

# *In vivo* tumor delivery of the green fluorescent protein gene to report future occurrence of metastasis

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The green fluorescent protein (GFP) gene was administered to intraperitoneally (i.p.) growing human stomach cancer in nude mice to visualize future regional and distant metastases. GFP retroviral supernatants were injected i.p. from day 4 to day 10 after i.p. implantation of the cancer cells. Tumor and metastasis fluorescence was visualized every other week with the use of fluorescence optics *via* a laparotomy on the tumor-bearing animals. At 2 weeks after retroviral GFP delivery, GFP-expressing tumor cells were observed in gonadal fat, greater omentum, and intestine, indicating that these primary i.p. growing tumors were efficiently transduced by the GFP gene and could be visualized by its expression. At the second and third laparotomies, GFP-expressing tumor cells were observed to have spread to lymph nodes in the mesentery and other regional sites. At the fourth laparotomy, widespread tumor growth was visualized by GFP expression, inducing liver metastasis. No normal tissues were found to be transduced by the GFP retrovirus. Thus, reporter gene transduction of the primary tumor enabled detection of its subsequent metastasis. This gene therapy model could be applied to primary tumors before resection or other treatment to have a fluorescent early detection system for metastasis and recurrence. **Cancer Gene Therapy (2000) 7, 1336–1340**

**Key words:** metastasis; green fluorescent protein; reporter gene therapy; early detection.

A number of approaches have been taken to label tumor cells to visualize and track them *in vivo*. Previous attempts to genetically label tumor cells for tracking purposes used the *Escherichia coli*  $\beta$ -galactosidase (*lacZ*) gene to detect micrometastases.<sup>1,2</sup> However, detection of *lacZ* requires extensive histological preparation, with sacrifice of the tissue and/or animal; therefore, it was not possible to image, visualize, and study tumor cells in real-time in viable fresh tissue or in the live animal.

The ability to confer real-time visualization and imaging of tumor growth and progression in viable fresh tissue and in the live animal would be an important factor in the development of a real-time reporter gene for metastasis and recurrence. Several approaches have been developed with this goal in mind: Fukumura et al<sup>3</sup> and Chambers et al<sup>4</sup> labeled tumor tissue with fluorescent dyes. However, these methods are not suitable for long-term metastasis studies. Weissleder et al<sup>5</sup> have infused tumor-bearing animals with protease-activated near-infrared fluorescent probes. Tumors with appropriate proteases could activate the probes and could be

imaged externally. The limits to such a system include a much higher liver to tumor background precluding liver metastasis imaging, which is among the most important metastatic sites; the stated time limit of 96 hours, which precludes growth and efficacy studies; the requirement of appropriate tumor protease activity; and the requirement of selective tumor delivery of the probes.

Another attempt involved insertion of the luciferase gene into tumor cells such that they emit light.<sup>6</sup> However, luciferase enzymes transferred to mammalian cells require the exogenous injected delivery of their luciferin substrate, an invasive and impractical requirement in an intact animal. The image resolution of this approach is slow, requiring anesthesia due to the long periods needed for image acquisition. Also, it is not known whether luciferase genes can function stably over significant time periods in tumors and in the metastases derived from them.

It became clear that higher specificity, resolution, and physiological conditions were necessary to report the natural course of tumor progression and metastasis on a real-time basis. The green fluorescent protein (GFP) gene, cloned from the bioluminescent jellyfish *Aequorea victoria*,<sup>7</sup> was chosen to satisfy these conditions because it has demonstrated its great potential for use as a cellular marker.<sup>8,9</sup> GFP cDNA encodes a 283-aa monomeric polypeptide with a molecular mass of 27 kDa<sup>10,11</sup> that

Received December 28, 1999; accepted June 30, 2000.

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requires no other *Aequorea* proteins, substrates, or cofactors to fluoresce.<sup>12</sup> Recently, GFP gene gain-of-function bright mutants have been generated by various techniques<sup>13–16</sup> that have been humanized for high expression.<sup>17</sup>

We have developed technology that has enabled the stable transduction of the GFP gene into a large series of human tumor cell lines *in vitro*.<sup>18–25</sup> The tumor cell lines were able to stably express GFP at high levels both *in vitro* and *in vivo*. We have previously demonstrated the important parameter that GFP-expressing cancer cells could be directly visualized in fresh tissues of transplanted animals at a very high resolution down to the single-cell level.<sup>18–22</sup> With this technology, we were able to visualize tumor cells that had seeded with or without subsequent colonization in all the major organs, including the liver, lung, brain, spinal cord, axial skeleton, and lymph nodes.<sup>18–22</sup> Our recent results include the development of orthotopic GFP metastatic models of lung cancer,<sup>23</sup> prostate cancer,<sup>24</sup> melanoma,<sup>25</sup> and colon cancer.<sup>26</sup> These results demonstrated that GFP gene-transfected tumor cells represent a new tool to study tumor cell growth, dissemination, invasion, metastasis, and progression through all stages.

In this study, a GFP-gene tumor transduction system was developed as a potential clinical application to report the occurrence and recurrence of metastasis in real-time. Retroviral gene transfer leads to stable integration in the target cell genome that is limited only to dividing cells.<sup>27</sup> In this study, we have targeted GFP to primary cancer cells *in vivo* using a retroviral vector. We demonstrate here the GFP transduction of the primary tumor that results in GFP expression in subsequent metastases.

## MATERIALS AND METHODS

### *GFP retroviral vector*

pLEIN, a retroviral vector, was purchased from Clontech (Palo Alto, Calif). pLEIN expresses enhanced GFP and the neomycin resistance gene on the same bicistronic message.<sup>23</sup>

### *Cell culture*

PT67, an NIH 3T3-based packaging cell line expressing the 10A1 viral envelope, was purchased from Clontech. PT67 cells were cultured in Dulbecco's modified Eagle's medium (Irvine Scientific, Santa Ana, Calif) supplemented with 10% heat-inactivated fetal bovine sera (Gemini Bio-Products, Calabasas, Calif), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin.<sup>23</sup> NUGC-4 cells were a kind gift of Dr. Narita (Laboratory of Experimental Pathology, Aichi Cancer Center Research Institute, Nagoya, Japan).<sup>28</sup> NUGC-4 cells were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine sera, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin.

### *Retroviral transduction of packaging cells*

PT67 packaging cells were plated at a density of  $1 \times 10^5$  cells in a 6-well plate 24 hours before transfection. The cells were 70–80% confluent at the time of GFP retrovirus transduction. A total of 2.5  $\mu$ g of plasmid and 15  $\mu$ L of *N*-(1-[2,3-dioleoyl-

loxy]propyl)-*N,N,N*-trimethylammonium methylsulfate reagent (Boehringer Mannheim, Indianapolis, Ind) were mixed according to the manufacturer's protocol. The cells were examined by fluorescence microscopy 48 hours after transfection. For selection, the cells were cultured in the presence of 0.5–2.0 mg/mL G418 (Life Technologies) for 14 days.

### *Preparation of retroviral supernatant*

Retroviral GFP producer packaging cells were cultured at 80% confluence for 24 hours to generate retroviral supernatants. Supernatants were harvested, passed through a 0.45- $\mu$ m filter, and stored at  $-80^\circ\text{C}$ .<sup>29</sup>

### *Viral titer determination*

NIH 3T3 cells were plated at a density of  $1 \times 10^5$  cells in 10-cm dishes 24 hours before infection. The cells, at 70% confluence at the time of infection, were incubated in 5 mL of undiluted and diluted retroviral supernatants. Selection in G418 (1.0 mg/mL) began at 48 hours postinfection. After 14 days, cells were stained with 0.5% methylene blue dissolved in 50% methanol; G418-resistant colonies were counted.<sup>29</sup>

### *Tumor transplantation*

A total of  $1 \times 10^7$  NUGC-4 cells in 1 mL of RPMI 1640 were inoculated subcutaneously into 6- to 8-week-old BALB/c *nu/nu* female mice. At 5 weeks postinoculation, the subcutaneous tumor was excised and cut into pieces that could pass through an 18-gauge needle. Tumor pieces were suspended in RPMI 1640, and 1 mL of suspension was injected with an 18-gauge needle into the peritoneal cavity. All mice were engrafted at the same time.

### *In vivo transduction of GFP*

A total of 1 mL of the GFP retroviral supernatant, produced as described above, was supplemented with 8.0  $\mu$ g/mL polybrene and was administered to the tumor-bearing mice once per day intraperitoneally (i.p.) from day 4 to day 10 after tumor transplantation.<sup>30</sup>

### *Visualization of tumor growth, spread, and metastasis by GFP fluorescence*

Mice were anesthetized by isoflurane inhalation and put in a supine position. A laparotomy was performed *via* a midline incision. Fresh visceral organs were analyzed under a fluorescence microscope with GFP filters (Chromatechnology Corp., Brattleboro, Vt). After observation, the abdominal wall and the skin were closed with 6–0 silk sutures.

### *Histological examination*

For histological studies, GFP-expressing tissues were removed at the time of sacrifice and put into 10% buffered formalin. All of the tissues were subsequently processed through alcohol dehydration, chlorate, and paraffinization. Tissues were embedded in paraffin and sectioned at 3  $\mu$ m. All slides were stained by hematoxylin-eosin and examined microscopically.

## RESULTS

### *Packaging cells and viral titer*

The packaging cells were examined by fluorescence microscopy 48 hours after transfection and were visualized to

**Table 1. GFP-Reported Time Course of Metastatic Spread of Peritoneal Human Stomach Cancer NUGC-4 in Nude Mice**

| Week | Number of positive mice out of a total of 12 |             |             |                |       |           |        |        |
|------|--|-------------|-------------|----------------|-------|-----------|--------|--------|
|      | Omentum                                      | Gonadal fat | Mesenterium | Abdominal wall | Liver | Intestine | Kidney | Spleen |
| 2    | 5  | 6           | 0           | 2              | 0     | 7         | 0      | 0      |
| 4    | 5  | 7           | 1           | 3              | 0     | 11        | 0      | 0      |
| 6    | 6  | 9           | 8           | 11             | 0     | 12        | 0      | 0      |
| 8    | 6  | 9           | 9           | 12             | 1     | 12        | 2      | 1      |

Tumors were transplanted i.p. and then treated with a GFP retrovirus from 4 to 10 days posttransplantation. Tumors were visualized by GFP expression in laparotomized mice under fluorescence optics at the indicated times. See *Materials and Methods* for details.

highly express GFP. After selection of the packaging cells in G418 for 2 weeks, the retroviral supernatants were harvested and titrated on NIH 3T3 cells. The supernatant viral titer was  $\sim 1.5 \times 10^5$  colony-forming units/mL. A total of 12 mice were injected with 1 mL of this virus daily from day 4 to day 10 after tumor transplantation. The experiment plan is outlined in Figure 1.

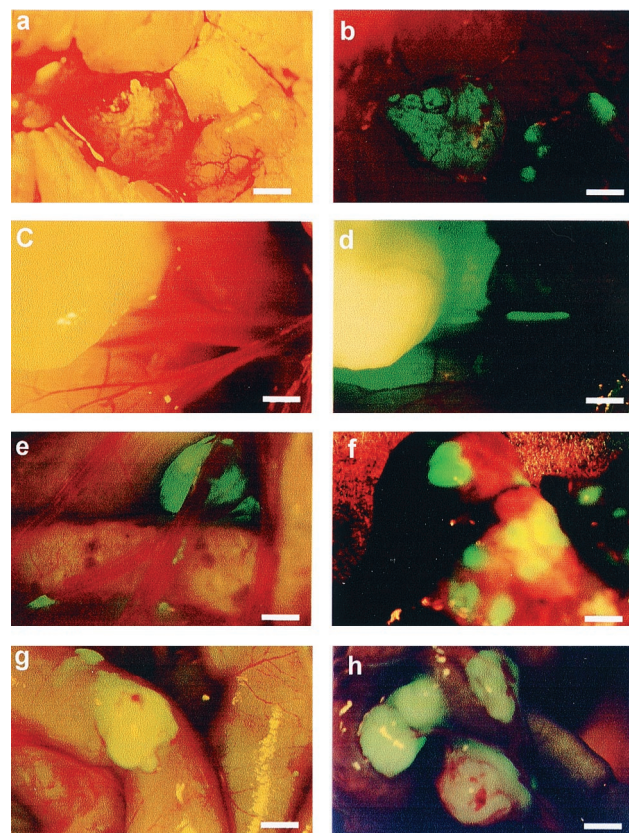
#### *In vivo GFP transduction of i.p. tumor tissue*

At 2 weeks after tumor transplantation, the first laparotomy was performed on the tumor-bearing mice that had been infected with GFP retroviral supernatants. The visceral organs were analyzed during the surgical procedure by fluorescence microscopy. The tumors growing i.p. were visualized to strongly express the GFP gene at the first laparotomy, mainly growing in gonadal fat, the greater omentum, and the intestine. The small disseminated tumors in gonadal fatty tissue could not be detected by bright-field microscopy (Table 1; Fig 2, a and b).

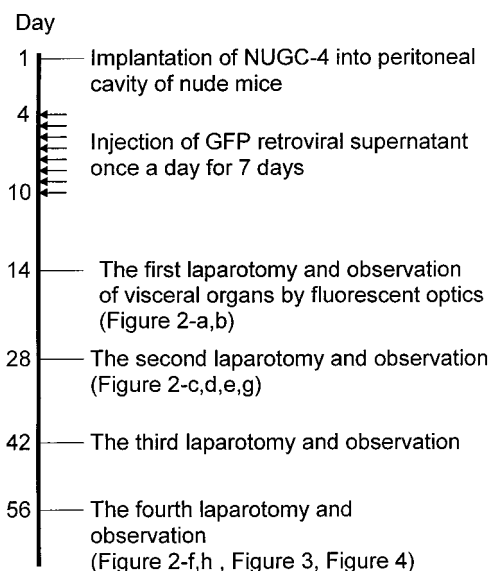
#### *Visualization of tumor spread and metastasis by GFP expression*

At 4 weeks posttransplantation, the second laparotomy revealed GFP-expressing tumors on the surface of the

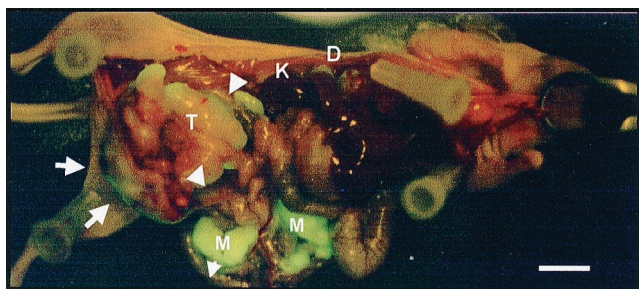
colon (Fig. 2g). At the second laparotomy (4 weeks posttransplantation), GFP-expressing tumor cells were visualized in lymph nodes in the mesentery (Table 1; Fig 2, c–e). The second laparotomy demonstrated that mesenteric lymph nodes, adjacent to GFP-expressing



**Figure 2.** Imaging of tumors after *in vivo* GFP: retroviral transduction. GFP fluorescence enabled visualization of small, disseminated tumors in gonadal fatty tissue 2 weeks after implantation; these tumors could not be detected by bright-field microscopy. **a**: bright field; **b**: fluorescence. Bar = 500  $\mu\text{m}$ . GFP-expressing tumor cells were visualized in lymphatic vessels and lymph nodes in the mesentery at 4 weeks posttransplantation. **c**: bright field; **d,e**: fluorescence. Bar = 500  $\mu\text{m}$ . At 4 weeks posttransplantation, GFP-expressing tumors were found on the surface of the colon (**g**). Bar = 1000  $\mu\text{m}$ . After 8 weeks, mesenteric lymph nodes were swelling with GFP-expressing tumors (**h**). Bar = 1000  $\mu\text{m}$ . At the time of death, multiple metastatic nodules expressing the GFP gene were observed on the surface of the liver (**f**). Bar = 1000  $\mu\text{m}$ . The experimental plan is outlined in Figure 1.



**Figure 1.** Experimental plan.



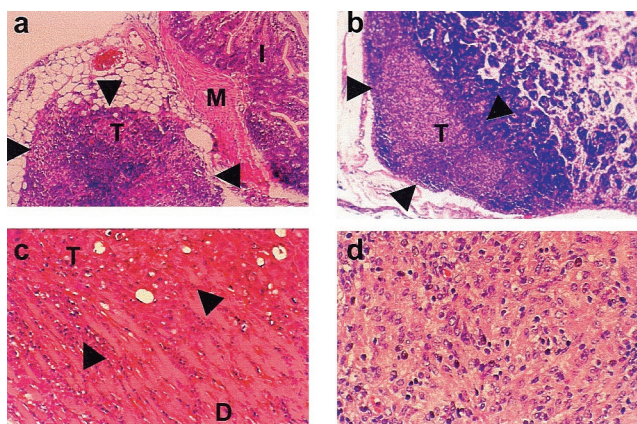
**Figure 3.** Peritoneal tumor dissemination was visualized 8 weeks after tumor implantation. D, tumor on diaphragm; K, tumor on the surface of the kidney; M, tumor nodule in mesentery; T, tumor on intestine and peritoneum; arrowhead, tumor nodule on intestine; arrow, tumor on parietal peritoneum. Bar = 5 mm.

tumors on the colon, were involved with GFP-expressing tumor cells (Table 1; Fig 2h). At 8 weeks, GFP-expressing tumors were found on the intestines, mesentery, and peritoneum as well as on the surface of the kidney and diaphragm (Table 1; Fig 3). In one mouse, multiple metastatic nodules were observed on the surface of the liver (Table 1; Fig 2f; all visualized by GFP expression).

#### Histological examination

GFP-expressing tissues were examined after hematoxylin-eosin staining, and these tissues were identified as poorly differentiated adenocarcinomas (Fig 4). All GFP-expressing tissue was found to be malignant, indicating that normal tissues were not transduced.

A total of 1 mL of retroviral supernatant of PT67 cells was injected into the peritoneal cavity of five nontransplanted nude mice once a day for 5 days. The nude mice were sacrificed 2 weeks after the last injection of retro-



**Figure 4.** Histological examination demonstrated that GFP-expressing tissues were poorly differentiated adenocarcinomas. **a:** on the intestine ( $\times 40$  magnification); T and arrowhead, tumor; M, mouse smooth muscle; I, mouse intestinal mucosa. **b:** in the lymphatic vessel of the mesentery of the intestine ( $\times 40$  magnification); T and arrowhead, tumor. **c:** in the diaphragm ( $\times 100$  magnification); T and arrowhead, tumor; D, diaphragm. **d:** on parietal peritoneum ( $\times 200$  magnification).

viral supernatants; no GFP expression could be detected in any organs and tissue.

#### DISCUSSION

This study demonstrated that the GFP gene was able to transduce primary i.p. growing tumor cells by injection of GFP retroviral supernatants, resulting in subsequent lymphatic, liver and other metastasis.

In the early phase of tumor progression in this model, tumor nodules were visualized by GFP expression; these tumors were observed to be growing on gonadal fat, on the intestine, and on the abdominal wall, but not in mesenteric lymph nodes or on the liver, which occurred only late in the course of the experiment. These experiments demonstrated that retroviral administration of the GFP gene to the primary tumors enabled the visualization of subsequent metastasis; this visualization did not extend to cells that could have been accidentally seeded at the time of transplantation. Although there have been reports of GFP-induced immunogenicity,<sup>31</sup> the aggressive tumor progression observed in the present experiment suggests that in the NUGC-4 model in nude mice, the T-cell-deficient host does not mount an immunological defense against the GFP-expressing tumor. This is similar to the results we obtained with B16-GFP melanoma in C57BL/6 mice.<sup>25</sup> Future experiments will compare NUGC-4 cells transduced both *in vitro* and *in vivo* with nontransduced NUGC-4 cells to determine whether GFP transduction affects the dissemination pattern of this tumor.

There are two major advantages to retroviral gene transfer compared with other gene delivery systems. First, retroviral gene transfer can lead to stable integration in the target cell genome, thus providing the possibility of long-term gene expression.<sup>27</sup> The GFP gene was continuously expressed in tumor cells for at least 7 weeks in the present study. Recently GFP on a herpes simplex virus-1 Epstein-Barr virus vector was administered to tumor-bearing animals. However, long-time GFP expression did not seem to be achieved.<sup>32</sup> Second, retroviral-mediated gene transfer is limited to the transduction of dividing cells. Retroviruses do not transduce nondividing cells. It has been reported that cancer cells attached to visceral organs begin to proliferate within 1 week of inoculation into the peritoneal cavity.<sup>33</sup> During this period in the present study, GFP retroviral-containing supernatants were injected. Most cells, such as mesothelial cells, muscle cells, liver cells, and fibroblasts in the peritoneal cavity are not usually dividing.<sup>27</sup> Histological examination confirmed retroviral GFP gene expression in cancer cells only. This result was confirmed by injecting non-tumor-bearing mice with GFP retrovirus, which resulted in no GFP transduction of normal tissue.

The use of GFP to visualize and track tumor growth and dissemination has advantages over other methods such as detection with *lacZ* and reverse transcriptase polymerase chain reaction in that for GFP, no tissue or



cell preparation is needed. Therefore, GFP cancer cells can be visualized *in vivo* in real time.<sup>26</sup>

The present study has demonstrated that GFP transduction of cancer cells *in vivo* can facilitate the detection of future subclinical metastasis, which could eventually be applied clinically as a reporter of tumor progression or recurrence.

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