Video Article

High-sensitivity Detection of Micrometastases Generated by GFP Lentivirus-transduced Organoids Cultured from a Patient-derived Colon Tumor

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URL: https://www.jove.com/video/57374
DOI: doi:10.3791/57374

Keywords: Cancer Research, Issue 136, Human CRC, patient-derived tumor xenograft (PDXs), GFP-labelled PDX-derived tumor organoids, lentiviral transduction, micrometastasis, 3D culture

Date Published: 6/14/2018

Citation: Okazawa, Y., Mizukoshi, K., Koyama, Y., Okubo, S., Komiyama, H., Kojima, Y., Goto, M., Habu, S., Hino, O., Sakamoto, K., Orimo, A. High-sensitivity Detection of Micrometastases Generated by GFP Lentivirus-transduced Organoids Cultured from a Patient-derived Colon Tumor. *J. Vis. Exp.* (136), e57374, doi:10.3791/57374 (2018).

Abstract

Despite current advances in human colorectal cancer (CRC) treatment, few radical therapies are effective for the late stages of CRC. To overcome this clinical challenge, tumor xenograft mouse models using long-established human carcinoma cell lines and many transgenic mouse models with tumors have been developed as preclinical models. They partially mimic the features of human carcinomas, but often fail to recapitulate the key aspects of human malignancies including invasion and metastasis. Thus, alternative models that better represent the malignant progression in human CRC have long been awaited.

We herein show generation of patient-derived tumor xenografts (PDXs) by subcutaneous implantation of small CRC fragments surgically dissected from a patient. The colon PDXs develop and histopathologically resemble the CRC in the patient. However, few spontaneous micrometastases are detectable in conventional cross-sections of affected distant organs in the PDX model. To facilitate the detection of metastatic dissemination into distant organs, we extracted the tumor organoid cells from the colon PDXs in culture and infected them with GFP lentivirus prior to injection into highly immunodeficient NOD/Shi-scid IL2Rγ^{null} (NOG) mice. Orthotopically injected PDX-derived CRC organoid cells consistently form primary tumors positive for GFP in recipient mice. Moreover, spontaneously developing micrometastatic colonies expressing GFP are notably detected in the lungs of these mice by fluorescence microscopy. Moreover, intrasplenic injection of CRC organoids frequently produces hepatic colonization. Taken together, these findings indicate GFP-labelled PDX-derived CRC organoid cells to be visually detectable during a multistep process termed the invasion-metastasis cascade. The described protocols include the establishment of PDXs of human CRC and 3D culture of the corresponding CRC organoid cells transduced by GFP lentiviral particles.

Video Link

The video component of this article can be found at https://www.jove.com/video/57374/

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer deaths worldwide[1]. The insufficient response to conventional therapies of patients with advanced stage disease indicates the ineffectiveness of attempts to radically cure CRCs. To develop more effective therapeutic approaches, various preclinical mouse models of cancer that mimic the characteristics of CRCs have been established. Various CRC cell lines have been widely used to generate tumor xenografts due to their convenience and ease of manipulation. Long-term culture of cancer cell lines, however, often causes selection of unique cell populations that are quite proliferative under a particular culture condition, thereby resulting in unreliable outcomes and crucial limitations in preclinical drug development.

Without being cultured *in vitro*, patient-derived tumor xenografts (PDXs) have also been generated by implantation into animal models of human CRC tissues surgically dissected from patients[2,3,4]. PDXs are widely recognized as recapitulating the major histopathological features and genetic alterations originally present in tumors of the patients from which they were derived. Moreover, patient-derived tumor organoids composed of tumor cell clusters were established by culture under 3D conditions that closely mimicked the biological properties of the original tumors[5,6]. These tumor organoids were also applied to high-throughput drug screening, thereby allowing personalized therapies to be designed[5]. However, markedly heterogeneous CRC populations are assumed to be present within a tumor mass. Particular CRC populations might selectively proliferate and expand during the *in vivo* and *in vitro* series of passages of PDX and tumor organoids, respectively. This may also

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allow the overall gene expression profiles and epi/genetic status of the affected CRCs to change, thereby resulting in minimal resemblance to the parental CRC.

Patient-derived CRC organoids and those extracted from noncancerous human colon engineered to harbor combinations of oncogenic mutations have also been employed to investigate the hallmarks of human tumor cells exemplified by tumor invasion and metastasis[6,7,8,9]. However, the very low incidence of spontaneous metastasis arising from orthotopic implantation of patient-derived CRC into immunodeficient mice, has made it difficult to study the multistep process of the invasion-metastasis cascade that includes local invasion, intravasation, transport in the bloodstream, extravasation and colonization of distant organs[4,10]. Micrometastasis as represented by tumor cell deposition of ≤2 mm, formed by patient-derived CRC organoids, has often been overlooked on histopathological analysis of sections from affected distant organs in experimental mouse models. Visualization of spontaneous micrometastases has also been minimal *in vivo* due to the difficulty of efficiently introducing fluorescent markers into tumor organoid cells prior to their injection into recipient mice. In this study, we developed a protocol to efficiently transduce GFP lentivirus into PDX-derived CRC organoid cells in 3D culture prior to injection into recipient mice and to allow highly sensitive detection, employing stereo-fluorescence microscopy, of their colonization of different organs to form micrometastases.

Protocol

The patient provided written informed consent and the project was approved by the Research Ethics Committee of the Juntendo University Faculty of Medicine. The mouse experiments were also approved by the Animal Research Ethics Committee of the Juntendo Faculty of Medicine.

1. Establishment of CRC PDXs in Immunodeficient Mice

Experimental procedures for establishing CRC PDXs (step 1) are outlined in **Figure 1A**.

1. Prepare the primary CRC tissue immediately after surgical resection of the tumor from the patient.
2. Wash the primary CRC tissue by gentle agitation several times in 30 mL of ice-cold sterile phosphate buffered saline (PBS) in a 50 mL conical tube and keep it on ice.
3. Place the tissue on a 10 cm Petri dish using sterile tweezers, remove the necrotic areas as extensively as possible using sterile scissors and cut the solid tumor tissues to small pieces (5 x 5 x 5 mm^3 in size) using sterile razor blades.
   NOTE: Necrotic areas are often whiter, softer and more friable than the surrounding areas.
4. Place the small pieces of tumor using sterile tweezers in 2 mL of ice-cold sterile PBS on a 6-well plate.
5. Breed 6 week-old NOD/Shi-scid IL2Rγ null (NOG) highly immunodeficient mice under germ-free and specific pathogen-free conditions.
6. Anesthetize the mice with isoflurane inhalation using the small animal inhalation anesthesia device and disinfect the skin with 70% ethanol. Assure the normal rate and depth of respiration and the absence of a toe pinch reflex for proper anesthetization. Vet ointment on the eyes is an option, to prevent ocular dryness while under anesthesia.
7. Sterilize all surgical tools using dry heat sterilization and then use these tools for all procedures to prevent wound infections in recipient mice.
8. Place the anesthetized mouse in a prone position on the laboratory bench. Make a small incision on the lower parts of the right and left flanks of the recipient mouse to generate subcutaneous pockets for tissue implantation using sterile scissors. Take care not to puncture the peritoneal membrane. There should be little bleeding with this procedure.
9. Gently dip the above prepared tumor fragments into 50 µL of artificial extracellular matrix and then implant them into the lower parts of the right and left subcutaneous pockets.
10. Suture the incision with wound clips in the mice undergoing surgical procedures. Make sure that the implanted tumor tissues are located at some distance from the incision under the skin.
    NOTE: In accordance with minimizing surgical procedures, no treatments for post-surgical pain and infection were administered in this study.
11. Place the mouse on a warm pad and maintain the sternal recumbent position until sufficient consciousness is restored. Once fully recovered and able to sit up on all four legs, the mice can be returned to their home cages. To prevent predation, do not allow breeding with non-operated animals in the same cage.
12. Check for suture leakage and confirm that the mice are in stable condition the next day, in the surgically treated group. Check the mice for signs of illness and measure the tumor size in each mouse, once a week, employing calipers.

2. Dissection of CRC PDXs from Mice

Experimental procedures for dissection of CRC PDXs (step 2) are outlined in **Figure 1B**.

1. Allow the tumor to grow subcutaneously to ~1 cm^3 in size, which usually takes approximately 1–3 months.
2. Anesthetize the mouse with isoflurane inhalation using the small animal inhalation anesthesia device and disinfect the skin with 70% ethanol.
3. Place the mouse in a prone position on the laboratory bench. Incise the skin, expose the tumor and carefully detach the skin from the tumor using sterile scissors. After removing the tumor, place it on a 10 cm Petri dish and remove any grossly necrotic regions from the tumor, then rinse twice with 5 mL of PBS.
   NOTE: Necrotic areas are often whiter, softer and more friable than the surrounding areas.
4. **Place the tumor on ice and divide it equally into at least 4 parts for different applications including 1) the CRC organoid culture, 2) transplantation into secondary mice, 3) histological examination and 4) freezing and storage of the tumor fragments.**
   1. For generation of the CRC organoids, place the solid tumor tissue on a 6 cm petri dish containing 1 mL of sterile PBS. Keep the tissue on ice until processing (step 3).
   2. To establish the PDX model for human CRC, passage the fresh CRC PDXs into a secondary mouse. Cut the tumor tissue into small pieces (5 x 5 x 5 mm^3 in size) using sterile razor blades and gently dip these tissue fragments into 50 µL of artificial extracellular matrix. Then, implant these fragments into the lower parts of the right and left subcutaneous pockets of the NOG mouse, as described in step 1.8–1.11.

3. For histological analysis, fix the samples in 5 mL of 10% neutral buffered formalin in a 15 mL conical tube at room temperature for 2 days.
4. For cryopreservation, cut the tumor tissue into small pieces (5 x 5 x 5 mm^3 in size) and gently dip the samples into 500 µL of cell freezing solution in 1–1.5 mL cryovial tubes. Then, transfer the tube to a -80 °C freezer.

3. Extraction of PDXs into the Cell Suspension for the CRC Organoid Culture

Experimental procedures for dissociation of CRC PDXs (step 3) are outlined in **Figure 1B**.

1. Place the above (step 2.4.1) prepared tumor fragments on a 10 cm Petri dish.
2. Mince the fragments using sterile razor blades and transfer them into a 15 mL conical tube containing 8 mL of 10% fetal calf serum (FCS)-Dulbecco's Modified Eagle's Medium (DMEM) with 80 µL of collagenase enzyme stock (see **Table of Materials**).
3. Close the tube tightly and wrap the cap with parafilm to prevent leakage. To dissociate the minced tumor fragments into the cell suspension, agitate it gently for 2 h at 37 °C. Note that there will still be many fragments of undigested tissue remaining in the tube.
4. Centrifuge the tube at 300 x *g* for 5 min at 4 °C and remove the supernatant. Resolve the cell pellet using 10 mL of 10% FCS-DMEM to make the cell suspension.
5. To eliminate undigested tissue debris, filter the cell suspension twice using the 40 µm cell strainer set on a 50 mL conical tube. Then, centrifuge the flow-through at 300 x *g* for 5 min at 4 °C. Remove the supernatant and keep the pellet on ice.

4. Generation of the CRC Organoids Cultured on Artificial Extracellular Matrix

Experimental procedures for the CRC organoid culture of the colon PDXs (step 4) are outlined in **Figure 1B**.

1. Establish a culture medium suitable for the PDX-derived CRC organoids (see **Table of Materials**, “the CRC organoid culture medium”) employing media previously described for human colon organoids[11].
2. Apply 150 µL of artificial extracellular matrix (see **Table of Materials**) per well on a 12-well plate on ice and then incubate it for 30-60 min in a 37 °C, 5% CO_2 incubator to solidify the gels.
3. Suspend the cell pellet from step 3.5 with the CRC organoid culture medium (from step 4.1) with 5% FCS to achieve adjustment to 3 x 10^5 cells/mL. Then, seed 1 mL of the medium onto the artificial extracellular matrix-coated plate prepared in step 4.2. and incubate overnight at 37 °C under 5% CO_2. Use a hemocytometer for counting the number of tumor cells including single cells and multicellular clusters (a group of adherent cells) in the cell suspension.
   NOTE: 5% FCS is used to quench the residual enzymatic activity of collagenase in this study.
4. Carefully collect the culture medium, including floating cells that have detached from the artificial extracellular matrix-coated plate, into a 1.5 mL centrifuge tube on the following day and centrifuge it at 1,400 x *g* for 5 min. Then, remove the supernatant and re-suspend the pellet in 70 µL of artificial extracellular matrix on ice.
5. To increase CRC cell viability, overlay the tumor cell-containing artificial extracellular matrix onto the tumor organoid cells attached to the artificial extracellular matrix-coated plate and incubate for 30 min in a 37 °C, 5% CO_2 incubator to solidify the artificial extracellular matrix coating. Then, incubate it with 1 mL of the CRC organoid cell culture medium with 1% FCS at 37 °C under 5% CO_2.
6. Change the culture medium every second day. As the CRC organoids fill the medium, it may become necessary to change the medium daily.

5. Generation and Enrichment of GFP Lentiviral Particles

Experimental procedures for generation and enrichment of GFP lentiviral particles (step 5) are outlined in **Figure 1C**.

1. Culture HEK293T cells with Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FCS and prepare six 10 cm dishes (2 x 10^6 cells per dish) for the following transfection procedure.
2. For transfection per dish, prepare 500 µL of the mixture including 20 µL of the transfection reagent in DMEM with a PRRL-GFP vector (5 µg)[12] and two lentiviral packaging plasmids, such as pCMV-VSV-G (1 µg) and pCMV-dR8.2 dvpr (5 µg), in a sterile 1.5 mL microtube. Keep the mixture at room temperature for 20 min and then overlay it onto each of the 10-cm dishes and incubate them for 18 h.
3. Remove the medium, add 5 mL of fresh 10% FCS-RPMI 1640 medium to each dish, and leave standing for 24 h.
4. Collect the conditioned medium from each dish into a 50 mL centrifuge tube and store a total of 30 mL of the medium at 4 °C overnight. Add 5 mL of the fresh RPMI 1640 onto each dish and leave standing for 24 h.
5. Collect the conditioned medium from each dish into a 50 mL centrifuge tube and keep, in total, 30 mL of the medium on ice. Then, discard the cells.
6. Filter 60 mL of the medium (from step 5.4–5.5) through a 0.45 µm filter to remove the cells and divide this quantity into twelve 5 mL polypropylene centrifuge tubes for ultracentrifugation.
7. To concentrate virus, centrifuge them using a swinging bucket rotor (see **Table of Materials**) at 85,327 x *g* for 1.5 h at 4 °C.
8. Immediately remove the medium. To resolve the concentrated virus pellet, place 250 µL of Nutrient Ham's Mixture F-12 (F12)/DMEM medium without FCS in each of the twelve tubes and maintain it at 4 °C overnight. Note that the virus pellet is often invisible.
9. Gently pipette the medium to collect 3 mL of the concentrated 5x GFP lentivirus stock, in total, presumably including GFP lentiviral particles with 10^8 transducing units per mL (TU/mL). Then, store the twelve 1.5 mL microtubes (including 250 µL per tube) under sterile conditions at -80 °C for up to a year. Prepare many small aliquots of the original solution to avoid multiple freeze-thaw cycles.

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6. Labelling of CRC Organoid Cells with GFP Lentiviral Particles Cultured on Artificial Extracellular Matrix

Experimental procedures for labelling the CRC organoid cells with GFP lentivirus (step 6) are outlined in **Figure 1C**.

1. Allow the CRC organoids prepared in step 4.6 to grow without interference for 7–10 days and harvest them mechanically using a sterile cell scraper and then transfer them into a 1.5 mL microtube.
   NOTE: The growth of CRC organoids in culture depends on the nature of the original tumors from patients. Avoid overgrowth of the CRC organoids, by maintaining them at 60–70% confluence prior to transfer at a 1:2 split ratio onto a new artificial extracellular matrix-coated 12-well plate.
2. Centrifuge the microtube for 3 min at 1,400 x *g*, remove the culture medium and resolve the cell pellet in 500 µL of PBS by gentle tapping.
3. Centrifuge the microtube for 3 min at 1,400 x *g* and eliminate PBS.
4. To dissociate the adherent CRC organoids, add 500 µL of a cell detachment solution of proteolytic and collagenolytic enzymes to the cell pellet in the tube and mix it by gentle tapping. Then, leave the tubes to settle for 10 min at room temperature.
5. Very gently pipette the cell suspension with an additional 500 µL of 1% FCS-DMEM several times in a 1.5 mL microtube to roughly dissociate the cells. Generation of single cells from the CRC organoids by harsh pipetting markedly reduces cell viability. Thus, it is essential to leave the mass of cells visually detectable in the cell suspension by very gently pipetting in a 1.5 mL microtube.
6. Centrifuge the cell suspension for 3 min at 1,400 x *g* and remove the supernatant.
7. Resolve the cell pellet with a mixture of the 100 µL of 5x GFP lentivirus stock (>10^8 TU/mL) and 400 µL of the CRC organoid culture medium in a 1.5 mL microtube by gentle tapping to adjust to a concentration of 5 x 10^5 dissociated tumor cells per 500 µL. Use a hemocytometer for counting the number of tumor cells, including single cells and groups of cells in the cell suspension, as described above (step 4.3).
8. Prepare an artificial extracellular matrix-coated 12-well plate, as described in step 4.2. Then, place 500 µL of the cell suspension prepared in step 6.7 on the plate and leave it for 18 h at 37 °C under 5% CO_2.
9. Collect the medium with the floating cells detached from the artificial extracellular matrix-coated plate into a 1.5 mL centrifugation tube. Centrifuge it at 1,400 x *g* and then remove the supernatant and re-suspend the pellet in 70 µL of artificial extracellular matrix on ice.
10. To increase CRC cell viability, overlay the tumor cell-containing artificial extracellular matrix onto the tumor organoids attached to the artificial extracellular matrix-coated 12-well plate, as described in step 4.5. Next, incubate the plate for 30 min in a 37 °C, 5% CO_2 incubator to solidify the artificial extracellular matrix coating and then culture it with 1 mL of the CRC organoid culture medium with 1% FCS at 37 °C under 5% CO_2.
11. Observe the cells at 3 days after infection under a fluorescence microscope to confirm nearly 100% GFP positivity due to the use of a high titer of lentiviral particles (see **Figure 2A**).
12. Culture the CRC organoids for periods of 7–10 days to expand cell growth prior to injection into recipient mice.

7. Generation of Metastases by GFP-labeled CRC Organoids in Recipient Mice

Experimental procedures for generation of metastases using the GFP-labelled CRC organoids (step 7) are outlined in **Figure 1C**.

1. To dissociate the GFP-labeled CRC organoids into the cell suspension, harvest them mechanically using a sterile cell scraper and transfer them into a 1.5 mL microtube, as described above (step 6.1).
2. Centrifuge the microtube for 3 min at 1,400 x *g*, remove the culture medium, and resolve the cell pellet in 500 µL of PBS by gentle tapping.
3. Centrifuge the microtube for 3 min at 1,400 x *g* and eliminate PBS.
4. Overlay 500 µL of the cell detachment solution onto the cell pellet in the tube and mix it by gentle tapping. Then, leave the mixture standing for 10 min at room temperature to enzymatically dissociate the adherent CRC organoids, as described above (step 6.4).
5. Very gently pipette the cell suspension with an additional 500 µL of 1% FCS-DMEM several times in a 1.5 mL microtube to roughly dissociate the cells, as described above (step 6.5). Generation of single cells from the CRC organoids by harsh pipetting markedly reduces cell viability. Thus, leave the mass of cells visually detectable in the cell suspension by very gently pipetting in a 1.5 mL microtube.
6. Centrifuge the cell suspension for 3 min at 1,400 x g and remove the supernatant.
7. **To establish a spontaneous metastasis mouse model of CRC PDXs:**
   1. Prepare a cell suspension including 5 x 10^5 cells in 50 µL of PBS with 50% artificial extracellular matrix per mouse. Maintain the suspension on ice before use. Use a hemocytometer for counting the cancer cells, as indicated in step 4.3.
   2. Anesthetize a mouse with isoflurane inhalation using the small animal inhalation anesthesia device and disinfect the skin with 70% ethanol, as indicated in step 1.6.
   3. Place the NOG mouse in a supine position on the laboratory bench. Grasp the rectal mucosa with sterile tweezers and gently pull it out of the anus. Then, immediately inject the cell suspension prepared in step 7.7.1 into the rectal submucosa of the mouse using a syringe (needle size: 22 G). Routinely, 4–6 mice per group are used to evaluate the results.

8. **To establish an experimental metastasis model of CRC PDXs:**
   1. Prepare a CRC organoid cell suspension including 4 x 10^4 cells in 50 µL of PBS per mouse. Maintain the suspension on ice before use. Use a hemocytometer for counting the cancer cells (step 4.3).
   2. Anesthetize a mouse with isoflurane inhalation using the small animal inhalation anesthesia device and disinfect the skin with 70% ethanol, as indicated in step 1.6.
   3. Place the anesthetized NOG mouse in a prone position on the laboratory bench. Make a small incision on the lower part of the left flank and then cut the peritoneum to carefully expose the spleen using sterile scissors and tweezers. Grasp the fat tissue attached to the spleen with sterile tweezers, then slowly inject 50 µL of the cell suspension (prepared as above, step 8.1) into the spleen. Be certain that the cell suspension is injected appropriately without outward leakage, from the spleen. Routinely, 4 mice per group are used to evaluate the results.

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9. Place all mice on a warm pad after surgery and maintain in a sternal recumbent position until sufficient consciousness is restored, as described in step 1.11. Once the mice have fully recovered, return them to their home cages. To prevent predation, do not allow breeding with non-operated animals in the same cage.
10. Check for suture leakage and confirm that the mice are alive, in the surgically treated group, the next day. Check the mice for signs of illness and measure tumor sizes, employing calipers, in all mice once a week.
11. Observe mice to detect spontaneous metastasis (for periods of up to 2.5 months) and experimental metastasis (1 month) after injection.
12. Sacrifice the mice via anesthesia and cervical dislocation on the indicated days. Dissect primary tumors and various tissues including the liver and lungs. Place the dissected tumors and organs in 5 mL of ice-cold PBS on a 10 cm Petri dish and store them on ice.
13. To detect GFP-positive CRC organoid cells, observe the dissected tissues under a stereo-fluorescence microscope. Acquire images with a CCD camera and prepare using standard image-processing software.

Representative Results

A primary colorectal adenocarcinoma diagnosed as moderately differentiated had been surgically resected from a 76 year-old female with TNM classification, stage IIIa, followed by post-operative chemotherapy. The primary CRC cells immunohistochemically stained positive for carcinoembryonic antigen, Ki-67, pan-cytokeratin and E-cadherin. Pieces of the resected tumor were also subcutaneously implanted into NOG mice to generate the colon PDX model. CRC organoid cells were then extracted for tissue culture from the colon PDX which had developed subcutaneously in these mice. The CRC organoids were capable of consistently forming multicellular clusters during a series of passages on artificial extracellular matrix. As tumor organoids histologically and genetically reflect the nature of primary tumors from patients[5,6], we attempted to develop a mouse model that would allow *in vivo* detection of CRC organoid cells with high sensitivity during metastatic dissemination. To achieve this goal, CRC organoids were infected with GFP lentivirus, thereby resulting in nearly all organoids showing very bright GFP (**Figure 2A**). These organoids were then injected orthotopically and intrasplenically into secondary NOG mice prior to detection of GFP-positive cells in distant organs under a fluorescence microscope. We observed formation of GFP-positive primary tumors at the orthotopic site in all four mice examined (**Figure 2B**, left). Moreover, microscopic metastases were found in the lungs of three out of four mice examined at 2.5 months after orthotopic injections (**Figure 2B,** middle). Furthermore, liver metastases were observed in response to intrasplenic injection into all four mice examined at 1 month after the injections (**Figure 2B**, right).

Taken together, these findings indicate that high-resolution GFP fluorescence on PDX-derived CRC organoid cells allows their detection in both spontaneous micrometastases and those developed experimentally, in distant organs, when introduced into recipient mice. This high-sensitivity detection of the disseminating PDX-derived CRC organoids *in vivo* also offers a versatile model not only for studying the biology of tumor metastasis, but also for developing targeted therapies in the preclinical setting.

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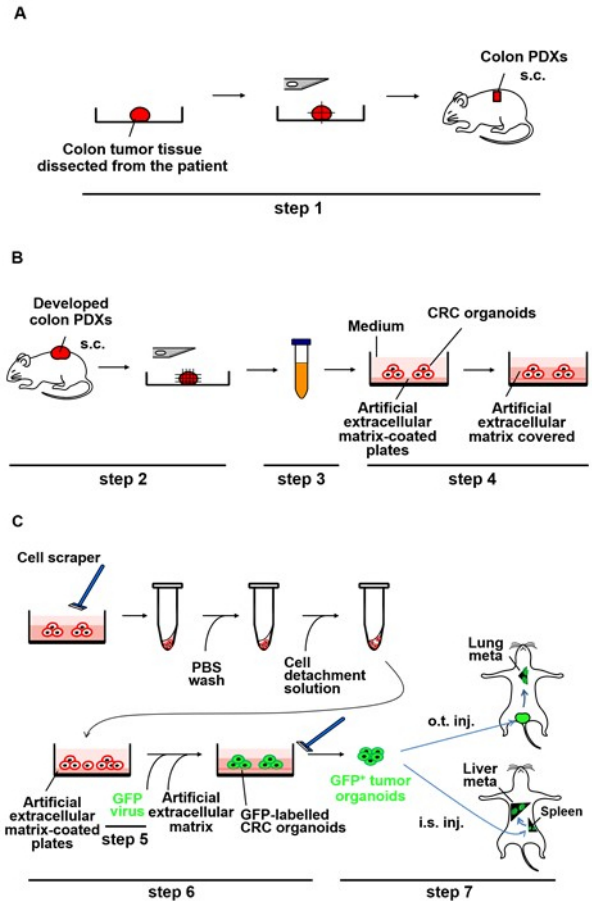

Figure 1: Schematic representation of generation of metastases by the PDX-derived CRC organoids labeled with GFP lentivirus in NOG mice. (A) Implantation of small pieces of the CRC tissue subcutaneously into NOG mice (step 1). The CRC tissue surgically dissected from the patient was cut into pieces and implanted subcutaneously into NOG mice. s.c.: subcutaneous implantation. **(B)** Generation of CRC organoids dissociated from PDXs (step 2–4). The developed CRC xenografts were minced (step 2) and transferred into a 15 mL tube containing the culture medium including collagenase (step 3). After incubation with slow agitation, the CRC cell suspension was filtered (step 3). Then, the organoid cell suspension in the CRC organoid medium was seeded onto an artificial extracellular matrix-coated plate and incubated overnight in a CO_2 incubator (step 4). The CRC organoid cells attached to the artificial extracellular matrix were also coated with additional artificial extracellular matrix and incubated in a CO_2 incubator (step 4). s.c.: subcutaneous implantation. **(C)** Generation of CRC organoids transduced by GFP lentiviral particles prior to employing injection into recipient mice (step 5–7). The GFP lentiviral particles were generated at a high titer (step 5). The PDX-derived CRC organoids grown on artificial extracellular matrix were directly harvested with a cell scraper and transferred into a microtube (step 6). After centrifugation, the cell pellet was re-suspended in PBS. The cell suspension was centrifuged and the cell pellet was dissociated. Then, the CRC organoid cell suspension was incubated with GFP virus stock in the CRC organoid culture medium on the artificial extracellular matrix-coated plate overnight in a CO_2 incubator (step 6). The CRC organoid cells attached to the artificial extracellular matrix were coated with additional artificial extracellular matrix and incubated in a CO_2 incubator to solidify the artificial extracellular matrix coating (step 6). The CRC organoids were then cultured for periods of 7–10 days to expand cell growth (step 6). To develop a spontaneous metastasis model, the dissociated 5 x 10^5 CRC organoid cells labelled with GFP suspended in 50 µL of PBS with 50% artificial extracellular matrix were injected orthotopically into NOG mice (step 7). To generate an experimental metastasis model, 4 x 10^4 CRC organoid cells labelled with GFP in 50 µL of PBS were injected intrasplenically into NOG mice (step 7). o.t.: orthotopic injection, i.s.: intrasplenic injection. Please click here to view a larger version of this figure.

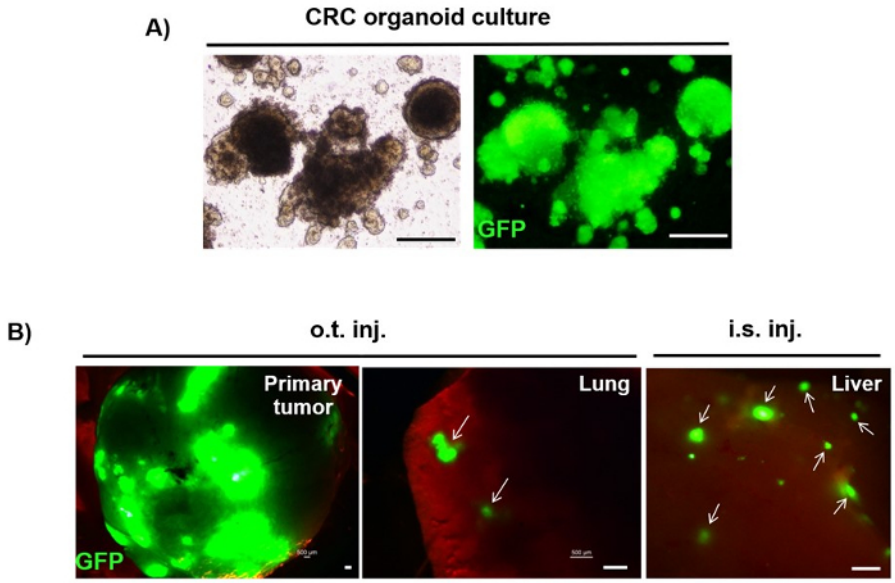

Figure 2: High-resolution of GFP visualization detected in cultured GFP-labeled CRC organoids, primary tumors and micrometastases. **(A)** Nearly 100% of cultured CRC organoids are GFP positive. The images were captured using a stereo-fluorescence microscope. Scale bar = 250 µm. **(B)** GFP-positivity in the primary tumor and micrometastases in the lungs and liver. The dissociated GFP-expressing CRC organoid cell suspension was injected into the rectal submucosa (o.t. inj.) of NOG mice. The majority of tumor cells are shown to be positive for GFP in the primary tumor. GFP-positive micrometastases colonizing the lungs (indicated by an arrow) are also shown. Moreover, GFP-positive micrometastases are detected in the liver (indicated by an arrow), when the cell suspension was intrasplenically injected (i.s. inj.) into mice. Scale bar = 500 µm. Please click here to view a larger version of this figure.

Discussion

Although the CRC PDX model has been widely employed to study primary tumor growth, whether this model is also applicable to investigating tumor metastasis has not yet been fully elucidated. Spontaneous metastases were also barely detectable in the liver and lungs of various reported colon PDX models[4,10]. To detect micrometastases with high sensitivity, we developed a protocol for transducing GFP lentiviral particles into PDX-derived CRC organoids prior to their orthotopic and intravenous injections into recipient mice. Of note, we were able to show that this method allows highly sensitive detection of CRC organoid-derived micrometastases, forming both spontaneously and experimentally, in distant organs.

Critical steps within the protocol include maintaining intact spheroid formation without induction of anoikis on the artificial extracellular matrix by gently pipetting the CRC organoids during the passaging series. The preparation of high titer lentivirus particles concentrated by ultracentrifugation is also important for achieving the nearly 100% GFP positivity of CRC organoids. Moreover, it is recommended that the GFP-labeled CRC organoids be injected within 10–14 days into recipient mice. Excessively long periods for expansion of the CRC organoids after infection may favor selection of weakly GFP-expressing organoids.

The injection of CRC organoids into the rectal submucosa of NOG mice showed their metastatic dissemination into the lungs, though not into the liver, presumably through the inferior vena cava. To generate liver metastases spontaneously, injection of CRC organoids into the colon with laparotomy would be required[13].

Note that the incidence of B cell lymphomas is on occasion increased in NOG immunodeficient mice implanted with tissues including lymphocytes, infected with Epstein-Barr oncovirus, from human patients[14]. It is therefore reasonable to recommend that whether the emerging tumors originate from the implanted human CRC be determined by performing immunohistochemistry using human CRC cell markers, such as carcinoembryonic antigen and cytokeratin 20.

As we investigated CRC organoids derived from only one patient, more samples from larger numbers of patients would need to be examined to generalize our findings in the future. If the implanted PDX-derived CRC organoids show minimal growth at the orthotopic site and rarely form metastases at distant sites, investigators are encouraged to consider extracting CRC organoids from different patient-derived PDXs.

The highly sensitive detection of GFP-labeled micrometastasis achieved with this method encourages us not only to further investigate several unresolved biological issues regarding transition of micrometastasis to macrometastasis, formation of the stromal niche for metastatic colonization and acquisition of drug resistance, but also to evaluate the efficacies of novel anti-cancer drugs by employing PDX-bearing animals as preclinical models.

The GFP-based FACS-sorting of living CRC organoids extracted from primary and distant sites also has the potential to allow single cell-based examinations of the gene expressions, as well as the epigenetic and metabolomic profiles, of a tumor. Such findings may well lead to elucidation of the molecular mechanisms underlying metastatic dissemination of the CRC organoids.

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Disclosures

The authors have no potential conflicts of interest to disclose.

Acknowledgements

This work was supported by the Juntendo University Young Investigator Award (2013, 2014 and 2015) to Y.O., the Joint Project Award (2013 and 2014) to K. M., and Grants in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (16K15625 to Y. K. and 16K15598 to M.G.). We are especially thankful to all members of the Dept. of Coloproctological Surgery and Molecular Pathology for useful discussions and technical support. We also thank Dr. Hiroyuki Konno (Hamamatsu University Schoolof Medicine) and Dr. Hideki Kitajima (International University of Health and Welfare) for generous technical guidance in the surgical procedures for orthotopic implantation into mice and Dr. Yoshitaka Hippo (Chiba Cancer Center) for technical advice on performing the tumor organoid culture.

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