[CANCER RESEARCH 64, 8651–8656, December 1, 2004]

Transgenic Nude Mouse with Ubiquitous Green Fluorescent Protein Expression as a Host for Human Tumors

Meng Yang,[1] Jose Reynoso,[1] Ping Jiang,[1] Lingna Li,[1] Abdool R. Moossa,[2] and Robert M. Hoffman[1,2]

[1]AntiCancer, Inc., San Diego, California; and [2]Department of Surgery, University of California, San Diego, California

ABSTRACT

We report here the development of the transgenic green fluorescent protein (GFP) nude mouse with ubiquitous GFP expression. The GFP nude mouse was obtained by crossing nontransgenic nude mice with the transgenic C57/B6 mouse in which the β-actin promoter drives GFP expression in essentially all tissues. In crosses between *nu/nu* GFP male mice and *nu/+* GFP female mice, the embryos fluoresced green. Approximately 50% of the offspring of these mice were GFP nude mice. Newborn mice and adult mice fluoresced very bright green and could be detected with a simple blue–light-emitting diode flashlight with a central peak of 470 nm and a bypass emission filter. In the adult mice, the organs all brightly expressed GFP, including the heart, lungs, spleen, pancreas, esophagus, stomach, and duodenum. The following systems were dissected out and shown to have brilliant GFP fluorescence: the entire digestive system from tongue to anus; the male and female reproductive systems; brain and spinal cord; and the circulatory system, including the heart and major arteries and veins. The skinned skeleton highly expressed GFP. Pancreatic islets showed GFP fluorescence. The spleen cells were also GFP positive. Red fluorescent protein (RFP)–expressing human cancer cell lines, including PC-3-RFP prostate cancer, HCT-116-RFP colon cancer, MDA-MB-435-RFP breast cancer, and HT1080-RFP fibrosarcoma were transplanted to the transgenic GFP nude mice. All of these human tumors grew extensively in the transgenic GFP nude mouse. Dual-color fluorescence imaging enabled visualization of human tumor–host interaction by whole-body imaging and at the cellular level in fresh and frozen tissues. The GFP mouse model should greatly expand our knowledge of human tumor–host interaction.

INTRODUCTION

Although much recent research has focused on the genetic makeup of tumors themselves, it has long been apparent that host tissues also participate in the phenomena of malignancy. Studies pioneered by Folkman (1) showed that the development of tumor-induced vasculature is essential for tumor growth beyond an initial small size. This knowledge has greatly increased our appreciation of the importance of tumor–host interaction.

One of the earliest indications of the importance of host tissue to tumor growth was the selectivity of metastatic seeding. Such metastasis was described in the "seed and soil" hypothesis by Paget (2) more than 100 years ago. Paget (2) proposed that tumor cells, or "seeds," were randomly disseminated by vascular routes but that metastatic deposits grew only on permissive organs, *i.e.*, the "soil." Fidler (3–6) developed the concept of the tumor microenvironment in the host tissue necessary for growth promotion. The metastatic host microenvironment consists of critical host endothelial cells that form new blood vessels, epithelial cells, lymphocytes, platelets, macrophages, fibroblasts, and other cell types interacting with tumor cells, thereby enabling a metastasis to grow. Fidler (3–6) noted that the microenvironments of different organs (the soil) are biologically unique and that the growth of potentially metastatic cells depends on interaction of these cells with host cells. The host may resist tumor growth by immune and other mechanisms (7).

Thus, solid tumors proliferate in a complex association with the stromal tissue, which, among other structures and functions, provides the vascular supply to the tumor. Unfortunately, the factors regulating stromal element induction, as well as the influences these elements have on tumor growth, are poorly understood. The paucity of information about the interaction between tumor and host has been due largely to the absence of suitable models that allow visualization and precise study of the tumor–host interaction in the living state.

A number of attempts have been made to visualize the tumor–host interaction. To study tumor angiogenesis, Fukumura *et al.* (8) and Brown *et al.* (9) have used transgenic mice that express the green fluorescent protein (GFP) under the control of the human vascular endothelial cell growth factor promoter. After implantation of solid tumors, highly fluorescent fibroblasts were observed surrounding and infiltrating the tumor mass. When spontaneous mammary tumors developed in these mice, GFP was visualized in fibroblasts surrounding the neoplastic nodules, but not in the tumor cells themselves. Thus, the vascular endothelial cell growth factor promoter of nontransformed cells is strongly activated by the tumor microenvironment, which in turn stimulates tumor angiogenesis (8, 9). However, these models did not enable simultaneous imaging of tumor and host cells.

Okabe *et al.* (10) produced transgenic mice with GFP under the control of a chicken β-actin promoter and cytomegalovirus enhancer. All of the tissues from these transgenic mice, with the exception of erythrocytes and hair, fluoresce green. Mouse tumor cells transplanted in the GFP transgenic mouse were made visible by transforming them with red fluorescent protein [RFP (11, 12)]. To gain further insight into the tumor–host interaction in the living state, including tumor angiogenesis and immunology, we visualized RFP-expressing tumors transplanted in GFP-expressing transgenic mice using dual-color fluorescence imaging and microscopy (12).

We report here the development and characterization of the transgenic GFP nude mouse with ubiquitous GFP expression. The GFP nude mouse, which is a unique construct, was obtained by crossing nontransgenic nude mice with transgenic C57/B6 mice, in which the β-actin promoter drives GFP expression in essentially all tissues. The GFP nude mouse was used to visualize the growth, metastasis, and tumor–host interaction of human tumor cell lines expressing RFP.

MATERIALS AND METHODS

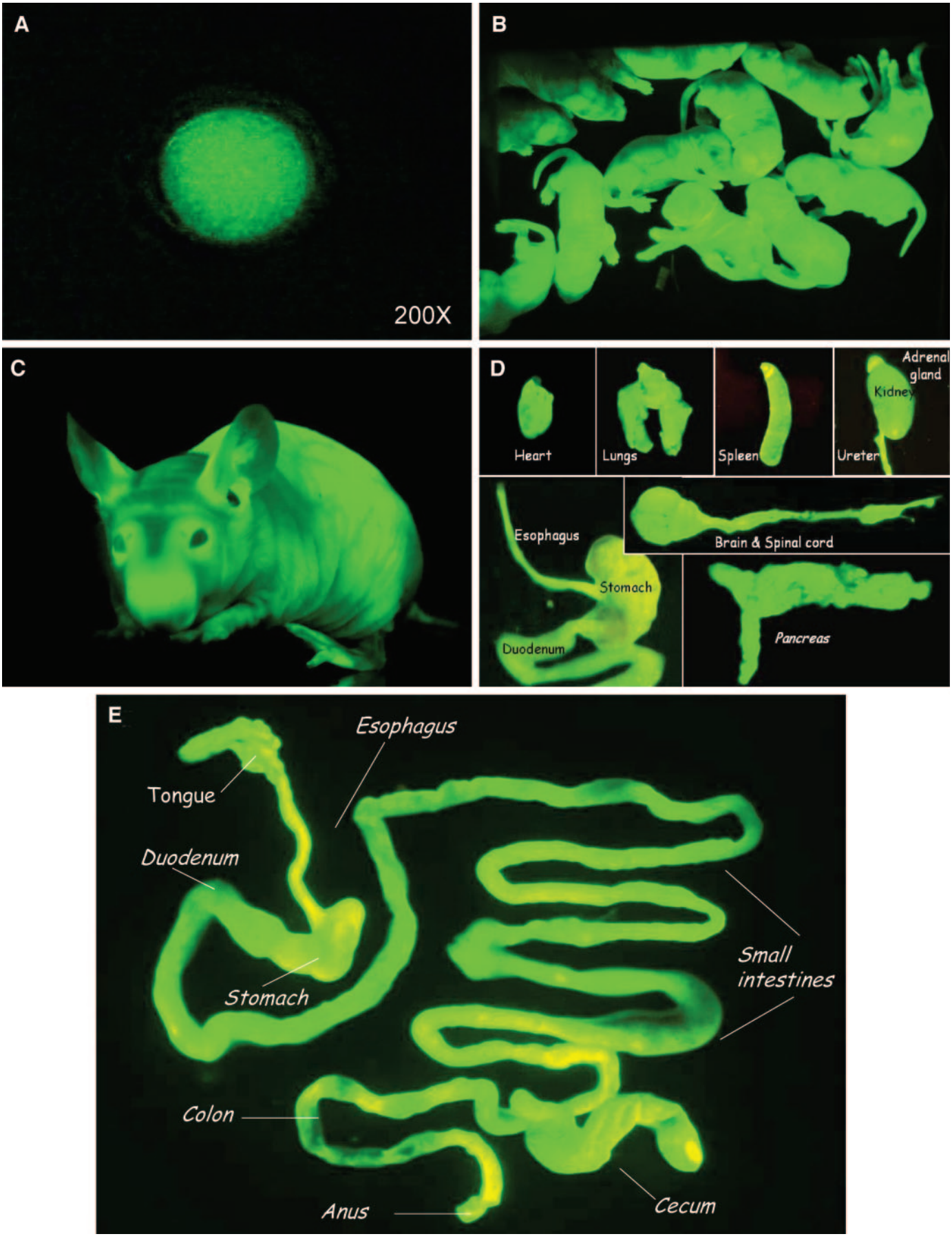
Transgenic Green Fluorescent Protein Nude Mice. Transgenic C57/B6-GFP mice were obtained from Prof. Masaru Okabe (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). C57/B6-GFP mice express GFP under the control of the chicken β-actin promoter and cytomegalovirus enhancer (10). All of the tissues from this transgenic line, with the exception of erythrocytes and hair, express GFP. Six-week–old transgenic GFP female C57/B6 mice were crossed with six- to eight-week–old BALB/c *nu/nu* or NCR *nu/nu* male mice (Harlan, Indianapolis, IN). Male F_1 mice were crossed with female F_1 C57/B6 GFP mice to obtain GFP nude mice. When

Received 8/27/04; revised 9/22/04; accepted 9/27/04.

Grant support: National Cancer Institute grants CA099258, CA103563, and CA101600.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Robert M. Hoffman, AntiCancer, Inc., 7917 Ostrow Street, San Diego, CA 92111. Phone: 858-654-2555; Fax: 858-268-4175; E-mail: all@anticancer.com.


A
200X
B
C
D
Heart
Lungs
Spleen
Adrenal gland
Kidney
Ureter
Esophagus
Brain & Spinal cord
Stomach
Duodenum
Pancreas
E
Esophagus
Tongue
Duodenum
Stomach
Small intestines
Colon
Anus
Cecum

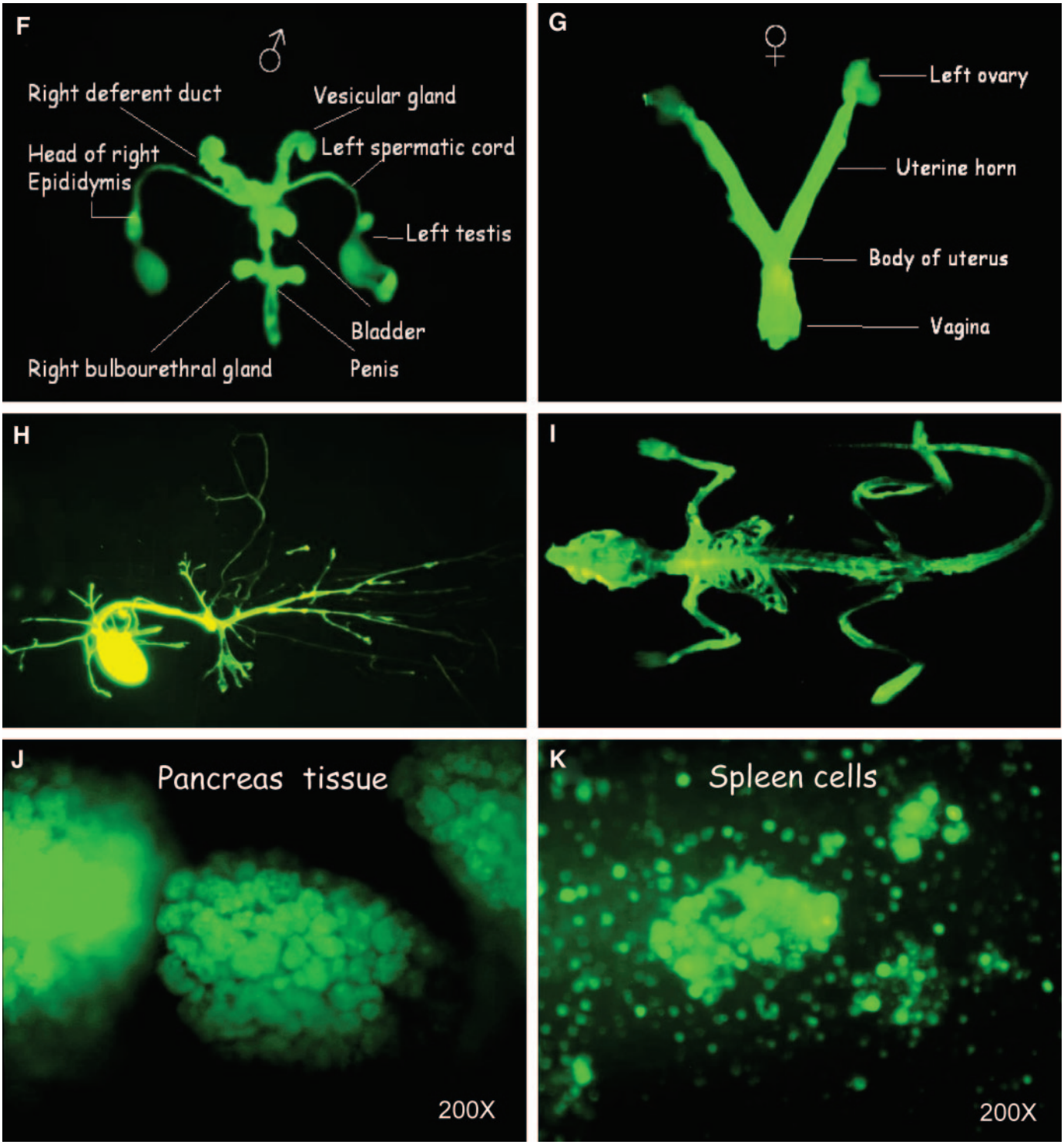


Fig 1. *Continued.*

Fig. 1. GFP expression in the tissues and cells of the transgenic GFP nude mouse. *A*, an embryo expresses GFP at the single-cell stage. Magnification, ×200. *B* and *C*. Newborn and adult mice fluoresce brilliant, bright green under blue light excitation. The fluorescence could be detected with a simple blue–light-emitting diode flashlight with a central peak of 470 nm and a bypass emission filter. *D*. The panel shows GFP expressed in major internal organs including the heart, lungs, spleen, adrenal grand, kidney, esophagus, stomach, duodenum, pancreas, brain, and spinal cord on blue light excitation. *E*. The entire digestive system was dissected out from tongue to anus and fluoresces brilliant green on blue light excitation. *F* and *G*. The male and female reproductive systems were dissected out, and all components fluoresced bright green on blue light excitation. *H*. The dissected circulatory system, including the heart and major arteries and veins, had brilliant green fluorescence on blue light excitation. *I*. The entire skeleton was dissected and could be seen to fluoresce brilliant green on blue light excitation. *J*. Pancreatic islets had brilliant green fluorescence. *K*. Spleen cells also could be seen to fluoresce brilliant green on blue light excitation.

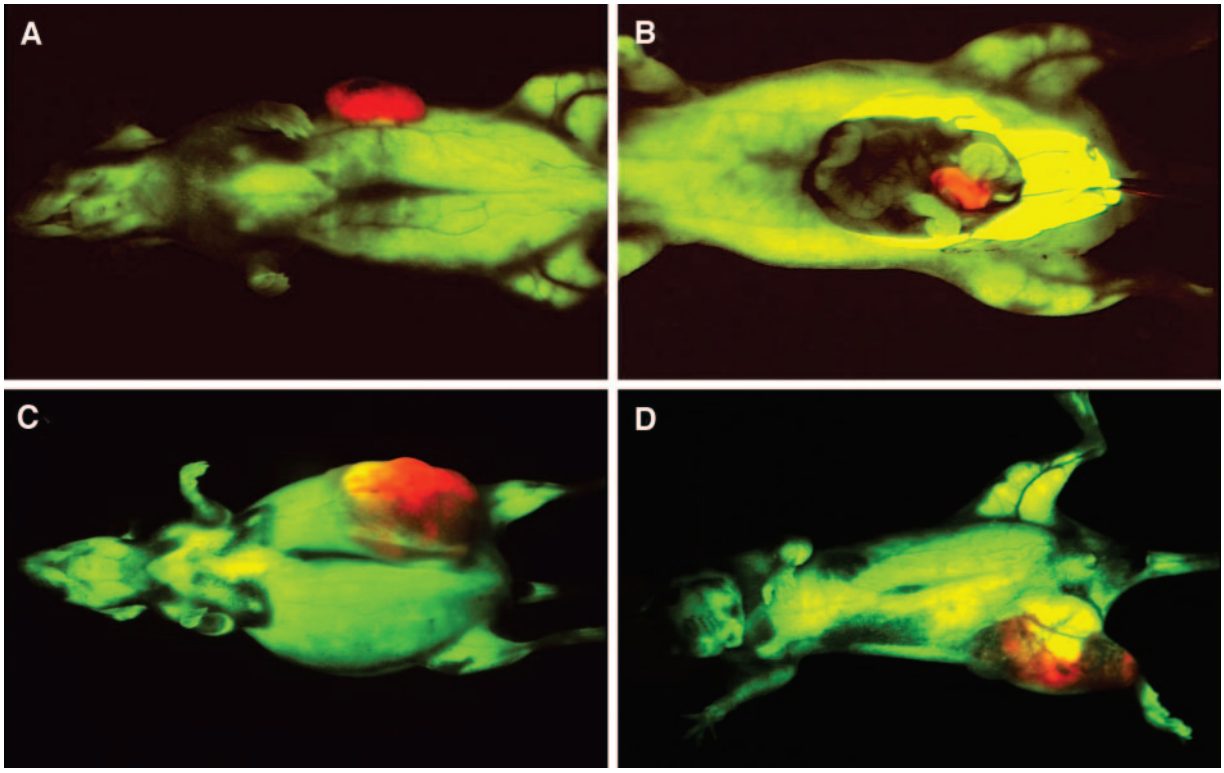


Fig. 2. RFP-expressing human tumors growing in transgenic GFP nude mice. *A*. Whole-body image shows the RFP-expressing MDA-MB-435 human mammary cancer growing orthotopically in the GFP nude mouse 4 weeks after implantation. *B*. Intra-vital image shows the RFP-expressing PC 3-RFP human prostate cancer growing orthotopically in the GFP nude mouse 2 weeks after implantation. *C*. Whole-body image shows the RFP-expressing HCT-116-RFP human colon cancer growing orthotopically in the GFP nude mouse 6 weeks after intratibial implantation. *D*. Whole-body image shows the RFP-expressing HT1080 human fibrosarcoma growing in the GFP nude mouse 6 weeks after intratibial implantation.

female F_2 immunocompetent GFP mice were crossed with male GFP nude mice or when F_2 GFP nude male mice were back-crossed with female F_1 immunocompetent GFP mice, approximately 50% of their offspring were GFP nude mice. GFP nude mice can be consistently produced by the methods described above.

Red Fluorescent Protein Expression Vector. The pLNCX2 vector was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). The pLNCX2 vector contains the neomycin resistance gene for antibiotic selection in eukaryotic cells (12). The RFP (DsRed2; Clontech Laboratories, Inc.) was inserted in the pLNCX2 vector at the *Egl*II and *Not*I sites.

Red Fluorescent Protein Vector Production in Packaging Cells. For retroviral transduction, PT67, a NIH3T3-derived packaging cell line expressing the 10 Al viral envelope, was purchased from Clontech Laboratories, Inc. PT67 cells were cultured in DME (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bioproducts, Calabasas, CA). For vector production, packaging cells (PT67), at 70% confluence, were incubated with a precipitated mixture of DOTAP reagent (Boehringer Mannheim, Indianapolis, IN) and saturating amounts of pLNCX2-DsRed2 plasmid for 18 hours. Fresh medium was replenished at this time. The cells were examined by fluorescence microscopy 48 hours after transfection. For selection, the cells were cultured in the presence of 500 to 2,000 μg/mL G418 (Life Technologies, Inc., Grand Island, NY) increased in a stepwise manner for 7 days (12).

Red Fluorescent Protein Gene Transduction of Tumor Cell Lines. For RFP gene transduction, 20% confluent human cancer cells, including PC-3 prostate cancer cells, HCT-116 colon cancer cells, MDA-MB-435 breast cancer cells, and HT1080 human fibrosarcoma cells, were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 cells and RPMI 1640 or other culture media (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Gemini Bioproducts) for 72 hours. Fresh medium was replenished at this time. Tumor cells were harvested with trypsin/EDTA and subcultured at a ratio of 1:15 into selective medium that contained 50 μg/mL G418. The level of G418 was increased to 800 μg/mL in a stepwise manner. Clones expressing RFP were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ) by trypsin/EDTA and amplified and transferred by conventional culture methods in the absence of selective agent (12).

Red Fluorescent Protein-Expressing Orthotopic Human Prostate Cancer Green Fluorescent Protein Host Model. Six-week–old male GFP nude mice received orthotopic injection with 10^6 RFP-expressing PC-3 human prostate carcinoma cells. The bladder and prostate were exposed through a lower midline abdominal incision. Twenty microliters containing 10^6 PC-3-RFP cells per mouse were injected in the lateral lobe with a 25-μL Hamilton syringe (Fisher Scientific, Two Rivers, WI), respectively. The incision in the abdominal wall was closed with a 6-0 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 $\times$7 magnification stereomicroscope.

Red Fluorescent Protein-Expressing Orthotopic Human Colon Cancer Green Fluorescent Protein Host Model. A 6-week–old male GFP nude mouse received orthotopic injection with 10^6 RFP-expressing HCT-116 human colon cells in 20 μL. After proper exposure of the colon through a lower left abdominal incision, HCT-116-RFP cells were injected under the serosa of the descending colon with a 25-μL Hamilton syringe (Fisher Scientific). The incision in the abdominal wall was closed with a 6-0 surgical suture in one layer.

Red Fluorescent Protein-Expressing Orthotopic Breast Cancer Green Fluorescent Protein Host Model. A 6-week–old female nude GFP mouse received orthotopic injection with 10^6 RFP-expressing MDA-MB-435 cells. Cells were injected in the mammary fat pad of the animal in a total volume of 30 μL.

Red Fluorescent Protein-Expressing Human Fibrosarcoma Green Fluorescent Protein Host Model. Six-week–old male GFP nude mice were inoculated with 10^6 RFP-expressing HT1080 human fibrosarcoma cells. Cells were inoculated by intra-bone marrow injection of the tibia of the animal in a total volume of 20 μL, using a 25-μL Hamilton syringe (Fisher Scientific).

Tumor Tissue Sampling. Tumor tissue was obtained at different time points after orthotopic inoculation of the tumor cells. Fresh tissues were cut into $\sim$1-mm^3 pieces or very thin slices under the microscope. Pressed sections were then made for observation and imaging. For analysis of tumor angiogen-

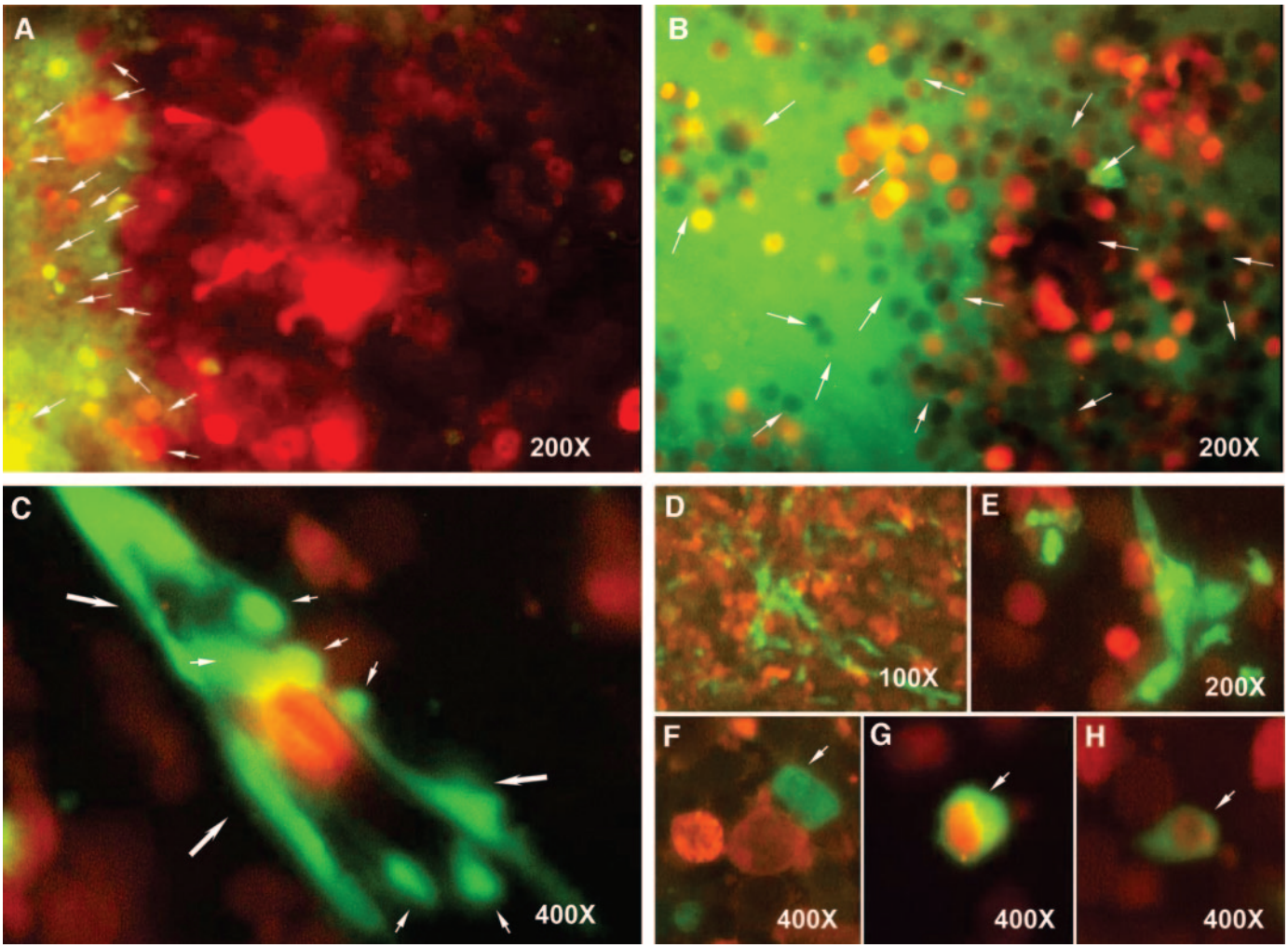


Fig. 3. Visualization of human tumor–host interaction. *A* and *B*, RFP-expressing HCT-116-RFP human colon cancer cells invading the GFP nude mouse host tissue 6 weeks after implantation. *C*, HT1080-RFP human fibrosarcoma cell surrounded by GFP nude mouse host stromal cells 6 weeks after implantation. *D*, HT1080-RFP fibrosarcoma with host GFP stromal cells among the tumor cells. *E*. High magnification of *D* showing intimate interaction of HT1080-RFP tumor cells with GFP-expressing host stromal cells. *F*−*H*, interaction and engulfment of HT1080-RFP tumor cells by host GFP-expressing macrophages.

esis, the tissues were digested with trypsin/EDTA at 37°C for 5 minutes before examination. After trypsinization, tissues were put on a precleaned microscope slide (Fisher Scientific) and covered with another microscope slide.

Fluorescence Microscopy. An Olympus BH 2-RFCA fluorescence microscope equipped with a mercury 100-W lamp power supply was used. To visualize both GFP and RFP fluorescence at the same time, excitation was produced through a D425/60 bandpass filter, 470 DCXR dichroic mirror, and emitted fluorescence was collected through a long pass filter (GG475; Chroma Technology, Brattleboro, VT). High-resolution images of 1024 × 724 pixels were captured with a Hamamatsu C5810 three-chip cooled color charge-coupled device camera (Hamamatsu Photonics Systems, Bridgewater, NJ) and directly stored on an IBM personal computer. Images were processed for contrast and brightness and analyzed with the use of Image Pro Plus 4.0 software (Media Cybernetics, Silver Springs, MD; ref. 12).

Whole-Body Fluorescence Imaging. Whole-body imaging (12) was performed in a fluorescent light box illuminated by fiber optic lighting at 470 nm (Lightools Research, Encinitas, CA). Emitted fluorescence was collected through a long pass filter (GG475; Chroma Technology) on a Hamamatsu C5810 three-chip cooled color charge-coupled device camera (Hamamatsu Photonics Systems). High-resolution images of 1,024/724 pixels were captured directly on an IBM personal computer. Images were processed for contrast and brightness and analyzed with the use of IMAGE PRO PLUS 3.1 software (Media Cybernetics).

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

RESULTS AND DISCUSSION

Development of the Green Fluorescent Protein Nude Mouse. In crosses between *nu/nu* GFP male mice and *nu/+* GFP female mice, the resultant embryos were green, apparently at the single-cell stage (Fig. 1*A*). Newborn mice (Fig. 1*B*) and adult mice (Fig. 1*C*) were very bright green. Green fluorescence could be detected with a simple blue–light-emitting diode flashlight with a central peak of 470 nm and a bypass emission filter (Chroma Technology).

Description of the Green Fluorescent Protein Nude Mouse. In the adult mice, the organs all brightly expressed GFP, including the heart, lungs, spleen, pancreas, esophagus, stomach, adrenal gland, kidney, and duodenum (Fig. 1*D*). The entire digestive system was dissected out from tongue to anus and could be seen to fluoresce brilliant green on blue light excitation (Fig. 1*E*). The male and female reproductive systems were dissected out, and all components fluoresced bright green on blue light excitation (Fig. 1*F* and *G*). The dissected brain and spinal cord also had brilliant GFP fluorescence (Fig. 1*D*). The dissected out circulatory system, including the heart

and major arteries and veins, had a brilliant green fluorescence (Fig. 1*H*). The skinned skeleton highly expressed GFP (Fig. 1*I*). Pancreatic islets showed GFP fluorescence (Fig. 1*J*). The spleen cells were also GFP positive (Fig. 1*K*).

Transplantation and Dual-Color Imaging of Red Fluorescent Protein-Expressing Human Tumors in the Green Fluorescent Protein Nude Mouse. Dual-color images visualized the tumor–host interaction of RFP-expressing human tumors in the GFP nude mice, including whole-body image of orthotopic growth of the MDA-MB-435 mammary tumor (Fig. 2*A*), intravital image of orthotopic growth of the PC-3-RFP prostate tumor (Fig. 2*B*), whole-body image of orthotopic growth of the HCT-116-RFP colon cancer (Fig. 2*C*), and whole-body image of growth in the tibia of the HT1080-RFP fibrosarcoma (Fig. 2*D*).

Dual-Color Imaging of Tumor–Host Cell Interaction in the Green Fluorescent Protein Nude Mouse. For examples at the cellular level, the HCT-116-RFP tumor can be seen to be heterogeneous, with RFP-expressing tumor cells invading the GFP host tissue (Fig. 3*A* and *B*). The HT1080-RFP tumor cells and GFP host fibroblasts and endothelial cells can be seen interacting. Stromal cells (Fig. 3*C–E*) expressing GFP can be seen interacting with the human HT1080-RFP cells. Macrophages expressing GFP were visualized engulfing HT1080-RFP tumor cells (Fig. 3*F–H*).

The GFP nude mouse enables visualization of human tumor–host interaction in live tissue. Both the tumor and the host cells are uniquely identified by their fluorescence color (RFP for the tumor, and GFP for the host cells). We have visualized tumor cells being specifically contacted by various different host cells.

The GFP nude mouse has many uses to study human tumor biology. With the use of RFP-expressing human tumor cells, it provides a system to study human tumor–host interaction of every type. All observed organs of the GFP nude mouse express a brilliant GFP-mediated fluorescence, thereby enabling each organ to be implanted with a corresponding tumor to study human tumor–host interaction at the organ and cellular level. Fluorescent proteins such as GFP and RFP have many advantages over all other known reporter genes in that no substrate is needed to observe their fluorescence and stable real-time images can be acquired with widely available, simple equipment.

Previously, the ROSA-26 mutant line was produced by infection of embryonic stem cells with the ROSA βgeo retrovirus that contains the *lac*-z gene, which expresses β-galactosidase (β-gal) (13). Widespread β-gal expression starts at the morula-blastocyst stage in this mouse line. Ubiquitous *lac*-z staining, indicating β-gal expression, was observed in the brain, bone marrow, cartilage, heart, intestine, kidney, liver, lung, pancreas, muscle (skeletal and smooth), skin (dermis and epidermis), spleen, submandibular gland, thymus, trachea, and urinary bladder (13). ROSA-26 also has ubiquitous *lac*-z expression in nucleated cells in the spleen as well as all of the major hematolymphoid lineages. Although the ROSA-26 mouse has proven useful to mark cells, preparation is necessary to visualize *lac*-z. In contrast, GFP can be visualized simply by applying blue light.

The GFP nude mice appear to have a life span similar to that of non-GFP nude mice, such that long-term tumor growth and metastasis studies can be carried out. The GFP nude mouse has a critical advantage over the GFP C57/B6 immunocompetent mouse in that human tumors can grow in the GFP nude mouse. In addition, the lack of hair in the GFP nude mouse makes imaging more facile.

The model can be used to identify and characterize cells within the tumor or host cells that play a role in malignancy. The model can also be used to develop specific therapeutic agents that target host cells as well as tumor cells that affect tumor growth and progression.

Recently, Duda *et al.* (14) transplanted nonfluorescent mouse tumor cells growing in transgenic immunocompetent mice with GFP-expressing stromal cells to nonfluorescent mouse hosts. They found that the fluorescent stromal cells continued to grow in the nonfluorescent transplanted host mice. Our approach, using RFP-expressing human tumor cells growing in GFP nude mice, can allow simultaneous visualization of the growing human tumor cells and the surviving stromal cells to give further information on the development of tumor–host interaction during growth and transplantation to a nonfluorescent host.

The introduction of the nude mouse to cancer research (15) led to a paradigm change in cancer biology, enabling human tumors to be consistently grown in a mouse model. The GFP nude mouse should lead to another paradigm change, enabling the visualization of the interaction of the human tumor and host in the living mouse.

REFERENCES

1. Folkman J. Angiogenesis and apoptosis. Semin Cancer Biol 2003;13:159–67.
2. Paget S. The distribution of secondary growths in cancer of the breast. Lancet 1889;1:571–3.
3. Fidler IJ. The pathogenesis of cancer metastasis: the "seed and soil" hypothesis revisited. Nat Rev Cancer 2003;3:453–8.
4. Fidler IJ. The organ microenvironment and cancer metastasis. Differentiation (Camb) 2002;70:498–505.
5. Fidler IJ. Angiogenic heterogeneity: regulation of neoplastic angiogenesis by the organ microenvironment. J Natl Cancer Inst (Bethesda) 2001;93:1040–1.
6. Fidler IJ. Seed and soil revisited: contribution of the organ microenvironment to cancer metastasis. Surg Oncol Clin N Am 2001;10:257–69.
7. Krutovskikh V. Implication of direct host-tumor intercellular interactions in non-immune host resistance to neoplastic growth. Semin Cancer Biol 2002;12:267–76.
8. Fukumura D, Xavier R, Sugiura T, et al. Tumor induction of VEGF promoter activity in stromal cells. Cell 1998;94:715–25.
9. Brown EB, Campbell RB, Tsuzuki Y, et al. In vivo measurement of gene expression, angiogenesis and physiological function in tumors using multiphoton laser scanning microscopy. Nat Med 2001;7:864–8.
10. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. "Green mice" as a source of ubiquitous green cells. FEBS Lett 1997;407:313–9.
11. Hoffman RM. Green fluorescent protein imaging of tumour growth, metastasis, and angiogenesis in mouse models. Lancet Oncology 2002;3:546–56.
12. Yang M, Li L, Jiang P, et al. Dual-color fluorescence imaging distinguishes tumor cells from induced host angiogenic vessels and stromal cells. Proc Natl Acad Sci USA 2003;100:14259–62.
13. Zambrowicz BP, Imamoto A, Fiering S, et al. Disruption of overlapping transcripts in the ROSA betageo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. Proc Natl Acad Sci USA 1997;94:3789–94.
14. Duda DG, Fukumura D, Munn LL, et al. Differential transplantability of tumor-associated stromal cells. Cancer Res 2004;64:5920–4.
15. Rygaard J, Povlsen CO. Heterotransplantation of a human malignant tumour to "Nude" mice. Acta Pathol Microbiol Scand 1969;77:758–60.