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

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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) in 1889. 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.

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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 gland, 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.
female F1 immunocompetent GFP mice were crossed with male GFP nude mice or when F2 GFP nude male mice were back-crossed with female F1 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 EcoRI and NotI 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 \( \mu \text{g/mL} \) G418. The level of G418 was increased to 800 \( \mu \text{g/mL} \) after intratibial implantation.

**Red Fluorescent Protein-Expressing Human Fibrosarcoma 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 into the lateral lobe with a 25-\( \mu \)L 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 female nude GFP mouse received orthotopic injection with \( 10^6 \) RFP-expressing HCT-116 human colon cells in 20 \( \mu \)L. 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-\( \mu \)L 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 \( \mu \)L.

**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-
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 1024/724 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 (Teklad 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. 1A). Newborn mice (Fig. 1B) and adult mice (Fig. 1C) 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. 1D). The entire digestive system was dissected out from tongue to anus and could be seen to fluoresce brilliant green on blue light excitation (Fig. 1E). The male and female reproductive systems were dissected out, and all components fluoresced bright green on blue light excitation (Fig. 1F and G). The dissected brain and spinal cord also had brilliant GFP fluorescence (Fig. 1H). The dissected out circulatory system, including the heart...
and major arteries and veins, had a brilliant green fluorescence (Fig. 1H). The skinned skeleton highly expressed GFP (Fig. 1I). Pancreatic islets showed GFP fluorescence (Fig. 1J). The spleen cells were also GFP positive (Fig. 1K).

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. 2A), intravital image of orthotopic growth of the PC-3-RFP prostate tumor (Fig. 2B), whole-body image of orthotopic growth of the HCT-116-RFP colon cancer (Fig. 2C), and whole-body image of growth in the tibia of the HT1080-RFP fibrosarcoma (Fig. 2D).

Dual-Color Imaging of Tumor–Host Cell Interaction in the Green Fluorescent Protein Nude Mouse. For examples at the cellular level, the HT-116-RFP tumor can be seen to be heterogeneous, with RFP-expressing tumor cells invading the GFP host tissue (Fig. 3A and B). The HT1080-RFP tumor cells and GFP host fibroblasts and endothelial cells can be seen interacting. Stromal cells (Fig. 3C–E) expressing GFP can be seen interacting with the human HT1080-RFP cells. Macrophages expressing GFP were visualized engulfing HT1080-RFP tumor cells (Fig. 3F–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 mouse appears 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 nonfluorescence mouse tumor cells growing in transgenic immunocompetent mice with GFP-expressing stromal cells to nonfluorescence mouse hosts. They found that the fluorescence stromal cells continued to grow in the nonfluorescence 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 non-fluorescence 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.

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