Visualization of the Metastatic Process by Green Fluorescent Protein Expression

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Abstract. We demonstrate here the visualization of the cancer metastatic process in live tissue in vivo by green fluorescent protein (GFP) expression. The human lung adenocarcinoma cell-line Anip 973 was transfected with the humanized GFP-S65T cDNA and stable high-level GFP-expressing transfectants were established. GFP transfectants were initially inoculated subcutaneously in nude mice. Five weeks after transplantation, when the tumor had reached 1.2 cm in diameter, fragments of subcutaneously tumor were implanted onto the visceral pleura of nude mice by surgical orthotopic implantation (SOI) as a spontaneous metastatic model. GFP expressing cells were injected intravenously in nude mice as an experimental hematogenous metastasis model. Mice were sacrificed four and eight weeks after treatment. At eight weeks, SOI-treated mice had lymphogenous (3/4 mice) and direct seeding (3/4) metastasis in the pulmonary hilum, cervical lymph nodes, the mediastinum and contralateral pleural cavity as detected by GFP expression in live tissue. All intravenously injected mice had metastases in the lung (4/4) and some of them had metastases in the brain (2/4) and other organs (1/4) as detected by GFP expression in fresh tissue. Some of the lung metastases produced by intravenous injection remained as dormant small colonies even eight weeks after treatment. These different metastatic patterns after SOI and intravenous injection visualized by GFP expression indicates that initial steps of the metastatic cascade influence the subsequent progression of metastasis.

Metastasis is the growth of secondary tumors at sites distant from a primary tumor and very frequently a lethal aspect of malignant tumor progression. The process of metastasis consists of a series of interactions between tumor cells and host cells or tissues (1). However, the early stages of tumor progression and micrometastasis formation in the seeded distant target organs have been difficult to directly analyze. These studies have been hampered by the inability to identify small numbers of tumor cells in a background of host cells. Several approaches have been developed to study particular aspects of metastasis, including a variety of in vivo and in vitro assays. However these approaches are not sufficiently informative as to how tumor cells metastasize and progress at sites distant from a primary tumor leaving us with a relatively poor understanding of metastasis. To understand how tumor cells metastasize and progress in the target organs, what has been needed is a way to directly visualize the process in vivo in fresh tissue as it occurs over time.

To enhance the resolution of the visualization of micrometastasis, we have utilized the green fluorescent protein (GFP) gene mutant hGFP-S65T which was isolated to develop higher gene expression in human cells (2,3,4). We have isolated stable transfectants of human lung adenocarcinoma cells (Anip 973) that express high level GFP fluorescence in vitro (24). The stable transfectants are also stably highly fluorescent in vivo in tumors formed from the cells. Using these fluorescent transfectants, two metastatic animal models were utilized for studying the metastatic processes, one for experimental hematogenous metastasis and the other using surgical orthotopic implantation (SOI) (II) to study spontaneous metastasis.

Abbreviations: GFP, green fluorescent protein; FCS, fetal calf serum; MTX, methotrexate; PBS, phosphate-buffered saline; DHFR, dihydrofolate reductase; SOI, surgical orthotopic implantation.


Key Words: Metastatic process, experimental hematogenous metastasis, surgical orthotopic implantation (SOI), green fluorescent protein (GFP).
Table I. Local and regional spreading four (Group 1) and eight (Group 2) weeks after surgical orthotopic implantation of clone-26 tumor pieces.

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<th>Tumor vol (mm³)</th>
<th>Chest wall</th>
<th>Mediastinum</th>
<th>Intralateral pleural cavity</th>
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* SE ± 72.22

Group 2

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* SE ± 329.23

Materials and Methods

Cell line and animals. Human lung cancer cell line (Anip 973) was obtained from Harbin Medical University in China. Six-week-old BALB/c nu/nu male mice were used for in vivo animal experiments.

DNA manipulations and expression vector. The dicistronic expression vector (pED-mtx²) was obtained from Genetics Institute (Cambridge, MA). The mammalian expression vector containing codon-optimized hGFP-S65r gene was purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA). To construct the hGFP-S65T containing expression vector, phGFP-S65T was digested with Hind III, blunted at the end. The hGFP entire coding region was then excised with Xba I. The pED-mtx² vector was digested with Pst I, blunted at the end, and further digested with Xba I. The hGFP-S65T cDNA fragment was then unidirectionally subcloned into pED-mtx² (24).

Cell culture, transfection, subcloning. Anip 973 cells were cultured in RPMI 1640 (GIBCO) containing 10% fetal calf serum (FCS) (Gemini Bio-products, Calabasas, CA), 2 mM L-glutamine and 100 µM non-essential amino acids (Irvine Scientific, Santa Ana, CA). For transfection, near-confluent Anip 973 cells were incubated with a precipitated mixture of LipofectAMINE™ reagent (GIBCO), and saturating amounts of plasmids for 6 hours before being replenished with fresh medium. Anip 973 cells were harvested with trypsin / EDTA 48 hours post-transfection, and subcultured at a ratio of 1:15 into selective medium which contained 50 nM methotrexate (MTX). Cells with stably integrated plasmids were selected by growing transiently-transfected cells in the MTX-containing medium. Clones were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ) by trypsin/EDTA. The clones were amplified and transferred with conventional culture methods. Clone-26 was chosen because of its high-intensity GFP fluorescence and stability (24).

Tumor growth. Nude mice were inoculated subcutaneously with a single dose of 10³ Clone-26 cells. Cells were first harvested by trypsinization and washed 3 times with cold serum-containing medium, then kept on ice. Cells were inoculated in a total volume of 0.4 ml within 40 minutes of harvesting. Mice were sacrificed to harvest the tumor pieces when the tumor had reached approximately 1.2 cm in diameter.

Orthotopic implantation. Tumor pieces (1-mm³) derived from the nude mouse subcutaneous tumor were implanted by surgical orthotopic implantation (SOI) onto the left visceral pleura in eight nude mice (5,6). The mice were anesthetized by isotflurane inhalation. A small 1-cm transverse incision was made on the left-lateral chest of the nude mice via the fourth intercostal space. A small incision provided access to the pleural space, and resulted in total lung collapse. Five tumor pieces were sewn together with a 8-0 nylon (Look, Norwell, MA) surgical suture and
Figure 1. GFP stable-expression cell line Anip 973 -hGFP-S65T-Clone 26 (Clone-26). Anip 973 cells were transfected with the pED-mtx vector in which the hGFP-S65T and DHFR genes were transcribed in a dicistronic message. The stable high expression Clone-26 was selected in 50 nM MTX. (Original magnification × 100.)

Figure 2. GFP-expressing subcutaneous tumor in nude mouse formed from Clone-26. The tumor was harvested when the tumor had reached approximately 1.2 cm in diameter. The tumor formed with well-developed vessels (white arrows) and intense expression of GFP. (Original magnification × 8.)
Table II. *Hematogenous dissemination in the systemic organs four (Group 1) and eight (Group 2) weeks after tail vein injection of clone-26 cells.*

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<th>Mouse #</th>
<th>Brain</th>
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**Results**

**Transfection of GFP expression vector to human lung cancer cells** (24). The GFP expression-vector transfected cells were able to grow in levels of MTX up to 50 nM. The subcloned MTX-resistant Anip 973 cells showed a striking increase in GFP fluorescence compared to the transiently-transfected cells. A subclone which expressed the strongest GFP was isolated and termed Anip 973-hGFP-S65 Clone-26 (Clone 26) (Figure 1) (24).

Stable high-level expression of GFP in Clone-26 tumors in nude mice. Five weeks after inoculation of $1.0 \times 10^7$ Clone-26 cells, an approximately 1.2 cm in diameter tumor was formed.

**Figure 3.** Nude mice were sacrificed after four (Group-1) and eight (Group 2) weeks intravenous injection of $1.0 \times 10^7$ Clone-26 cells. The fresh lung tissue was sliced at approximately 1-mm thickness and observed directly under fluorescence microscopy. The lung tissues were involved with many tumor cells. 3a (Group-1) and 3b (Group-2) show the lung tissue which was involved with GFP transfecants which ranged from 16.5 μm to 180.3 μm. There is no difference in the size of tumor colonies between the two groups. (Original magnification $\times 40$). 3c shows the developing colony (82.6 μm in diameter) in a Group-1 mouse. (Original magnification $\times 100$). 3d shows a dormant colony which had less than 10 cells (29.0 μm in diameter) in Group-2 mouse. (Original magnification $\times 200$).
Figure 3.
and the animal was sacrificed. The tumor tissue was strongly fluorescent (Figure 2). No obvious metastases were found in systemic organs.

**GFP-expressing metastases after surgical orthotopic implantation in nude mice.** Nude mice were implanted with 1-mm³ cubes of Clone-26 tumor in the visceral pleura and four mice each were sacrificed at four (Group 1) and eight (Group 2) weeks (Table I). Primary tumors grew in all mice. Pleural tumors in group 1 and 2, respectively, ranged from 244.40 mm³ to 522.88 mm³ and from 1279.08 mm³ to 2714.40 mm³ in volume. Mean volumes were respectively, 370.78 mm³ (SE ± 72.22) and 1799.33 mm³ (SE ± 329.23). All animals had evidence of chest wall invasion and local and regional spread (Table I). In Group-2 mice only, all tumors involved the mediastinum and contralateral pleural cavity and formed metastases on the visceral and parietal pleura, but not in Group-1 mice. Pulmonary hilar lymph nodes were involved in 3 / 4 mice in Group-1 and 4 / 4 mice in Group-2. Cervical lymph node involvement was detected in 1 / 4 mice in Group-2. No other metastases were observed in the other systemic organs.

**GFP-expressing metastases after intravenous injection in nude mice.** 1.0 × 10⁷ Clone-26 cells were injected in the nude mice tail vein and four mice were sacrificed at four (Group 1) and eight (Group 2) weeks (Table II). In both groups, numerous micrometastatic colonies were detected in the whole lung tissue by GFP expression (Figure 3a, b, c, d). Tumor colonies in Group-1 and -2, respectively, ranged from 15.6 µm to 158.5 µm, and 16.5 µm to 180.5 µm. Even eight weeks after injection, most of the colonies in Group-2 mice were not obviously further developed compared with Group-1 mice sacrificed at four weeks. Numerous small colonies which ranged in number down to less than 10 cells were detected at the lung surface in both groups. Brain metastases were detected in 1/4 mice which ranged from 23.4 µm to 67.5 µm in Group-1, and in 2/4 mice ranging from 48.5 µm to 818.2 µm in Group-2 (Figure 4). A mouse in Group-2 had systemic metastases in the brain, submandibular gland, whole lung, pancreas, bilateral adrenal glands, peritoneum and pulmonary hilar lymph nodes. All metastases were detected by GFP expression in fresh tissue.

**Discussion**

We have demonstrated the visualization of the different metastatic patterns of GFP-gene transfected Anip 973 human lung cancer cells after SOI and intravenous injection. Cancer metastasis is a highly coordinated and dynamic multistep process in which cancer cells undergo extensive interactions with various host cells before they establish a secondary metastatic colony. Chambers et al (7,8) utilized intravital videomicroscopy with fluorescent-tagged cells and directly observed the fate of tumor cells after intravenous injection. They demonstrated that more than 80% of injected cells
survive and extravasated by 24 hours. Their results suggested that the most important process of metastasis was the migration and growth of colonies at a distant site after extravasation. However, tumor cells tagged with dyes decrease their fluorescence after a few generations and are, therefore, not useful for the long term needed to study metastasis. Romer et al. (9) demonstrated the dissemination pattern of cancer cells in vivo with lacZ transfected tumor cells. In this study, GLC-2 lung cancer cells produced multiple metastases in several organs after subcutaneous inoculation but not after intravenous injection. This result indicated that the earliest processes, such as detachment from the primary tumor and intravasation, play a important roles in cancer metastasis. The detection of lacZ transfecants, however, requires extensive histologic preparation and results in a high background due to endogenous beta-galactosidase activity in certain cells (10). Studies in fresh tissue are not possible with lacZ as a marker.

To overcome these problems, we visualized cancer metastasis at the single-cell level in fresh tissue and progression of the colonies over time by GFP expression. GFP-gene transfecants can be followed in the primary and target organs since the fluorescence gene has been integrated and is passed on to the daughter cells. The GFP-expressing cells can be visualized in fresh live tissues without any histologic preparation and interference from endogenous GFP.

In the present study, we used two different metastatic models. The SOI model produced lymphogenous and direct-seeding metastasis. The intravenous injection model produced metastases by hematogenous dissemination in several organs. The SOI model allows full expansion of the tumor’s metastatic potential (11,12) and represents the entire process of the metastasis, including local tumor growth as well as vascular and lymphatic invasion at the local site. In contrast, the intravenous injection model bypasses these earliest stages. Jahroudi et al. (13) suggested only a small percentage of tumor cells (<0.01%) can survive in the circulation when they intravasated spontaneously. Injection of tumor cells allows sufficient numbers of cells to extravasate and produce metastases in several organs compared with the spontaneous metastatic cascade.

In the present study, we visualized actively colonizing as well as dormant tumor cells in the lung. Many tumor cells in the lung have remained as small, but live, colonies more than eight weeks after intravenous injection. Dormant micrometastasis is one of the most important steps to understand in tumor progression (14-17). In recent studies, the mechanism of this important phenomenon was studied with regard to angiogenesis and other chemical regulators of tumor colonization (18-22). However, these experimental models did not allow direct observation of the dormant colonies in fresh live tissue as it occurs over time as do the present studies.

GFP fluorescence will facilitate the understanding of metastatic processes including each step of the metastatic cascade and the mechanism of tumor dormancy. These results provide us with new insights into the metastatic process which should contribute to the therapy of cancer metastasis. The results with GFP-transfected tumor cells demonstrate a fundamental new technology to effectively study, for the first time, the “seed” and “soil” process of cancer metastasis in fresh tissue.

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