

Orthotopic Implantation of Intact Tumor Tissue Leads to Metastasis of OCUM-2MD3 Human Gastric Cancer in Nude Mice Visualized in Real Time by Intravital Fluorescence Imaging

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Abstract. *Background/Aim:* Orthotopic (literally “correct place”) implantation of cancer in nude mice has long been known to be superior to subcutaneous transplantation because the orthotopic tumor can metastasize. We reported previously on surgical orthotopic implantation (SOI) of gastric cancer tissue in nude mice resulting in the formation of metastases in 100% of the mice with extensive primary growth to the regional lymph nodes, liver, and lung. In contrast, when cell suspensions were used to inject gastric cancer cells orthotopically, metastases occurred in only 6.7% of the mice with local tumor formation, emphasizing the importance of orthotopically implanting intact tissue to allow full expression of metastatic potential. However, the different behavior of tumors implanted orthotopically by the two methods has not been visualized in real time. *Materials and Methods:* OCUM-2MD3 human gastric cancer cells labeled with the fluorescent protein Azami-Green were implanted orthotopically as cells or tissue in nude mice. *Results:* Orthotopic implantation of cells resulted in local spread on the stomach. In contrast, SOI of tumor tissue of

OCUM-2MD3 resulted in vessel spread of the Azami-Green-expressing cancer cells. Metastasis was also observed in the left lobe of the liver after SOI. *Conclusion:* These results demonstrate the physiological importance of intact cancer tissue for orthotopic implantation in order for tumors to properly grow and express their metastatic potential.

Gastric cancer is the fourth most common cancer in the world, especially frequent in Asia (1). The overall survival of patients with gastric cancer is approximately 20% (2). The frequency of distant metastasis is approximately 35% at diagnosis (3). Recurrence after attempted curative resection ranges from 22-51% (4). Understanding cancer progression by tracking cancer-cell spread and metastatic routes in real time is necessary to improve outcome in patients with gastric cancer (5).

Orthotopic (literally “correct place”) implantation of cancer in nude mice has long been known to be superior to subcutaneous transplantation because the orthotopic tumor can metastasize (6, 7). We previously described the development of surgical orthotopic implantation (SOI) of tumors in immunocompetent mice, such as nude mice (6, 7). We found that SOI of histologically intact human gastric cancer in the sub-serosa of the stomach resulted in extensive orthotopic growth and metastases in 100% of the nude mice. In contrast, we observed metastases in only 6.7% of the mice with local growth resulting from inoculation of cell suspensions into the stomach wall (5). These results are similar to the results with bladder cancer (8) and kidney cancer (9), where SOI of intact tissue resulted in extensive metastases, and orthotopic implantation of a cell suspension did not.

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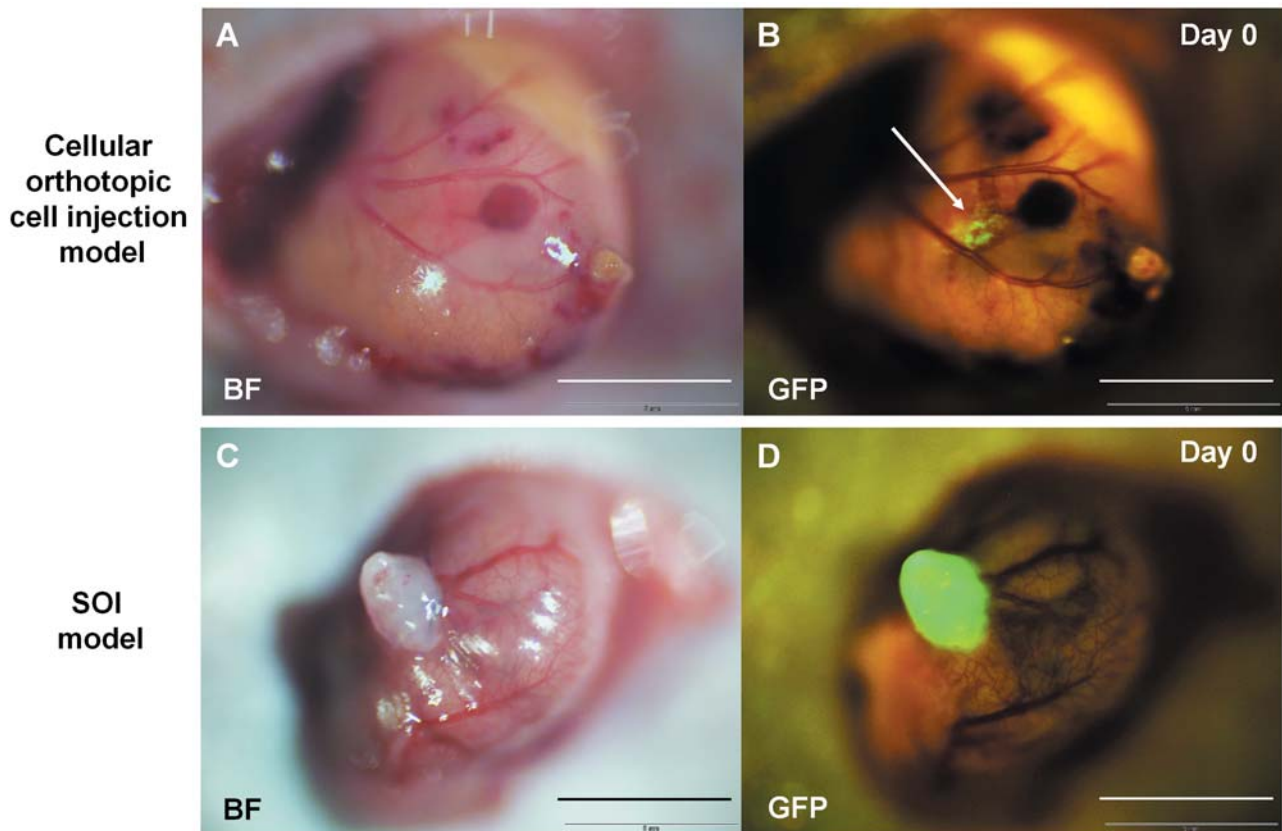


Figure 1. Orthotopic models using the human gastric cancer cell line OCUM-2MD3. To establish an orthotopic cell-injection model, a suspension of OCUM-2MD3–Azami-Green cells (2×10^6 cells/30 μ l RPMI-1640 medium with 50% Matrigel) was injected into the anterior and posterior wall of the stomach antrum of nude mice. A, B: Immediately after injection, Azami-Green-expressing OCUM-2MD3 cancer cells were identified in the stomach wall (arrow). To establish the surgical orthotopic implantation (SOI) model, a cell suspension of OCUM-2MD3 Azami-Green cells (1×10^6 cells/30 μ l cell-cultured medium with 50% Matrigel) was first injected subcutaneously into nude mice. Two weeks later, the resulting subcutaneous tumors were resected and cut into fragments (8 mm³). Each fragment was then implanted to the anterior wall of the antrum of other nude mice using SOI. C, D: Immediately after SOI, the implanted tumor clearly expressed Azami-Green. Fluorescence imaging was with the OV100. Scale bars: 5 mm. BF, Bright field; GFP: Azami-Green, green fluorescent protein.

In the present study, OCUM-2MD3 gastric cancer cells were labeled with the fluorescent protein Azami-Green, enabling us to demonstrate, in real time using intravital imaging, the different behavior of a cancer-cell suspension implanted orthotopically compared with SOI of tumor tissue.

Materials and Methods

Mice. All animal studies were conducted with an AntiCancer Institutional Animal Care and Use Committee-protocol specifically approved for this study and in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873-1. In order to minimize any suffering of the animals, anesthesia and analgesics were used for all surgical experiments. Animals were anesthetized by intramuscular injection of a 0.02 ml solution of 20 mg/kg ketamine, 15.2 mg/kg xylazine, and 0.48 mg/kg acepromazine maleate. The response of animals during surgery

was monitored to ensure adequate depth of anesthesia. The animals were observed on a daily basis and humanely sacrificed by CO₂ inhalation when they met the following humane endpoint criteria: prostration, skin lesions, significant body weight loss, difficulty in breathing, rotational motion and body- temperature drop. The use of animals was necessary to visualize routes of cancer metastasis by real-time imaging. Animals were housed with no more than five per cage in a barrier facility on a high-efficiency particulate arrestance (HEPA)-filtered rack under standard conditions of 12-hour light/dark cycles. The animals were fed an autoclaved laboratory rodent diet.

Cell line. A scirrhous gastric cancer cell line, OCUM-2MD3, was established from a peritoneal-metastatic nodule after orthotopic implantation of OCUM-2M, which was derived from a 49-year-old female (10, 11). OCUM-2MD3 cells were cultivated in a humidified incubator at 37°C in an atmosphere of 5% CO₂ and 95% air, in culture medium comprising Dulbecco's modified Eagle's medium (DMEM) (Bioproducts, Walkersville, MD, USA) with 10% heat-

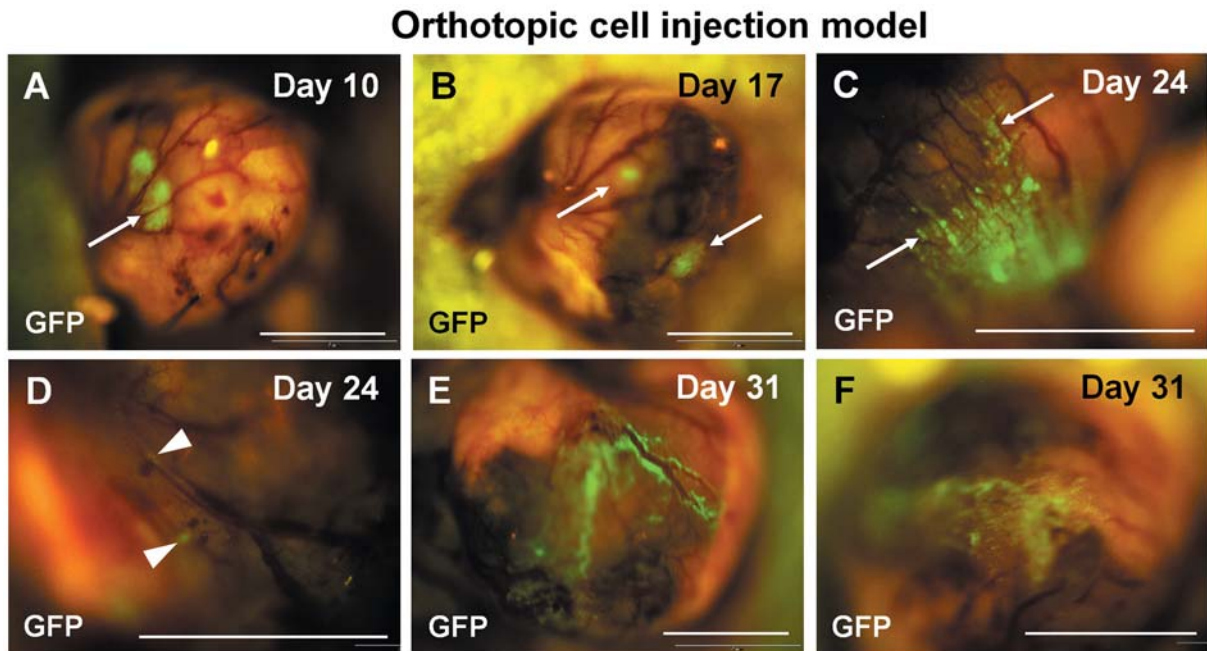


Figure 2. Longitudinal imaging of the migration of cancer cells in the orthotopic cell-injection model using OCUM-2MD3–Azami-Green gastric cancer cells. A, B: By days 10 and 17, Azami-Green-expressing tumor cancer cells still remained at the injection site on the stomach of nude mice (arrows). C, D: By day 24, the stomach cancer cells had migrated along vessels (arrows) and disseminated into the distal stomach wall from the injection site (arrowheads). E, F: By day 31, cancer cells had more broadly spread along vessels. Scale bars: 5 mm. Fluorescence imaging was performed with the OV100 imaging system. GFP: Azami-Green, green fluorescent protein.

inactivated fetal-calf serum (Gibco, Grand Island, NY, USA), 100 U/ml penicillin (ICN Biomedicals, Costa Mesa, CA, USA), 100 g/ml streptomycin (ICN Biomedicals), 2 mM glutamine (Bioproducts) and 0.5 mM sodium pyruvate (Bioproducts) (10, 11).

Azami-Green expression vector production. The OCUM-2MD3 cell line was transformed with the fluorescent protein Azami-Green (Biologics Research Laboratories, Kyowa Hakko Kirin Co., Ltd., Shizuoka, Japan) using standard transfection and cell-selection technology (12–14).

Orthotopic models of the OCUM-2MD3–Azami-Green stomach cancer cell line. Cultured OCUM-2MD3–Azami-Green cells were harvested. To establish the orthotopic cell-injection model, a cell suspension of 2×10^6 cells/30 μ l RPMI-1640 medium (Corning, Corning, NY, USA) with 50% Matrigel was injected into the anterior and posterior wall of the antrum of the stomach of three nude mice. Immediately after injection, Azami-Green-expressing cancer cells were observed in the stomach wall (Figure 1A and B).

To establish the surgical orthotopic implantation (SOI) model, a cell suspension of 1×10^6 cells/100 μ l in RPMI-1640 with 50% Matrigel was first injected subcutaneously in nude mice. Two weeks later, the resulting subcutaneous tumors were resected and cut into fragments (8-mm³). One fragment was implanted onto the wall of the antrum in the stomach of nude mice (Figure 1C and D) using an 8-0 suture.

Imaging. The mice were imaged with an Olympus OV100 variable-magnification imaging system (Olympus, Tokyo, Japan) with a sensitive CCD camera and four objective lenses, parcentered and parfocal,

enabling cellular imaging *in vivo* (15). With the fluorescent cancer cells and the highly sensitive variable magnification imaging system described above, the cellular dynamics of tumor growth and spread was observed intravitaly in live mice in real time in the present study.

Results and Discussion

Longitudinal imaging of migration of cancer cells in the orthotopic cell-injection model. For the orthotopic cell-injection model, the tumor status was visualised weekly from day 10 to day 31 by laparotomy and intravital imaging. On day 10 and 17, Azami-Green-expressing OCUM-2MD3 gastric cancer cells remained at the injection site after orthotopic implantation of a cell suspension (Figure 2A and B). On day 24, the cancer cells migrated along vessels and had disseminated into the distal stomach wall from the injection site (Figure 2C and D). On day 31, cancer cells more broadly spread along vessels within the stomach (Figure 2E and F).

Longitudinal imaging of migration and metastasis in the SOI model. For the SOI model, the tumor status was visualized weekly from day 12 to day 33 by laparotomy and intravital imaging. On days 19 and 26, an Azami-Green-expressing OCUM-2MD3 daughter nodule was visualized near the site of the orthotopically-implanted tumor (Figure 3A and B). On

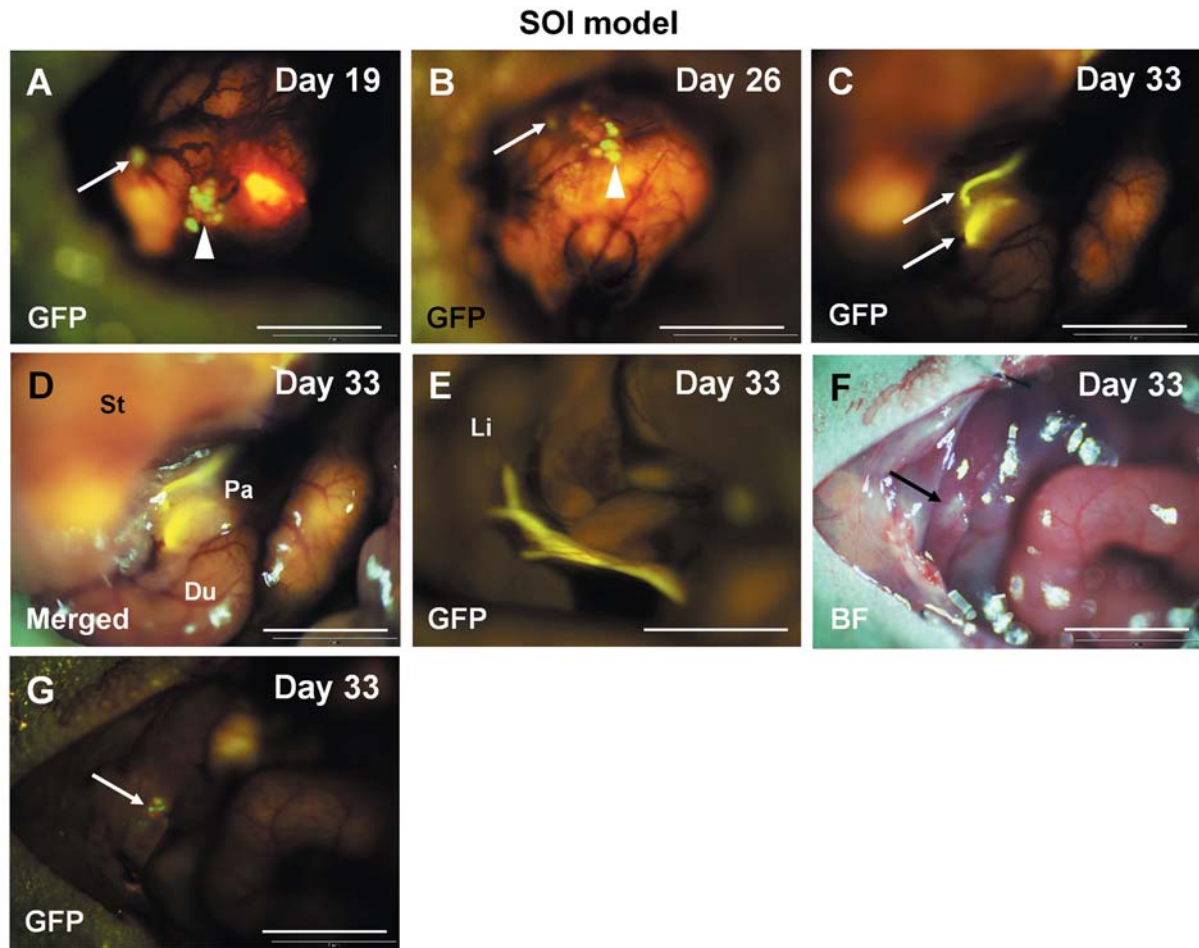


Figure 3. Longitudinal imaging of tumor growth, migration and metastasis in the surgical orthotopic implantation (SOI) model using OCUM-2MD3-Azami-Green stomach-cancer cells. A, B: By days 19 and 26, an Azami-Green-expressing OCUM-2MD3 daughter nodule (arrow) was observed near the implantation site on the stomach of a nude mouse (arrowheads). C: By day 33, branches of the splenic vein filled with Azami-Green-expressing OCUM-2MD3 cancer cells were visualized behind the pancreas (arrows). D: Merged image of (C) and bright field. E: The portal vein was filled with OCUM-2MD3 cancer cells in another mouse. F, G: A single liver metastasis expressing Azami-Green was observed in the left lobe of the liver of one nude mouse (arrows). Scale bars: 5 mm. St, Stomach; Du, duodenum; Pa, pancreas; Li, liver. Imaging was carried out with the OV100 imaging system.

day 33, the portal vein and branches of the splenic vein behind the pancreas were filled with Azami-Green-expressing OCUM-2MD3 cancer cells (Figure 3C-E). A single OCUM-2MD3 liver metastasis expressing Azami-Green was observed in the left lobe of the liver of one mouse (Figure 3F and G).

Local OCUM-2MD3 tumor formation was shown in four out of five of the SOI mice. There were three mice with intrastomach metastasis, two with cell migration to the portal or splenic venous systems, and one mouse with liver metastasis.

Orthotopic mouse models of cancer enable the implanted tumor to metastasize unlike subcutaneous-transplant models (6, 7). However, where suspensions of cancer cells are used for orthotopic implantation, they may not be able to express the full metastatic potential of the original tumor compared

with orthotopic implantation of histologically-intact tissue by SOI (6-8). The SOI approach avoids disruption of tumor integrity by implanting histologically-intact tumor tissue directly (6, 7). With this overall strategy, we have constructed models of human cancer in nude mice that can show the variety of clinical behavior that occurs in human patients (6, 7, 16-43). In a recent study, we demonstrated multiple metastatic routes from the prostate where a tumor fragment of prostate cancer was orthotopically implanted (44).

The imaging technology and patient-like mouse model of gastric cancer demonstrated in the present report will enable further understanding of the critical steps of metastasis and provide visible targets for anti-metastasis therapy of this recalcitrant cancer.

Conclusion

With the use of brightly fluorescent stomach cancer cells and orthotopic mouse models of gastric cancer and a highly sensitive variable-magnification imaging system with both macro-optics and micro-optics, we were able to image the dynamics of cancer-cell invasion and metastasis *in vivo* and further understand the importance of using intact tumor tissue for orthotopic implantation.

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