



## A highly metastatic Lewis lung carcinoma orthotopic green fluorescent protein model

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### Abstract

The Lewis lung carcinoma has been widely used for many important studies. However, the subcutaneous transplant or orthotopic cell-suspension injection models have not allowed the expression of its full metastatic potential. A powerful new highly metastatic model of the widely-used Lewis lung carcinoma is reported here using surgical orthotopic implantation (SOI) of tumor fragments and enhanced green fluorescent protein (GFP) transduction of the tumor cells. To achieve this goal, we first developed *in vitro* a stable high-expression GFP transductant of the Lewis lung carcinoma with the pLEIN retroviral expression vector containing the enhanced *Aequorea victoria* GFP gene. Stable high-level expression of GFP was found maintained *in vivo* in subcutaneously-growing Lewis lung tumors. The *in vivo* GFP-expressing tumors were harvested and implanted as tissue fragments by SOI in the right lung of additional nude mice. This model resulted in rapid orthotopic growth and extensive metastasis visualized by GFP-expression. 100% of the animals had metastases on the ipsilateral diaphragmatic surface, contralateral diaphragmatic surface, contralateral lung parenchyma, and in mediastinal lymph nodes. Heart metastases were visualized in 40%, and brain metastases were visualized in 30% of the SOI animals. Mice developed signs of respiratory distress between 10–15 days post-tumor implantation and were sacrificed. The use of GFP-transduced Lewis lung carcinoma transplanted by SOI reveals for the first time the high malignancy of this tumor and provides an important useful model for metastasis, angiogenesis and therapeutic studies.

### Introduction

The Lewis lung carcinoma was first isolated by Dr Margaret R. Lewis in 1951 from a spontaneous epidermoid carcinoma of the lung in mouse [1]. The Lewis lung carcinoma has been an important tumor model for metastatic and angiogenesis studies and neoadjuvant chemotherapy [2–5]. Folkman's group demonstrated that removal of the subcutaneously implanted Lewis lung carcinoma tumor increases metastatic growth [2].

Li et al. reported that orthotopic implantation of Lewis lung cell suspension did not increase tumorigenicity [12]. Doki et al. demonstrated, after an orthotopic injection of tumor cells, limited metastasis localized only in mediastinal lymph nodes [13].

We have previously demonstrated, however, that orthotopic potential of implantation of tumor fragments allows the full metastatic potential of tumors to be expressed [6–11]. Our hypothesis was that the Lewis lung carcinoma had far greater metastatic potential than has previously demonstrated. In order to investigate the metastatic potential of Lewis lung carcinoma, the tumor was transplanted to nude mice using SOI in the present study. To fully visualize

metastases in the SOI Lewis lung carcinoma model, it was transduced with the jellyfish *Aequorea victoria* green fluorescent protein (GFP) gene [14, 15].

### Materials and methods

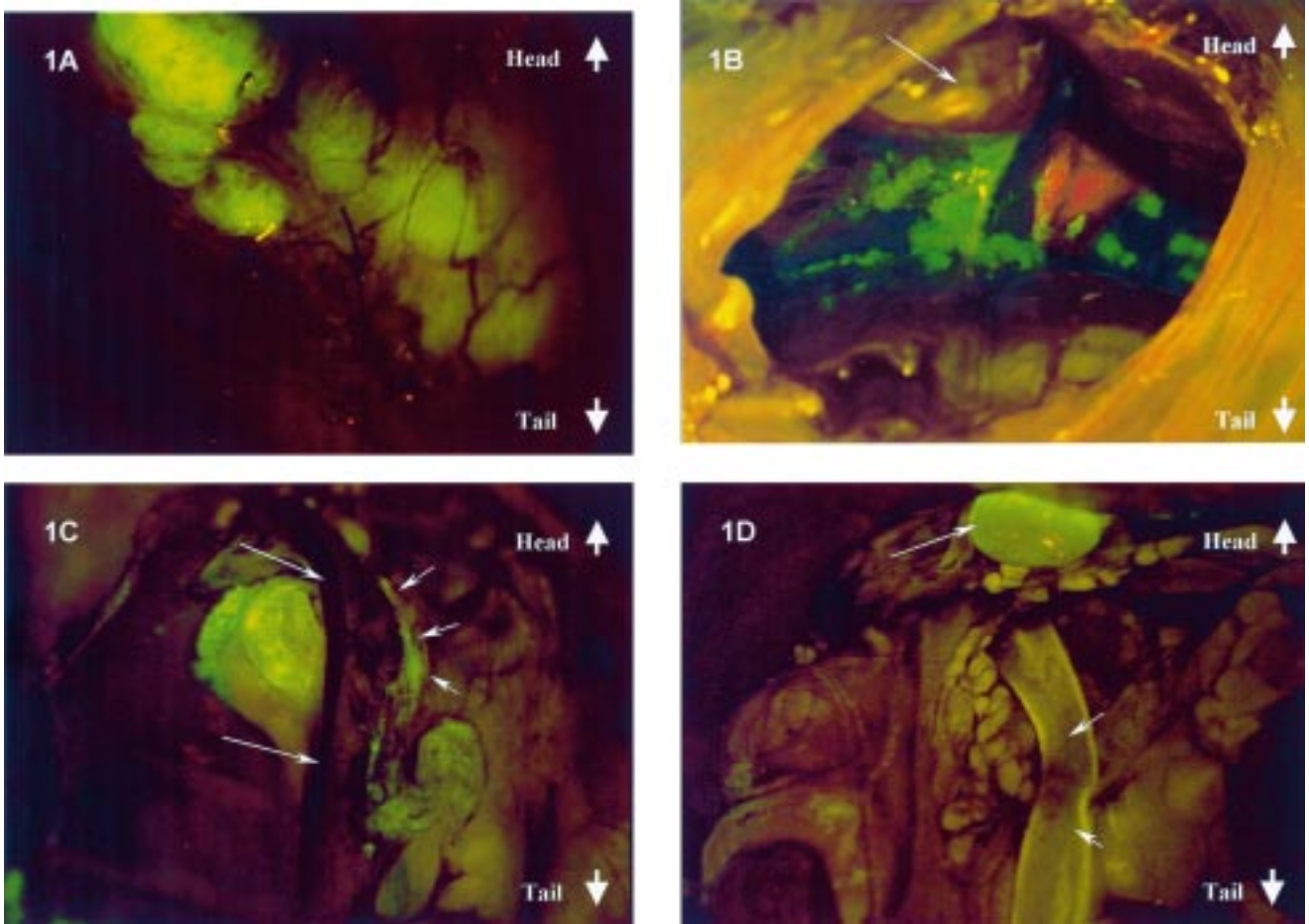
#### *GFP DNA expression vector* [13]

The retroXpress vector GFP pLEIN was purchased from Clontech Laboratories, Inc. (Palo Alto, California). The pLEIN vector expresses enhanced GFP and the neomycin resistance gene on the same bicistronic message, which contains an IRES site [13].

#### *GFP vector production* [13]

PT67, an NIH3T3-derived packaging cell line expressing the 10 A1 viral envelope, was purchased from Clontech Laboratories, Inc. PT67 cells were cultured in DMEM (Irvine Scientific, Santa Ana, California) supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bio-products, Calabasas, California). For vector production, packaging cells (PT67), at 70% confluence, were incubated with a precipitated mixture of DOTAP reagent (Boehringer Mannheim) and saturating amounts of pLEIN plasmid for 18 h. Fresh medium was replenished at this time. The cells

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**Figure 1.** (A) Primary GFP-Lewis lung tumor implanted in the right lung visualized by GFP expression 14 days after SOI. (B) Multiple metastatic sites of the GFP-Lewis lung carcinoma in the contralateral lung by GFP expression through a window in the thoracic wall 14 days after SOI (white arrow indicates the heart). (C) Superior mediastinal lymph nodes involved with GFP-Lewis lung carcinoma metastases around a thoracic vein (large white arrows) visualized by GFP expression 14 days after SOI. Tumor cells in the lymphatic duct (small white arrows) connected to a metastatic lymph node are clearly visualized by GFP expression. (D) A lymph node in the superior mediastinum massively involved with GFP Lewis lung carcinoma metastasis (large white arrow), close to the great vessels (small white arrows), visualized by GFP expression 14 days after SOI.

were examined under fluorescence microscopy after 48 h. For the selection of the GFP transductants, the cells were cultured in the presence of 500–2000  $\mu\text{g/ml}$  of G418 (Life Technologies, Inc, Grand Island, New York) for 7 days.

#### *GFP transduction of Lewis lung carcinoma cells*

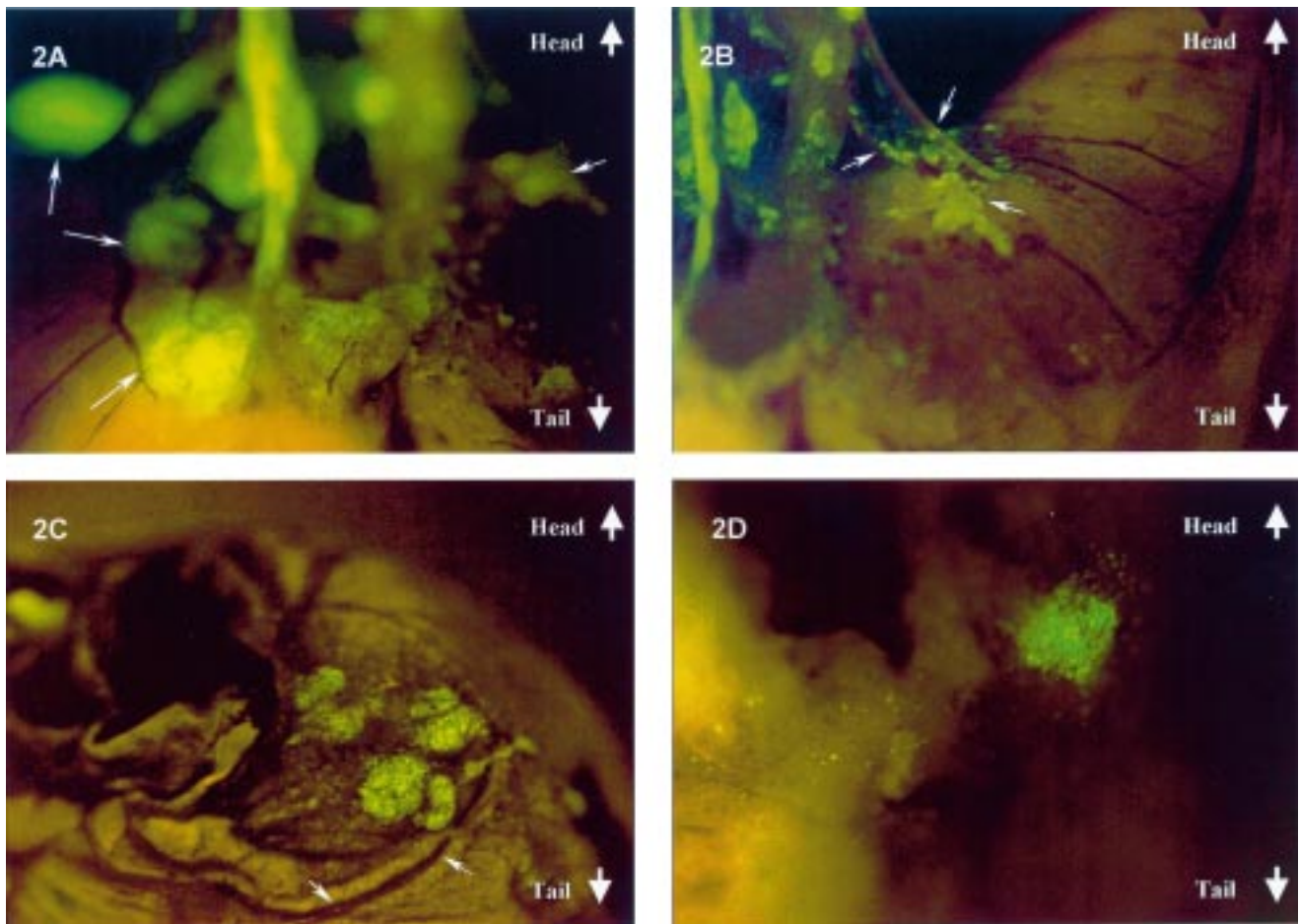
Confluent Lewis lung carcinoma cells from the National Cancer Institute were incubated with a 1–1 precipitated mixture of retroviral supernatants of PT67 cells and RPMI 1640 (Life Technologies, Inc.) containing 10% fetal bovine serum (Gemini Bio-products, Calabasas, California) for 72 h. Fresh medium was replenished at this time. Lewis lung carcinoma cells were harvested by trypsin/EDTA 72 h after infection and subcultured at a ratio of 1:15 into selective medium that contained 200  $\mu\text{g/ml}$  of G418. The level of G418 was increased to 400  $\mu\text{g/ml}$  gradually. Lewis lung carcinoma clones highly expressing GFP were isolated with cloning cylinders (Bel-Art products, Pequannock, New Jersey) by trypsin EDTA and were amplified and transferred by conventional culture methods.

#### *Subcutaneous tumor transplantation of GFP-Lewis lung cells*

Three BALB/c nu/nu female mice, 6 weeks of age, were injected s.c. with a single dose of  $5 \times 10^6$  of Lewis lung-GFP cells that were previously selected in G418 as described above. Cells were first harvested by trypsinization and washed three times with cold serum free medium and then injected in a total volume of 0.2 ml within 40 min of harvesting.

#### *Surgical orthotopic implantation (SOI) of GFP-Lewis lung tissue fragments*

Tumor fragments (1 mm) derived from the Lewis lung-GFP s.c. tumors growing in nude mice were implanted by SOI on the right lung in ten nude mice [10, 11]. The mice were anesthetized by isofluran inhalation. The animals were put in a position of left lateral decubitus. A 0.8-cm transverse incision of skin was made in the right chest wall. Chest muscles were separated by sharp dissection, and costal and intercostal muscles were exposed. A 0.5-cm intercostal incision between the third and fourth rib on the chest wall was



**Figure 2.** (A) The inferior mediastinum is massively involved with GFP-Lewis lung carcinoma metastases visualized by GFP expression 14 days after SOI. Large arrows indicate ipsilateral diaphragmatic surface metastases. Small arrows indicate the contralateral diaphragmatic surface involved with metastases. (B) Contralateral diaphragmatic surface involved with GFP-Lewis lung carcinoma metastases visualized by GFP expression 14 days after SOI (white arrows). (C) The heart is involved with GFP-Lewis lung carcinoma metastases around the posterior coronary artery visualized by GFP expression 14 days after SOI (white arrows). (D) Lewis lung carcinoma brain metastasis visualized by GFP expression 14 days after SOI.

made, and the chest wall was opened. The right lung was taken up by a forceps, and one tumor fragment was sewn promptly into the upper lung using one 8-0 suture. The lung was then returned into the chest cavity. The incision of the chest wall was closed with a 6-0 surgical suture. Closure of the chest wall was examined immediately and, if a leak existed, it was closed by additional sutures. After closing the chest wall, an intrathoracic puncture was made by using a 3-ml syringe and 25-gauge 1/2 ml needle to withdraw the remaining air in the chest cavity. After the withdrawal of air, a completely inflated lung could be seen through the thin chest wall of the mouse. The skin and chest muscles were then closed using a 6-0 surgical suture in one layer. All procedures of the operation described above were performed under a 7X microscope (Olympus).

#### *Analysis of GFP-Lewis lung carcinoma metastases*

After tumor progression in the SOI animals, the performance of the mice began to decrease, at which time the animals were sacrificed and autopsied. The orthotopic primary tumor and all major organs were explored under fluorescence microscopy.

#### *Fluorescence microscopy of GFP-Lewis lung carcinoma*

Light and fluorescent microscopy were carried out using a Nikon microscope equipped with a xenon lamp power supply. A Leica stereo fluorescent microscope model LZ12 equipped with a mercury lamp power supply was also used. Both microscopes had a GFP filter set (Chroma Technology, Brattleboro, Vermont).

#### **Results and discussion**

##### *Isolation of stable high-level expression GFP transductants of Lewis lung carcinoma cells*

The retroviral-vector GFP-transduced Lewis lung carcinoma cells were able to grow *in vitro* at levels of G418 up to 400  $\mu\text{g/ml}$ . The selected G418-resistant Lewis lung-GFP cells had very bright GFP fluorescence (data not shown).

##### *Stable high-level expression of GFP in Lewis lung carcinoma subcutaneous tumors in nude mice*

Three weeks after s.c. injection of Lewis lung-GFP cells, the mice were sacrificed. Tumor tissue was strongly GFP

Table 1. Metastasis of GFP-Lewis lung carcinoma after SOI in nude mice.

| % of the animals involved with metastases | Bilateral      |                       |           |           |                  |
|---|----------------|-----------------------|-----------|-----------|------------------|
|   | Bilateral lung | diaphragmatic surface | Heart     | Brain     | Mediastinal L.N. |
|   | 100%           | 100%                  | 40%       | 30%       | 100%             |
|   | (10 of 10)     | (10 of 10)            | (4 of 10) | (3 of 10) | (10 of 10)       |

Ten mice were implanted in the right lung by SOI with one piece each of GFP-Lewis lung carcinoma tissue (1 mm<sup>3</sup>) derived from the GFP-Lewis lung tumor previously grown s.c. in a nude mouse. The implanted mice were sacrificed at 10–14 days post-SOI at the time of significant decline of performance status.

fluorescent, demonstrating stable high-level GFP expression *in vivo* during s.c tumor growth (data not shown). Lung metastases were visualized by GFP-expression in these mice (data not shown). The median survival of these mice was approximately 27 days.

#### Orthotopic growth of GFP-Lewis lung carcinoma

Beginning 10 days after SOI, the mice started to decline in performance. All animals were sacrificed by day 15 post tumor implantation. All sacrificed animals had highly fluorescent tumors (Figure 1A) in the right lung weighing from 144 mg to 466 mg.

#### Metastatic pattern of the SOI Lewis lung carcinoma

Table 1 summarizes the metastatic pattern of the Lewis lung-GFP carcinoma. One-hundred percent of the animals (10 of 10) had disseminated contralateral lung metastases (Figure 1B); mediastinal lymph node metastases (Figures 1C, 1D); and ipsilateral (Figure 2A) and contralateral (Figures 2A, 2B) diaphragmatic surface metastases. The heart was involved with metastases in 40% (4 of 10) of the animals (Figure 2C) and the brain in 30% (3 of 10) of the animals (Figure 2D). Metastases in the contralateral lung, contralateral diaphragmatic surface, heart, and brain were only detectable by GFP expression and not detectable under bright-field microscopy in fresh tissue. A major advantage of GFP-expressing tumor cells is that they can be visualized in fresh live tissue [14–16].

The Lewis lung-GFP carcinoma implanted by SOI diffusely metastasized locally and distantly and decreased the mean survival of the animals to approximately 12 days, far shorter than the mean survival of 27 days in the subcutaneous transplant model.

The developments described here have enabled the widely-used Lewis lung carcinoma to become a far more powerful model to study the mechanism of tumor progression including regional and distant metastasis representative of lung cancer. The data obtained in this study confirmed our hypotheses that SOI allowed the Lewis lung carcinoma to express its enormous metastatic potential. The new model should be of greater use to study metastasis, the role of angiogenesis, and for the discovery of agents which inhibit or reverse these processes.

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