interventions. Moreover, the extreme detection sensitivity afforded by the strong GFP fluorescence allows imaging of very early events in blood vessel induction.

GFP expression in primary tumors and in their metastases in the mouse models can be detected by an intense fluorescence seen by intravital or by whole-body imaging. The nonluminous angiogenic blood vessels appear as sharply defined dark networks against this bright background. The high image resolution permits quantitative measurements of total vessel length. These genetically fluorescent tumor models thereby allow quantitative optical imaging of angiogenesis in vivo. Tumor growth, vascularization, and metastasis can now be followed in real time (24).

*Intravital images of angiogenesis of orthotopic pancreas cancer*

The clarity of angiogenic blood vessel imaging was illustrated by intravital examination of the orthotopic growth of a Bx-PC-3-GFP pancreatic tumor. The nonluminous blood vessels were clearly visible against the GFP fluorescence of the primary tumor. Angiogenesis associated with metastatic growths was readily imaged by intravital examination (24) (Fig. 1).

*Intravital imaging of angiogenesis of orthotopic prostate cancer*

Because angiogenesis could be measured without animal sacrifice, it was possible to determine a time course for individual animals. Sequential intravital images of angiogenesis for the PC-3 human prostate tumor expressing GFP and growing orthotopically in a single nude mouse were acquired. The tumor-associated blood vessels were clearly visible by day-7 and continued to increase at least until day-20 (24).

*Whole-body imaging of angiogenesis in orthotopic breast cancer*

We have demonstrated whole-body images and quantitation of the time course of angiogenesis of the MDA-MB-435-GFP human breast cancer growing orthotopically in the mammary fat pad in a nude mouse. The development of the tumor and its angiogenesis could be imaged in a completely noninvasive manner (24). The mouse mammary fat pad is the orthotopic environment for the implanted MDA-MB-435-GFP breast
cancer and allows noninvasive, whole-body imaging of tumor angiogenesis. The quantitative angiogenesis data show that microvessel density increased over 20 weeks. Thus, tumors in their natural microenvironment, growing orthotopically in sites such as the fatpad, can be whole-body imaged for quantitative angiogenesis studies (24) (Fig. 2).

Skin flaps enable ultra-high resolution external imaging of tumor angiogenesis

Opening a reversible skin-flap in the light path markedly reduced signal attenuation, increasing detection sensitivity many-fold. The observable depth of tissue is thereby greatly increased (25). The brilliance of the tumor GFP fluorescence, facilitated by the reduced absorption through the skin-flap window, allowed imaging of the induced microvessels by their contrast against a bright background. The orthotopically growing BxPC-3-GFP human pancreatic tumor was externally visualized under fluorescence microscopy to be surrounded by its microvessels visible by their dark contrast (25) (Fig. 3).

Dual-color tumor-host models to differentially visualize the tumor and blood vessels (Model #2)

Okabe et al. (26) produced transgenic mice with GFP under the control of a chicken beta-actin promoter and cytomegalovirus enhancer. All of the tissues from these transgenic mice, with the exception of erythrocytes and hair, fluoresce green.

Tumor cells to be transplanted in the GFP mouse were made visible by transforming them with the red fluorescent protein (RFP) (22). In order to gain further insight into tumor-host interaction in the living state, including tumor angiogenesis, we have visualized RFP-expressing tumors transplanted in the GFP-expressing transgenic mice under dual-color fluorescence microscopy. The dual-color fluorescence made it possible to visualize the tumor growth in the host by whole-body imaging as well as to visibly distinguish interacting tumor and host cells in fresh tissue. The dual-color approach affords a powerful means of both visualizing and distinguishing the components of the host-tumor interaction (27).

Dual-color images of early events in tumor angiogenesis induced by a B16F10 mouse melanoma in the transgenic GFP-expressing mouse were acquired in fresh tissue preparations. Host-derived GFP-expressing fibroblast cells and endothelial cells from nascent blood vessels were visualized clearly against the red fluorescent background of the RFP-expressing mouse melanoma. Host-derived GFP-expressing mature blood vessels within the RFP-expressing mouse melanoma also became visible. The images were acquired 3 weeks after subcutaneous injection of B16F10-RFP melanoma cells in the GFP mouse (27) (Fig. 4).

Dual-color imaging with spectral resolution with ultra-high resolution whole-body imaging of angiogenesis

The RFP-expressing MDA-MB-435 breast tumors growing in the GFP-mice were visual-
alyzed using excitation centered at 470 nm and appropriate >500 nm emission filters. Using a spectral imaging system based on liquid crystal tunable filters, we were able to perform whole-body imaging yielding high-resolution spectral information at each pixel of the resulting image. Analysis algorithms allow the separation (unmixing) of individual spectral species on a pixel-by-pixel basis. Such techniques non-invasively visualized the presence of host GFP-expressing stroma within the RFP-labeled tumor. Moreover, fluorescence spectra emitted in the far-red allow the whole-body imaging of tumor angiogenesis. This new differential dual-colored fluorescence imaging tumor-host model, along with spectral unmixing, can non-invasively visualize in real-time the onset and progression of angiogenesis in a tumor. Other host cells and structures in the tumor may also be visualized by whole-body spectral imaging (28).

Fig. 2. Time course of whole-body fluorescence imaging of MDA-MB-435-GFP human breast cancer angiogenesis in orthotopic primary tumor. The GFP-expressing human tumor was transplanted by SOI in the fat pad of nude mice and whole-body imaged. (A) Week 1. (B) Week 4. (C) Week 8. (D) Week 20. (E) Quantitative graph of microvessel density as a function of time (6).
Imaging of nascent angiogenesis using nestin-driven GFP transgenic mice (Model #3)
The intermediate filament protein, nestin, marks progenitor cells of the CNS. Such CNS stem cells are selectively labeled by placing GFP under the control of nestin regulatory sequences in transgenic mice. During early anagen or growth phase of the hair follicle, nestin-expressing cells, marked by GFP fluorescence in nestin-GFP transgenic mice, appear in the permanent upper hair follicle immediately below the sebaceous glands in the follicle bulge. This is where stem cells for the hair follicle are thought to be located. The relatively small, oval-shaped, nestin-expressing cells in the bulge area surround the hair shaft. In the nestin-GFP transgenic mice, the hair follicles are linked by a network of nestin-GFP-expressing blood vessels (Fig. 5) (Amoh Y, Li L, Hoffman RM, Yang M, Jiang P, unpublished data). When RFP-expressing B16 melanoma was transplanted to the nestin-GFP mice, nestin-expressing vessels grew from the hair follicles into the growing melanoma (Figs. 5 & 6) (Amoh Y, Li L, Hoffman RM, Yang M, Jiang P, unpublished data). RFP-tumor cells can be visualized filling the GFP-blood vessels (Fig. 6). Immunohistochemical staining showed that endothelial-cell-specific markers CD31 as well as von Willebrand factor (vWF) and nestin colocalize in the nestin-GFP-expressing nascent vessels. The data suggest hair follicles give rise to blood vessels in the skin. This model enables very early events in skin angiogenesis, including skin-tumor angiogenesis to be visualized, and to be used for antiangiogenesis drug screening.

Materials and Methods

Fluorescence optical imaging (24)
Whole-body imaging was performed in a fluorescent light box illuminated by fiberoptic lighting at
Fig. 5. B16 mouse melanoma expressing RFP subcutaneously implanted in nestin-GFP mice (day-21). Blood vessels, expressing nestin-GFP (white arrows), are seen growing from the bulge area of hair follicles into the melanoma, expressing RFP, which is highlighted in (B) with green excitation light. Fresh tissue, fluorescence microscopy (Amoh Y, Li L, Yang M, Jiang P, Hoffmann RM, unpublished data).

Fig. 6. RFP B16 melanoma cells in GFP-nestin-expressing vessels. GFP-expressing blood vessels in the nestin-GFP transgenic mouse are visualized by dual-color fluorescence microscopy in a transplanted RFP-expressing B16 melanoma. RFP-expressing cancer cells are present in GFP-expressing blood vessels under both blue (A) and green (B) excitation. Fluorescence microscopy in fresh tissue acquired at 7 days after transplantation (Amoh Y, Li L, Hoffman RM, Yang M, Jiang P, unpublished data).

470 nm (Lightools Research, Encinitas, CA). A Leica fluorescence stereo microscope model LZ12 equipped with a mercury lamp and a 50-W power supply was used. Selective excitation of GFP was produced through a D425y60 band-pass filter and a 470 DCXR dichroic mirror. Emitted fluorescence was collected through a long-pass filter (GG475; Chroma Technology, Brattleboro, VT) on a Hamamatsu C5810 3-chip cooled color charge-coupled device camera (Hamamatsu Photonics, Bridgewater, NJ). Images were processed for contrast and brightness and analyzed with the use of IMAGE PRO PLUS 3.1 software (Media Cybernetics, Silver Spring, MD). High-resolution images of 1024×724 pixels were captured directly on an IBM PC or continuously through video output on a high-resolution Sony VCR (model SLVR1000; Sony, Tokyo).

GFP and RFP vector production (24, 27)

GFP expression vector. The pLEIN retroviral vector (CLONTECH) expresses enhanced GFP and the neomycin resistance gene on the same bicistronic message, which contains an internal ribosome entry site.

RFP expression vector. The pLNCTX2 vector was purchased from Clontech Laboratories (Palo Alto, CA).
The pLNCX₂ vector contains the neomycin resistance gene for antibiotic selection. The RFP gene (DsRed2; Clontech Laboratories) was inserted in the pLNCX₂ vector at the Egl II and Not I sites.

Retroviral GFP and RFP transduction of tumor cells (24)

For GFP and RFP gene transduction, 25% confluent cells were incubated with a 1:1 precipitated mixture of supernatants of PT67 packaging cells, containing either pLEIN-GFP or pLNCX₂-RFP, and RPMI 1640 (GIBCO) containing 10% FBS (Gemini Biological Products) for 72 h. Fresh medium was replenished at this time. Cells were harvested by trypsin EDTA 72 h after transduction and subcultured at a ratio of 1:15 into selective medium, which contained 200 mg/ml of G418. The level of G418 was increased stepwise up to 1000 mg/ml. Clones stably expressing GFP or RFP were isolated with cloning cylinders (Bel- Art Products) with the use of trypsin/EDTA and were then amplified and transferred by conventional culture methods.

Animals (24)

GFP transgenic mice. Transgenic C57/B6-GFP mice (26) were obtained from the Research Institute for Microbial Diseases, Osaka University, Osaka, Japan. The C57/B6-GFP mice express GFP under the control of the chicken beta-actin promoter and cytomegalovirus enhancer. All of the tissues from this transgenic line, with the exception of erythrocytes and hair, fluoresce green under excitation light. The GFP gene, regulated as described above, was crossed in to nude mice on the C57/B6 background. Both immunocompetent and nude GFP transgenic mice were used.

Nestin-GFP transgenic mice. Nestin is an intermediate filament (IF) gene that is a marker for CNS progenitor cells and neuroepithelial stem cells. GFP transgenic mice carrying GFP under the control of the nestin second-intron enhancer were used. We have previously observed that hair follicle stem cells strongly express nestin as evidenced by nestin-regulated GFP expression (29).

All animal studies were conducted in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals under assurance number A3873-1. Animals were kept in a barrier facility under HEPA filtration. Mice were fed with autoclaved laboratory rodent diet (Teklad LM-485, Western Research Products, Orange, CA).

SOI tumor models (23)

Tumor fragments (1 mm³), stably expressing GFP or RFP, previously grown s.c. in nude mice, were implanted by SOI on the appropriate organ in nude mice. After proper exposure of the organ to be implanted, 8-0 surgical sutures were used to penetrate the tumor pieces and attach them to the appropriate orthotopic organ. The incision in the skin was closed with a 7-0 surgical suture in one layer. The animals were kept under isoflurane anesthesia during surgery. All procedures of the operation described above were performed with a 37 magnification microscope (MZ6; Leica, Nussloch, Germany).

Cutaneous melanoma model (27)

Six-week-old male C57/B6-GFP or nestin-GFP transgenic mice were injected subcutaneously with 10⁶ RFP-expressing mouse B16F0 melanoma cells. Cells were first harvested by trypsinization and washed three times with cold serum-containing medium, then kept on ice. Cells were inoculated by intradermal injection of the dorsal skin of the animal in a total volume of 50 μl within 40 min of harvesting.

Quantitative analysis of angiogenesis (24)

Periodically, the tumor-bearing mice were examined by intravital or whole-body fluorescence imaging. The extent of blood vessel development in a tumor was evaluated based on the total length of blood vessels (L) in chosen areas: areas containing the highest number of vessels were identified by scanning the tumors by intravital or whole-body imaging. To compare the level of vascularization during tumor growth, the “hot” areas with the maximum development of vessels per unit area were then quantitated for L expressed in pixels. Captured images were corrected for unevenness in illumination. Then the total number of pixels derived from the blood vessels was quantified with IMAGE PRO PLUS software.

Spectral resolution

Spectral imaging is the generation of images containing a high-resolution optical spectrum at every pixel, to “unmix” the autofluorescence signal from that of the fluorescent protein-labeled tumors and bacteria. The standard GFP imaging system (long-pass emission filter) is modified by replacing the usual color camera with a cooled monochrome camera and a liquid crystal tunable filter (CRI, Inc., Woburn, MA) positioned in front of a conventional macro lens. Typically, a series of images is taken every 10 nm from 500 to 650 nm and assembled automatically in memory into a spectral “stack”. Using pre-defined GFP or RFP and autofluorescence spectra, the image can be resolved into spectra using an algorithm (28).

Skin-flap windows (25)

Tumor cells on the various internal organs were visualized through the body wall through a skin-flap window over the abdomen. The animals were anesthetized with a ketamine mixture. An arc-shaped incision was made in the skin, and s.c. connective tissue was separated to free the skin flap. The skin flap could be opened repeatedly to image tumor cells on
the internal organs through the nearly transparent mouse body wall simply closed with a 6-0 suture. This procedure greatly reduced the scatter of fluorescent photons.

**Tumor tissue sampling (27)**

Tumor tissue biopsies were processed from 3 days to 4 weeks after inoculation of tumor cells. Fresh tissue was cut into \( \sim 1 \text{ mm}^3 \) pieces and pressed on slides for fluorescence microscopy. For analyzing tumor angiogenesis, the tissues were digested with trypsin/EDTA at 37°C for 5 min before examination. After trypsinization, tissues were put on pre-cleaned microscope slides (Fisher Scientific, Pittsburgh, PA 15219) and covered with another microscope slide.

**Visualization of nestin-expression vessels in anagen mouse skin (29)**

Nestin-regulated GFP transgenic mice, 6–8 weeks old, with almost exclusively telogen (resting) hair follicles were anesthetized with tribromoethanol (i.p. injection of 0.2 ml/10 g body weight of a 1.2% solution). The mice were depilated by a hot mixture of rosin and beeswax. The mice were anesthetized with tribromoethanol, and samples were excised from dorsal skin before depilation and at 48 and 72 h in early anagen after depilation. The skin samples were used for fluorescence microscopy.

**Immunohistochemical staining (29)**

CD31 and von Willebrand factor (vWF), in air-dried skin and frozen sections from nestin-GFP transgenic mice, were detected with the anti-rat Ig HRP detection kit (BD Pharmingen, San Diego, CA) for CD31 or anti-rabbit Ig HRP detection kit (BD Pharmingen) for vWF following the manufacturer’s instructions. The primary antibodies used were: CD31 mAb (1:50) and vWF pAb (1:100). Chromogen 3,3’-diaminobenzidine (DAB) was used for detection. CD31 mAb (CBL1337) was purchased from Chemicon (Temecula, CA). vWF pAb (A082) was purchased from Dako (Glostrup, Denmark).

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