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Multi-organ metastatic capability of Chinese hamster ovary cells revealed by green fluorescent protein (GFP) expression

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Abstract

Stable high-level green fluorescent protein (GFP)-expressing Chinese hamster ovary cells (CHO) were used to visualize the degree of metastatic behavior of this cell line in nude and SCID mice. A stable GFP high-expression CHO clone, selected in 1.5 μM methotrexate, was injected subcutaneously in nude and severe combined immunodeficient (SCID) mice and implanted orthotopically in the ovary of nude mice. CHO proved to be highly metastatic from both the subcutaneous and orthotopic sites as brightly visualized by GFP fluorescence. High-level GFP-expression allowed the visualization of metastatic tumor in fresh live host tissue in great detail. Metastases were visualized by GFP expression in the lung, pleural membrane, spleen, kidney, ovary, adrenal gland, and peritoneum after orthotopic implantation in nude mice. Metastases were visualized by GFP expression mainly in the lung, pleural membrane after subcutaneous implantation in nude mice. Metastases were visualized in the lung and pleural membrane, liver, kidney, and ovary after subcutaneous implantation in SCID mice. The construction of highly fluorescent stable GFP transfectants of CHO has revealed the multi-organ metastatic capability of CHO cells. CHO has such a high degree of malignancy that it is metastatic from both the orthotopic and subcutaneous transplant sites. This highly malignant GFP-expressing cell-line with multi-organ metastatic affinity should serve as a powerful tool to study tumor-host interaction.

Introduction

The green fluorescent protein (GFP) gene, cloned from the bioluminescent jellyfish *Aequorea victoria* has great potential for cell visualization after transfection [1–11]. The Chinese hamster ovary (CHO) cell line has proven to be an excellent model for transfection of exogenous genes, including those related to cancer [12]. Therefore, we chose GFP-transfected CHO as a candidate to visualize tumor cell behavior after transplantation to nude and SCID mice.

In order to visualize CHO metastases, we have previously transfected CHO cells with a dicistronic expression vector containing humanized GFP cDNA [13]. Stable transfectants were obtained after selection in methotrexate. The stable transfectants were subsequently implanted in nude mice subcutaneously and orthotopically where metastases were subsequently visualized by GFP expression [13]. However, Esko et al. [14] reported that although CHO was tumorigenic in nude mice, no metastases were observed. In this report, with the use of stable high-expression GFP transfectants of CHO cells that express GFP, we performed a systematic study of the metastatic behavior of CHO in nude and SCID mice using both orthotopic and subcutaneous transplantation. We demonstrate here the general multi-organ metatastic potential of CHO suggesting the use of GFP-CHO to study the relationship of cancer cells with multiple host organs.

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Materials and methods

Cell line and animals

CHO K1 cells were purchased from the American Type Cell Culture Collection. Six-week-old female BALB/c *nu/nu* and severe combined immunodeficient (SCID) mice were used for transplantation.

GFP expression vector

The dicistronic expression vector (pED-mtx^r) was obtained from the Genetics Institute (Cambridge, MA) [15]. The expression vector containing the codon-optimized hGFP-S65T 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

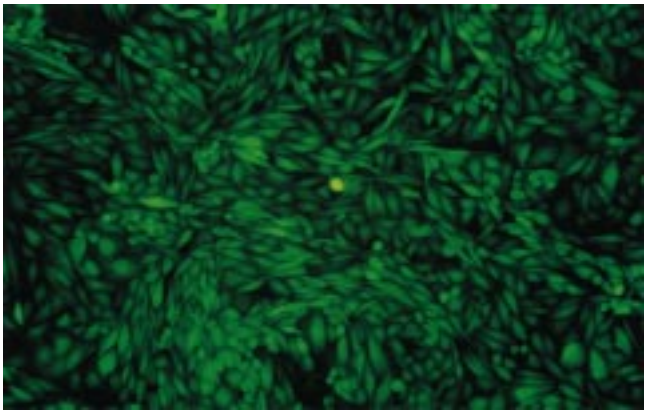

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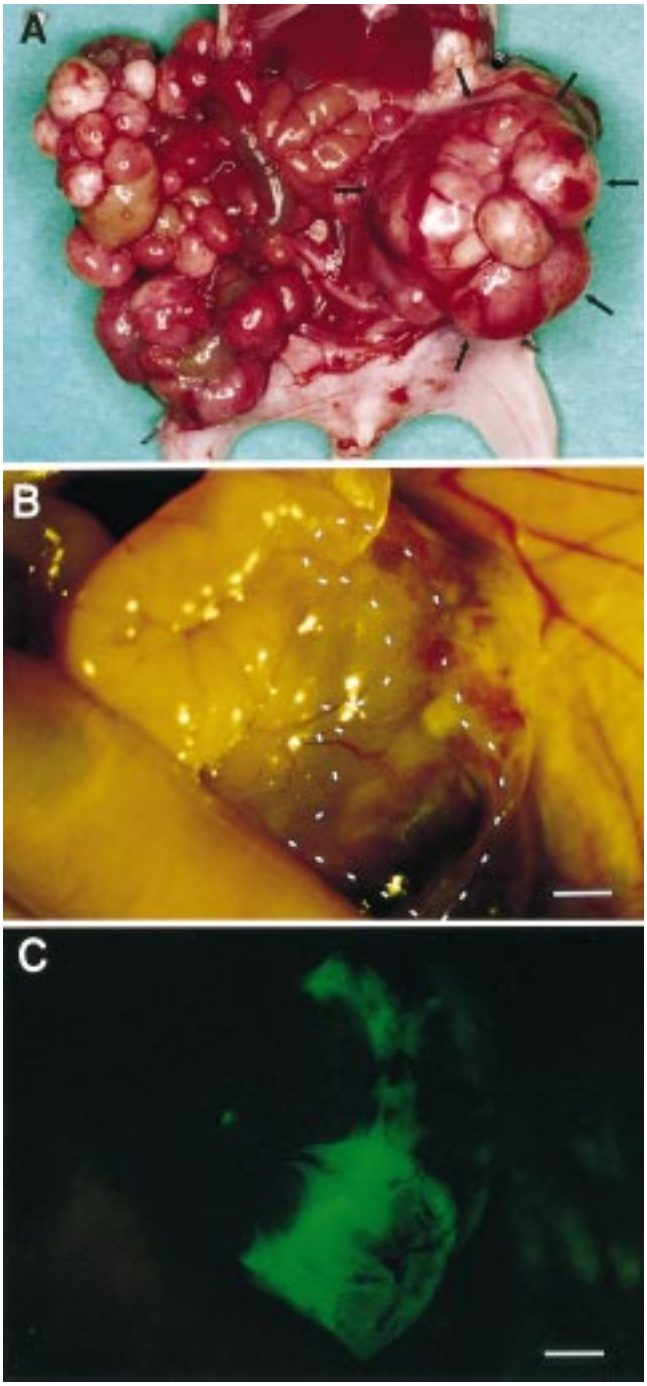
Figure 1. Stable high-level expression GFP-CHO transfectants *in vitro*. GFP stable expression cell line CHO-K1-GFP 38 (Clone-38). CHO cells were transfected with the pED-mtx^r vector in which the hGFP-S65T and DHFR genes were transcribed in a dicistronic message. The stable high-expression Clone-38 was selected in 1.5 μM MTX (original magnification $\times$ 100).



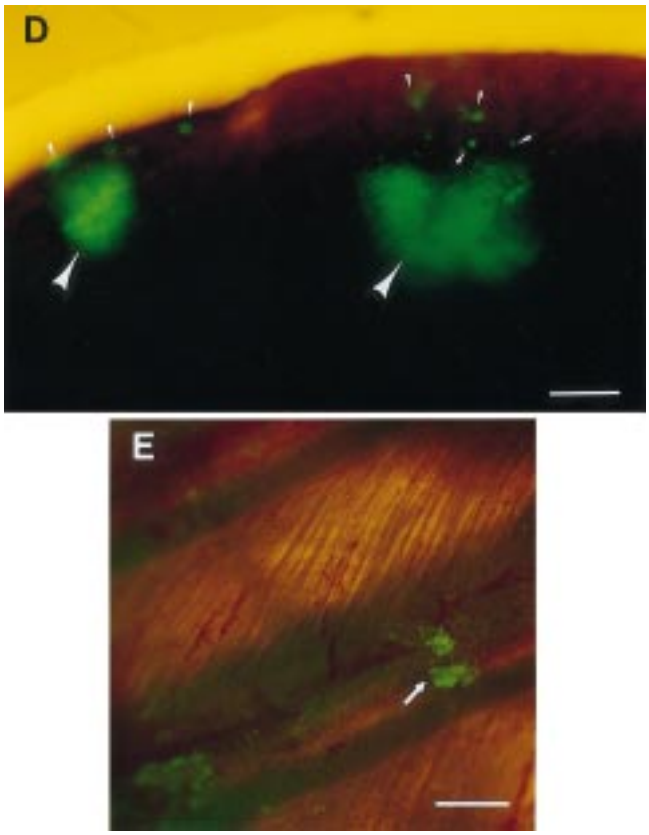


Figure 2. Visualization of CHO metastasis by GFP expression in fresh nude mouse tissue. (**A**) Extensive GFP-CHO metastases in the nude mouse peritoneal cavity after orthotopic transplantation. Orthotopic implantation (see text for details of surgical orthotopic transplantation) resulted in extensive tumor growth and metastases in the nude mice. Arrowheads indicate primary tumor, which grew segmentally in the left ovary. Many seeded metastases were seen on the visceral and parietal peritoneum. These metastases were strongly fluorescent (data not shown). (**B, C**) GFP-CHO tumor spreading on the fresh nude mouse visceral peritoneum after orthotopic transplantation. No tumor is visible under bright-field microscopy. Broken lines show the area where the tumor spread but could not be seen under bright field light (**B**). GFP-CHO tumor cells spread on the surface of mesentery of the small intestine, were brightly visualized by fluorescence (**C**). Bars: 1000 μm. (**D**) GFP-CHO metastases visualized in fresh nude mouse lung tissue after orthotopic transplantation. Metastatic CHO tumor colonies (approximately 400–700 μM in diameter) were visualized in fresh lung tissue of the nude mouse. These tumor colonies grew along with other small satellite colonies (20–30 μM). Bar: 300 μM. (**E**) GFP-CHO tumor spreading on the nude mouse pleural membrane after orthotopic transplantation. Highly fluorescent GFP-CHO tumor colonies grew on the pleura of the nude mouse. The vessels on the pleural membrane were interrupted by the tumor colony (arrowhead). Other small satellite colonies were visualized around the major tumor colonies. These colonies were not visible under bright-field microscopy (data not shown). Bar: 300 μM.

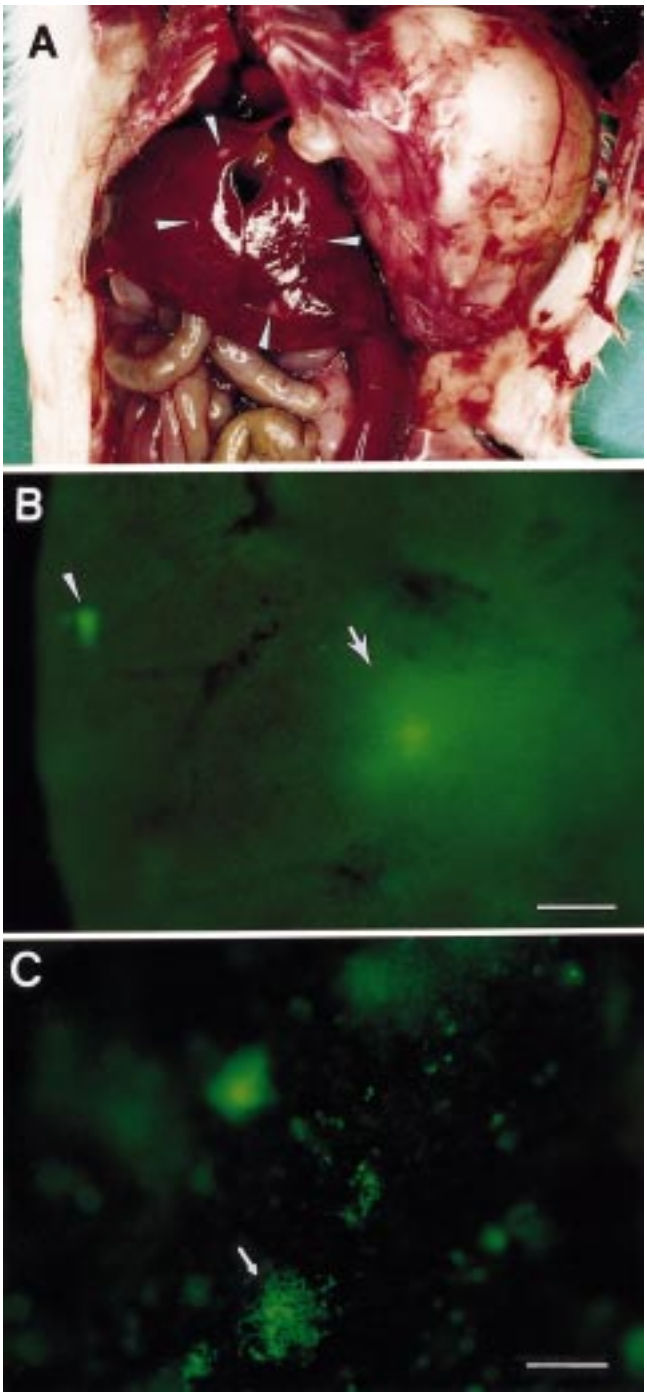


Figure 3. Visualization of CHO metastases by GFP in fresh SCID mouse tissue after subcutaneous transplantation. (**A, B**) GFP subcutaneous tumor and liver colonization in SCID mice. Metastatic CHO colonies in the liver are indicated by arrowheads (**A**). These liver colonies were strongly fluorescent (**B**). Bar: 300 μM. (**C**) Numerous GFP-CHO metastases in the fresh SCID mouse lung after subcutaneous transplantation. Many CHO micrometastases were visualized at the apparent single-cell level by GFP fluorescence. Arrowhead indicates a developing colony which is invading the lung tissues of the SCID mouse with pseudopodia-like structures. Bar: 300 μM.

and blunted at the end. The entire hGFP coding region was then excised with *Xba*I. The pED-mtx^r 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-mtx^r. The resulting vector was used to transfect the CHO cells with the GFP gene [13].

Cell culture, transfection, subcloning

CHO-K1 cells were cultured in DMEM (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 CHO K1 cells were incubated with a precipitated mixture of LipofectAMINE^{TM} reagent (GIBCO) and saturating amounts of plasmids for 6 h before being replenished with fresh medium [13]. CHO K1 cells were harvested with trypsin/EDTA 48 h post-transfection, and subcultured at a ratio of 1:15 in selective medium which contained 1.5 μM methotrexate (MTX). Cells with a stably integrated expression vector containing the GFP gene were selected by growing transiently-transfected cells in the MTX-containing medium. Clones were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ) with trypsin/EDTA. The clones were amplified and transferred with conventional culture methods. Clone-38 was chosen because of its high-intensity GFP fluorescence and stability [13].

Initial GFP-CHO tumor growth

Nude mice were injected subcutaneously with a single dose of 10^7 Clone-38 cells. Cells were first harvested by trypsinization and washed 3 times with cold serum-containing medium, then kept on ice. Cells were injected in a total volume of 0.4 ml within 40 min of harvesting. The mice were sacrificed to harvest tumor fragments two weeks after tumor cell injection.

Orthotopic implantation

Tumor fragments (1 mm^3) derived from the CHO clone-38 nude mouse subcutaneous tumor were implanted on the ovarian serosa in 10 nude mice by the surgical orthotopic implantation (SOI) technique developed by us [16]. The mice were anesthetized by isofuran inhalation. An incision was made through the left lower abdominal pararectal line and peritoneum. The left ovary was exposed and part of the serosal membrane was scraped with a forceps. Four 1-mm^3 tumor pieces were fixed on the scraped site of the serosal surface with an 8-0 nylon suture (Look, Norwell, MA). The ovary was then returned into the peritoneal cavity, and the abdominal wall and the skin were closed with 6-0 silk sutures [16].

Subcutaneous implantation

GFP-expressing tumor fragments were implanted subcutaneously in 10 nude mice and 5 SCID mice. The mice were

anesthetized and a 5 mm incision was made in the skin. Four 1-mm^3 tumor pieces were put in the subcutaneous space and the wound was closed with 6-0 silk sutures.

Analysis of metastasis

Six weeks after transplantation with GFP CHO clone-38, the mice were sacrificed and the lung and the other organs were removed. The fresh samples were sliced at approximately 1 mm thickness and observed directly under fluorescence microscopy.

Microscopy

Light and fluorescence microscopy were carried out using a Nikon microscope equipped with a xenon lamp power supply and a Leica stero fluorescence dissecting microscope (model LZ12) equipped with a mercury lamp power supply. Both fluorescence microscopes were equipped with a GFP filter set (Chromatechnology Corp., Battleboro, VT).

For photomicroscopy, a Nikon (Japan) FX-35A camera was used with Fuji (Japan) Color Superia film. The photomicrographs were scanned with a Hewlett Packard (USA) model scanner and digitally processed with Image ProPlus software from Media Cybernetics (Silver Springs, MD).

Results and discussion

GFP-fluorescent CHO cells and tumors

The CHO cells that were selected in 1.5 micromolar methotrexate (Figure 1) were implanted in three different tumor transplantation models using immunodeficient mice (please see below).

Stable high-level expression of GFP in subcutaneous CHO tumors of approximately 1.0 cm in diameter in nude mice was observed two weeks after inoculation of 1.0×10^7 Clone-38 cells. The tumor tissue was strongly and stably fluorescent without MTX selection [13]. This GFP-expressing subcutaneous tumor served as tissue stock for subsequent subcutaneous and orthotopic transplantation.

Metastasis of GFP-expressing CHO cells after orthotopic transplantation in the nude-mouse ovary

Tumor pieces previously grown in subcutaneous nude mice were implanted orthotopically into the left ovary of additional nude mice. Orthotopic transplantation resulted in extensive tumor growth and metastases of the CHO tumor in the animal as can be seen in Figure 2A. Figure 2B shows the mouse peritoneum in which no tumor is visible under bright-field microscopy of fresh tissue. However, fluorescent microscopy reveals an extensive GFP-fluorescing CHO tumor growing on the surface of the peritoneum (Figure 2C). The high expression of GFP in the metastasizing CHO tumor cells enabled the metastases to be very clearly visualized . For example, Figure 2D shows a highly fluorescent lung metastasis. GFP fluorescence revealed two CHO tumor colonies growing on the pleura along with other small satellite metastases that have seeded nearby (Figure 2E). The orthotopically-growing ovarian tumor also metastasized to the lung, pleural membrane, spleen, kidney, contralateral ovary, adrenal gland, and peritoneum (Table 1).

Metastasis of GFP-CHO cells after subcutaneous transplantation in nude mice

Table 2 demonstrates that GFP-CHO cells can also metastasize from the subcutaneous site of nude mice with all the animals having metastases in the lung and the majority of the animals having metastases in the pleural membrane. Two animals had a metastasis in the kidney, one of them also had an adrenal gland metastasis. In another animal, there was a metastasis in the ovary.

Metastasis of GFP-CHO cells after subcutaneous transplantation in SCID mice

The GFP-CHO cells were implanted subcutaneously in SCID mice. Extensive metastases resulted with the animals having tumors in the lung, pleural membrane, liver, spleen, kidney, adrenal gland, and peritoneum (Table 3). Figure 3A shows the subcutaneous tumor grown in the SCID mouse as well as metastasis in the liver in which some lesions can be seen under normal light. However, when the liver was observed by fluorescent microscopy, Figure 3B shows a diffuse metastatic tumor as well as a much smaller micro-metastasis, both visualized by their strong GFP fluorescence. Figure 3C shows a relatively large GFP-fluorescing CHO metastasis tumor with pseudopodia-like structures expanding into the lung tissue. In addition, one can visualize by GFP many small CHO seedings in the mouse lung that have not grown out, which are either dormant colonies or recently-migrated satellite colonies.

With the stably transfected GFP gene, we have demonstrated that CHO is a very highly malignant tumor that has affinity for at least seven organs in nude and SCID mice. Not only could the tumor metastasize after transplantation to the orthotopic site but had metastases six weeks after subcutaneous transplantation in nude mouse although not as extensively as after orthotopic transplantation. In the case of SCID mouse, the subcutaneous tumors could metastasize to the all sites in which metastases occurred in nude mice. In addition, tumors even metastasized to the liver in all of the transplanted SCID mice.

These phenomena demonstrated that CHO has a high metastasizing ability when it is implanted into nude or SCID mice. The orthotopically-transplanted CHO-GFP tumor grew extensively in the ovary of nude mice and metastasized to sites such as to the peritoneum and lung as well as the contralateral ovary which are typical of human ovarian cancer. GFP visualization of CHO metastases from both the ectopic and orthotopic sites suggest that CHO is highly metastatic to most major organ sites with very high affinity to lung, pleural membrane, and peritoneum. In contrast, related studies of GFP transfected human lung cancer cells

Table 1. Metastasis of GFP-expressing CHO Clone-38 cells after orthotopic transplantation in nude mice.

		Tumor spread							
Mouse #	Primary tumor weight (mg)	Lung	Pleural membrane	Liver	Spleen	Kidney	Ovary	Adrenal gland	Peritoneum
1	4445.9	+	+	−	+	+	−		+
2	9802.0	+	−	−	+	+	+	−	+
3	2983.2	+	+	−	+	−	+	+	+
4	2432.0	+	−	−	−	−	−	−	+
5	2526	+	+	−	−	+	−	+	+
6	4092	+	+	−	+	−	+	−	+
7	6750	+	+	−	−	−	−	−	+
8	1835	+	−	−	−	−	−	−	+
9	3882	+	+	−	−	+	−	+	+
10	3705	+	−	−	−	−	−	−	−

10^7 GFP-transfected CHO clone-38 cells were initially injected into the subcutaneous space of nude mice. The subcutaneous tumor grew for approximately two weeks, at which time tissue fragments of approximately 1 mm^3 were transplanted to the ovary of nude mice using the technique of surgical orthotopic implantation developed in our laboratory [15]. The tumors were allowed to grow and spread from the orthotopic space in nude mice for six weeks, at which time the animals were sacrificed and explored by fluorescence microscopy to determine the presence of fluorescent CHO-GFP cells in the indicated organs. A Leica dissecting fluorescence microscope (Model LZ 12) was used. Tumor weights were calculated by the equation: tumor weight (mg) = length (mm) × (width(mm))^2/2. Mean = 4245.3 SD ± 2387.1.

Table 2. Metastasis of GFP-expressing CHO clone-38 cells after subcutaneous transplantation in nude mice.

		Tumor spread							
Mouse #	Primary tumor weight (mg)	Lung	Pleural membrane	Liver	Spleen	Kidney	Ovary	Adrenal gland	Peritoneum
1	5315.9	+	+	−	−	−	+	−	−
2	6682.5	+	+	−	−	+	−	−	−
3	1318.2	+	−	−	−	−	−	−	−
4	8956.4	+	+	−	−	−	−	−	−
5	7160	−	−	−	−	−	−	−	−
6	5583	−	−	−	−	−	−	−	−
7	7267	+	+	−	−	+	−	+	−
8	4173	+	−	−	−	−	−	−	−
9	6932	+	−	−	−	−	−	−	−
10	7435	+	−	−	−	−	−	−	−

Fragments of a CHO-GFP clone-38 subcutaneous tumor were harvested and minced into pieces of approximately 1 mm^3. The resulting fragments were then reimplanted into the subcutaneous space of additional nude mice. Six weeks after implantation, the animals were sacrificed and the fresh tissues were explored for tumor spread by GFP-expression using a dissecting fluorescence microscope. Tumor weights were calculated as in Table 1. Mean = 6082.3 SD ± 2132.2.

Table 3. Metastasis of GFP-expressing CHO clone-38 cells after subcutaneous transplantation in SCID mice.

		Tumor spread							
Mouse #	Primary tumor weight (mg)	Lung	Pleural membrane	Liver	Spleen	Kidney	Ovary	Adrenal gland	Peritoneum
1	3702.8	+	+	+	+	+	−	+	−
2	7590.2	+	+	+	−	+	−	+	+
3	5571	+	+	+	+	+	−	+	+
4	6750	+	+	+	+	+	−	+	+
5	7267	+	+	+	+	+	−	+	+

Tumor fragments of a CHO-GFP clone-38 subcutaneous tumor in nude mice were harvested and minced into pieces of approximately 1 mm^3. The resulting fragments were then re-implanted into the subcutaneous space of SCID mice. Six weeks after implantation, the animals were sacrificed and the fresh tissues were explored for tumor spread by GFP-expression using a dissecting fluorescence microscope. Tumor weights were calculated as in Table 1. Mean = 6176.2 SD ± 1581.2.

revealed that these cells are much more selective in their metastatic targeting [17, 18].

Margolis et al. visualized the migration of externally fluorescently-labeled lung tumor cells in host mouse lung in three-dimensional histoculture [19]. Chambers et al. visualized externally-labels cancer cells by video microscopy [20, 21]. Both the Chambers [20, 21] and Margolis [19] studies were not able to visualize the long-term growth progression of metastatic colonies in host organs because they were not heritably labeled with fluorescence. On the other hand, the GFP gene transfectants described here have stable high-level GFP heritable expression.

When the CHO cells are stably transfected with a high-expression GFP vector, they become a particularly powerful tool to study metastasis, especially the types of metastases which have the propensity to target numerous metastatic organs which is typical of very advanced high-grade human neoplasms including ovarian cancer. In addition, GFP serves as a marker to distinguish the tranplanted tumor cells from endogenous mouse tumors which occasionally occur.

In conclusion, CHO is a highly and generally multi-organ malignant cell-line. Orthotopic transplantation yielded higher metastatic activity than subcutaneous transplantation in nude mice. However, the resutls of this report indicate that CHO is so metastatic that metastasis occurs after subcutaneous transplantation, especially in SCID mice where many organs are metastically targeted, including the liver. When transfected with GFP, the CHO line offers an important new tool to help us undertstand the key events of cancer metastasis especially interaction of the metastatic tumor with a wide array of normal organs and tissues.

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