

Color-coded Imaging of the Fate of Cancer-cell-derived Exosomes During Pancreatic Cancer Metastases in a Nude-mouse Model

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Abstract. *Background/Aim:* Tumor-derived exosomes play important roles in tumor metastases. In this report, we observed the fate of tumor-derived exosomes in pancreatic cancer metastatic nude-mouse models using color-coded imaging. *Materials and Methods:* Mia-PaCa-2 human pancreatic cancer cells expressing red fluorescent protein (RFP) were transduced by exosome-specific pCT-CD63-green fluorescent protein (GFP) and injected in the spleen of nude mice. *Results:* Four weeks after injection of these cells into the spleen, liver metastases developed and tumor-derived exosomes were observed within the metastatic cancer cells and in Kupffer cells. Furthermore, tumor-derived exosomes diffused to bone marrow and lung cells, especially macrophages, without any metastases present. *Conclusion:* In the present study, we visualized the distribution of cancer-derived exosomes for the first time at the cellular level, in a pancreatic-cancer metastatic model.

Exosomes are small membrane vesicles of approximate 30-150 nm in diameter (1). Tetraspanins, such as CD9, CD63, and CD81, are enriched in the membrane of exosomes and serve as exosomal markers (2). Exosomes contain bioactive molecules including DNA, mRNA, microRNA, and proteins

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(3-5). These components have a role in cell-to-cell communication. Exosomes have an important function in metastasis (6-8). Exosomes derived from tumors and also cancer-associated fibroblasts (CAFs) enhance metastasis. They may also affect angiogenesis, epithelial to mesenchymal transition (EMT), and immune modulation (9-12). We previously reported that breast-cancer-derived exosomes were incorporated into tumor-associated cells and contributed to developing a metastatic niche visualized by using color-coded imaging (13). Macrophages also have an important role in metastasis. For instance, tumor-associated macrophages (TAMs) have been shown to promote tumor growth in the tumor microenvironment (TME). Tumor-derived exosomes have been shown to induce polarization of macrophages, which enhanced tumor metastasis (14-18).

In the present report, we visualized the distribution of tumor-derived exosomes in a pancreatic cancer metastatic mouse model, and observed that tumor-derived exosomes were present in Kupffer cells in the liver, bone marrow and lung without any metastases.

Materials and Methods

Cell line and culture conditions. Mia-PaCa-2 human pancreatic adenocarcinoma cells, were engineered to stably express red fluorescent protein (RFP) as previously reported (19). The cells were maintained in RPMI 1640 medium (Gibco-BRL, Grand island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin and streptomycin (Gibco-BRL). The cells were cultured in a humidified atmosphere containing 5% CO₂ at 37°C.

Lentiviral exosome labeling. Mia-PaCa-2 cells were transduced with lentiviral vector, pCT-CD63-green fluorescent protein (GFP) (System Biosciences, CA, USA), which contains the tetraspanin CD63 gene fused to GFP for tracking exosomes (20).

Mice. All experiments were conducted in accordance with the institutional guidelines of Gifu University, Gifu, Japan, and

approved by the animal research committee and the committee on living modified organisms of Gifu University (approval number 26-37). In order to minimize any suffering of the animals, anesthesia and analgesics were used for all surgical experiments. Animals were anesthetized by subcutaneous injection of a 0.02 ml solution of 20 mg/kg ketamine. The response of animals during surgery was monitored to ensure adequate depth of anesthesia. Animals were housed in a barrier facility on a high-efficiency particulate arrestance (HEPA)-filtered rack under standard conditions of 12-h light/dark cycles. Mice were fed with an autoclaved laboratory rodent diet.

Pancreatic adenocarcinoma metastasis model. Balb-c/nu-nu mice (8 weeks old) were used as hosts. Mia-PaCa-2 cells, expressing RFP and transduced with pCT-CD63-GFP, were harvested by trypsinization, washed three times with cold serum-free medium, and then resuspended in serum-free RPMI 1640 medium. Mia-PaCa-2 cells (2.0×10^6) were injected into the spleen of mice. Four weeks later, all the mice were sacrificed and had primary tumors as well as liver metastases. Liver metastases as well as lung and bone marrow were cultured and observed for tumor-derived exosomes by fluorescence imaging.

Tumor imaging. The SZX microscope and FV1000 confocal microscope (Olympus Corp. Tokyo, Japan) were used for imaging.

Results and Discussion

In order to track the distribution of tumor-derived exosomes, pCT-CD63-GFP (System Biosciences), which contains the tetraspanin CD63 gene fused to GFP was used to transduce Mia-PaCa-2 human pancreatic adenocarcinoma cells expressing RFP. Tumor-derived exosomes were observed with a confocal microscope using color-coded imaging (Figure 1).

Mia-PaCa-2 human pancreatic cancer cells expressing exosome-specific pCT-CD63-GFP were injected into the spleen of Balb-c/nu-nu mice. Four weeks later, all mice were sacrificed. Primary tumor in the spleen (Figure 2A) and multiple metastases in the liver were imaged (Figure 2B). Metastases were not observed in the lung (Figure 2C). Mia-PaCa-2 cells in the metastases contained GFP-expressing exosomes (Figure 3A).

Liver metastases as well as lung and bone marrow were cultured to observe tumor-derived exosomes. Interestingly, although there were no cancer cells expressing RFP present, tumor-derived exosomes were observed in bone marrow cells, especially in macrophages (Figure 4B). Kupffer cells derived from the liver contained tumor-derived exosomes (Figure 3B). In addition, tumor-derived exosomes were occasionally observed in lung macrophages without any metastases (Figure 4A).

Tumor-derived exosomes develop a pre-metastatic niche and can determine metastasis (21). Tumor-derived exosomes also have immune suppressive effects and promote metastasis (22, 23). On the other hand, tumor-derived exosomes from poorly metastatic cells may induce

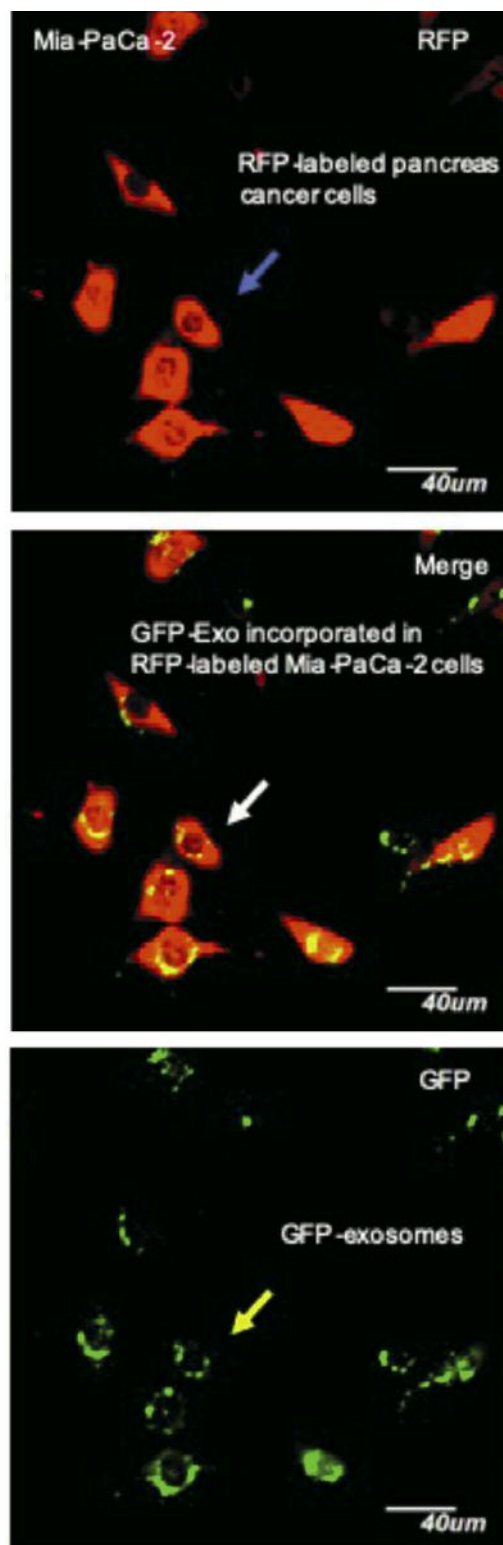


Figure 1. RFP expressing Mia-PaCa-2 cells were transduced with pCT-CD63-GFP for tracking exosomes. Blue arrow indicates RFP-labeled pancreatic cancer cells. Yellow arrow indicates GFP-exosomes. White arrow indicates GFP-exosome incorporated in RFP-labeled Mia-PaCa-2 cells. (Bar=40 μ m).

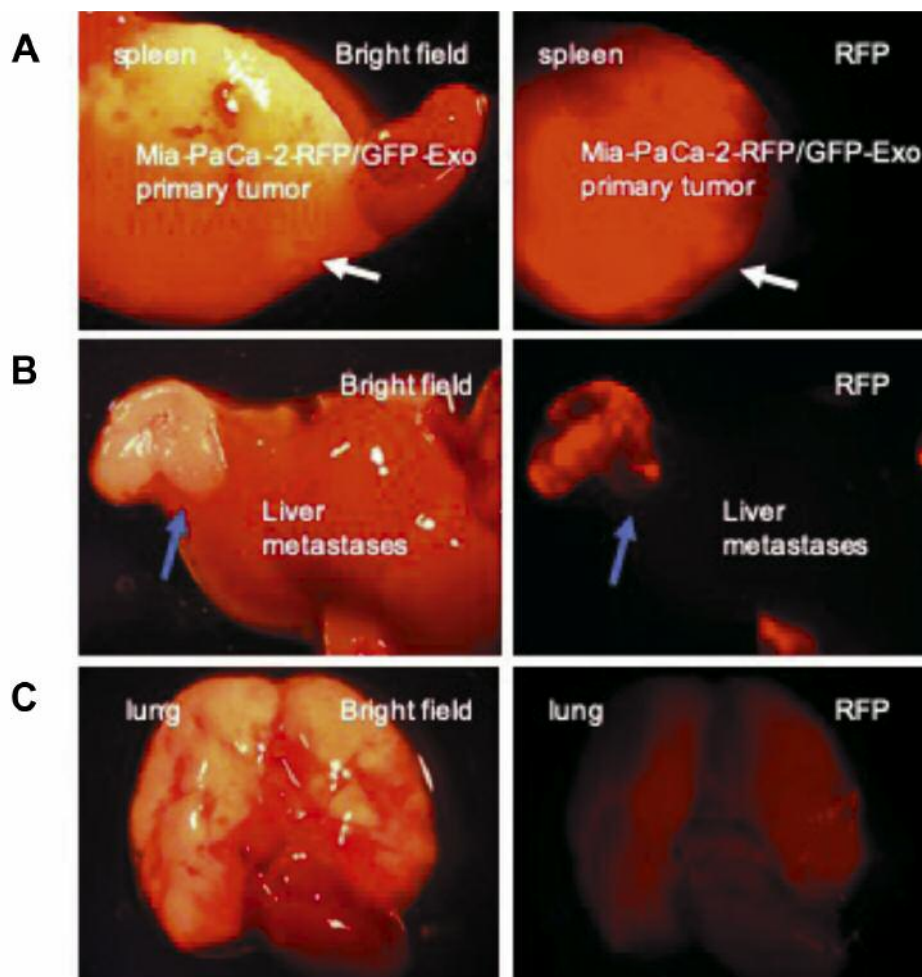


Figure 2. Pancreatic cancer experimental metastases model established with red fluorescent protein (RFP) expressing Mia-PaCa-2 cells with green fluorescent protein (GFP) expressing exosomes. Primary tumor in spleen (white arrow) and metastatic tumors in liver (blue arrows). No metastases in lung.

immune surveillance in the pre-metastatic niche (24). These tumor promoting or suppressing mechanisms of exosomes may be related to bone marrow derived cells, including macrophages. Macrophages are polarized between M1 and M2 phenotypes. M2 macrophages have tumor-promoting effects, while M1 macrophages have tumor-suppressive effects (15, 17). In addition, myeloid-derived suppressor cells (MDSC) suppress anti-tumor immunity and promote tumor progression and metastases (25, 26). These immune-suppressive functions are related to tumor-derived exosomes. The present report is the first to demonstrate tumor-derived exosomes in both metastatic and non-metastatic organs *in vivo* in a tumor-bearing animal.

Further experiments are needed to determine how tumor-derived exosomes promote or suppress metastases, and characterize how macrophages are educated by tumor-derived exosomes.

Conflicts of Interest

None of the Authors have any conflicts of interest with regard to this study.

Authors' Contributions

T.S., A.S., and R.M.H. conceived and planned the experiments. T.S., M.N., and A.S. carried out the experiments. T.S., M.N., A.S., and R.M.H. contributed to the interpretation of the results. All Authors provided critical feedback and helped shape the research and manuscript.

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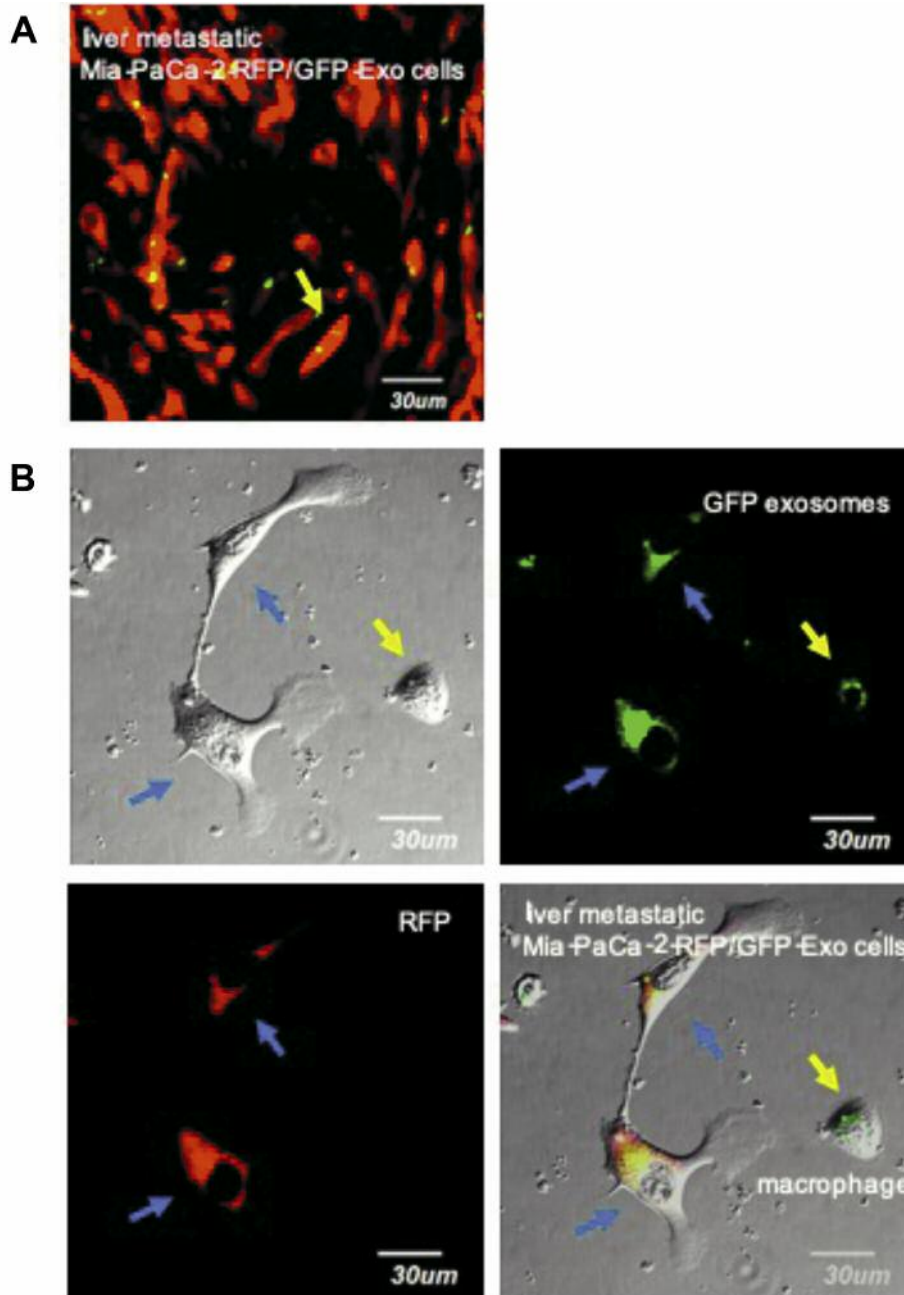


Figure 3. *Mia-PaCa-2* cells in liver metastasis contained GFP-expressing exosomes. (A) RFP-labeled *Mia-PaCa-2* cells producing GFP-labelled exosomes in liver metastases (yellow arrows). GFP expressing exosomes are present in metastasis (Bar=30 μm). (B) Blue arrows indicate GFP-exosomes incorporated in RFP-labeled *Mia-PaCa-2* cells. Yellow arrows indicate macrophages in liver containing tumor-derived exosomes (Bar=30 μm).

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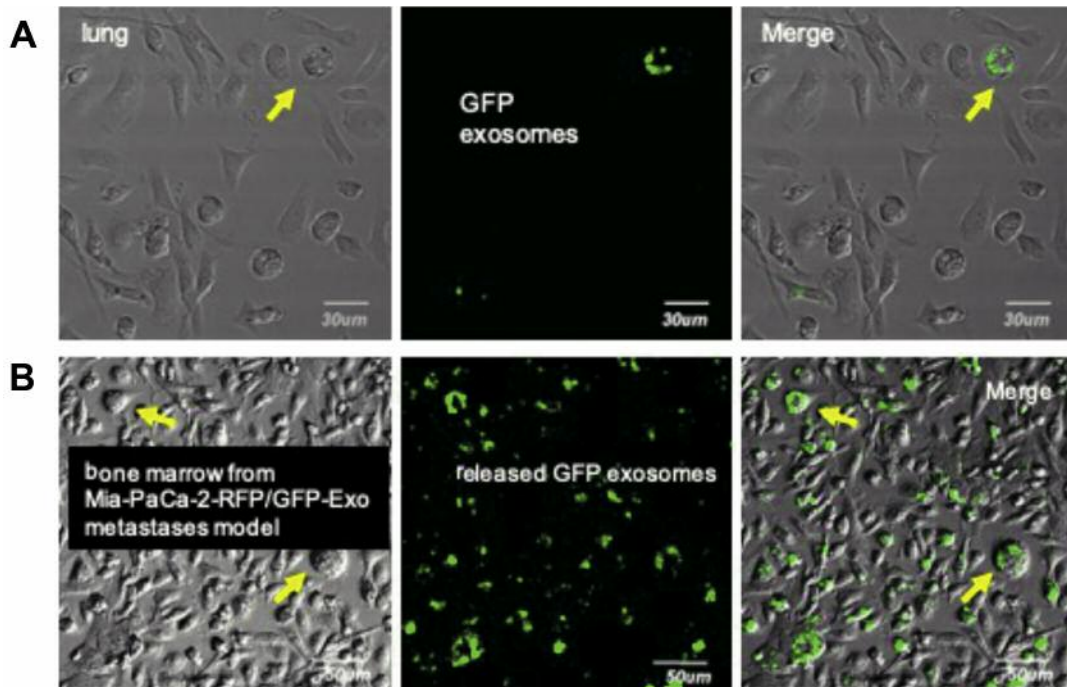


Figure 4. Distribution of tumor-derived exosomes. Yellow arrows indicate macrophages in lung (A) (Bar=30 μ m) and bone marrow (B) (Bar=50 μ m) containing tumor-derived exosomes.

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