Video Article

Experimental Metastasis Assay

Sonali Mohanty[1], Lei Xu[1,2]

[1]Department of Biomedical Genetics, University of Rochester Medical Center

[2]Department of Dermatology, University of Rochester Medical Center

Correspondence to: Lei Xu at Lei_xu@urmc.rochester.edu

URL: https://www.jove.com/video/1942
DOI: doi:10.3791/1942

Keywords: medicine, Issue 42, cancer, metastasis, experimental, mouse, intravenous injection, lung

Date Published: 8/24/2010

Citation: Mohanty, S., Xu, L. Experimental Metastasis Assay. *J. Vis. Exp.* (42), e1942, doi:10.3791/1942 (2010).

Abstract

Metastasis is the leading cause of death in cancer patients. To understand the mechanism of metastasis, an experimental metastasis assay was established using immunodeficient mice. This article delineates the procedures involved in this assay, including sample preparation, intravenous injection, and culturing cells from lung metastases. Briefly, a pre-determined number of human cancer cells were prepared *in vitro* and directly injected into the circulation of immunodeficient mice through their tail veins. A small number of cells survive the turbulence in the circulation and grow as metastases in internal organs, such as lung. The injected mice are dissected after a certain period. The tissue distribution of metastases is determined under a dissecting microscope. The number of metastases in a specific tissue is counted and it directly correlates with the metastatic ability of the injected cancer cells. The arisen metastases are isolated and cultured *in vitro* as cell lines, which often show enhanced metastatic abilities than the parental line when injected again into immunodeficient mice. These highly metastatic derivatives become useful tools for identifying genes or molecular pathways that regulate metastatic progression.

Video Link

The video component of this article can be found at https://www.jove.com/video/1942/

Protocol

1. Sample Preparation

1. Grow cells to ~70% confluent in their specific media with growth factors or FBS (e.g., DMEM with 10% FBS for MC-1 cells). Aspirate media from plate and gently wash several times with 1 x PBS (8 g/L of NaCl, 0.2 g/L of KCl, 1.15 g/L of $Na_2HPO_4.7H_2O$, 0.2 g/L of KH_2PO_4, pH 7.3).
2. Aspirate PBS and add 2 mL of 0.05% Trypsin in versene (0.014 g/L of phenol red and 0.2 g/L of EDTA-Na in 1 x PBS, pH 7.2). Gently rock plate to facilitate cell detachment from plate. Observe the cells under a microscope. It usually takes 2-5 minutes for the cells to detach.
3. Add an ample volume of media containing FBS (or soybean trypsin inhibitor) to quench the trypsin activity and collect cells in a 50mL falcon tube. Count cells using a hemacytometer. Load 10µL of cell suspension onto a clean hemacytometer. The number of cells per mL is equal to the average # of cells in each of the five squares multiplied by 10^4.
4. Centrifuge the cells at 1000 RPM (or ~200 xg) in a benchtop centrifuge for 3 minutes.
5. Carefully remove the media without disturbing the cell pellet. Resuspend cells in an appropriate volume of Hanks Balanced Buffer Solution (HBSS) to reach a final concentration of ~5 x 10^6 cells/mL.
6. Filter cells through a Falcon 70 µm cell strainer to exclude large cell aggregates. Place the tip of the pipette directly onto the filter over a labeled 5mL Falcon culture tube. Quickly eject the suspension through the filter into the culture tube.
7. Count cells again using a hemacytometer and dilute them to a final concentration of 2.5 x 10^6/mL. Keep cells on ice.
8. Determine the viability of the cells. Mix some cells with trypan blue and measure the percentage of dead (blue) cells over the total cells using a hemacytometer. The viability of cells should be ≥ 90% prior to injection.

2. Intravenous Injection

1. Change gloves. Gently grab the tail of an immunodeficient mouse (nude, NOD-SCID, or NSG, Jackson's Laboratory) and pull it into the mouse restrainer, with its back against the slit and its tail sticking out of the small opening in the back of the restrainer.
2. Slowly slide the ring inward along the slit and lock it in place once the ring catches the mouth of the mouse. The mouse should not be able to move freely, but should have normal rate of breathing.
3. Find the major tail veins. Four major blood vessels are present in a mouse tail. Blood vessels on the dorsal and ventral sides of the tail are arteries. Veins are on the lateral sides of the tail.
4. Draw more than 200 µL of the prepared cells into a 1mL syringe. Attach the 30 G1/2 inch needle and push out any air bubbles that may exist. The final volume of cells in the syringe should be 200 µL (i.e., 5 x 10^5 cells in total).
5. Inject cells into the tail vein.
    a. Start from the distal end of the tail, so if the first trial fails, a more proximal region of the tail could be used for a second try.

b. Wipe the tail with 70% ethanol. Pull the tail straight. Hold the tip of tail with thumb and support the point for injection with the index finger.
c. Insert the needle to the vein and inject cells. Make sure the needle and syringe are parallel to the vein during injection, otherwise the needle will poke through the vessel wall and inject the cells into the adjacent tail tissues.
d. Withdraw the needle after injection. Blood should profuse from the injection site if the injection went successfully. Press a clean piece of paper towel or cotton swab on the injection site to facilitate clotting, and palpate the tail upwards to push any residual sample in the vein into circulation.
e. Release the mouse from the restrainer and return it to the cage. Record the injection process (e.g., how many trials it took to inject the cells and how much cells were injected) in a lab notebook.
f. Determine the viability of cells after injection, as described in the Section of Sample Preparation, Step 9. This step provides reassurance that cells stay alive throughout the injection process. At the end of an injection experiment, the viability of cells will decline, but should be above 80%.
g. (optional) Spin down the leftover cells and rinse the pellets with PBS once. Freeze the pellets at -80 oC for future analyses (e.g., western blots to confirm gene expression or knockdowns).

6. Typically after one or two months, the mice will be dissected and the locations of metastases are grossly determined. Lung is the primary site for metastasis, since it contains the first capillary bed that the cells encounter after they enter the circulation.
7. After rinsing with PBS, each lobe of the lung (or other tissues containing metastases) is observed under a dissecting microscope (Figure 1). The number of detectable metastases on both sides of the lung is counted and the numbers of lung metastases on all the four lobes are added together as the total number of lung metastases.[1] Lung metastases are more easily detected if the lungs are fixed in formalin overnight, since the metastases will apppear as whitish spots in contrast to the adjacent dark brown lung tissues.

3. Culturing Cells from Lung Metastases

1. Lung metastases are isolated from injected mice. Each should be cultured separately.
2. Each metastasis is minced by the end of a needle cap (sterile) on a 70 μm cell strainer.
3. Rinse the cell strainer with several mLs of medium and collected cells that pass through the filter in a culture dish.
4. Incubate the cells at 37 °C for at least four days without disturbance.
5. Wash away blood or tissue debris on the dish with PBS.
6. Add fresh medium. At the beginning, both cancer cells and fibroblast cells grow on the plates, but gradually, the fibroblast cells will die out and be replaced by cancer cells. If the cancer cells carry any drug resistant genes, select the cells with corresponding antibiotics.
7. The purity of the derived cells is assessed by immunostaining using antibodies against human-specific proteins. We use an anti-human vimentin antibody (NCL-VIM-V9, Novocastra). The cells we derived contain typically over 99% of human cells, even in the absence of drug selection. The newly derived cells could be injected again into immunodeficient mice to test their metastatic abilities, as described above.

4. Representative Results

1. At the end of this assay, a highly metastatic cancer cell line typically gives rise to many lung metastases (Figure 1),[1] while very few lung metastases will come from a poorly metastatic cancer cell line.
2. The cells derived using this method usually give rise to more metastases than the parental line, when they are tested again using this assay.[1-3]

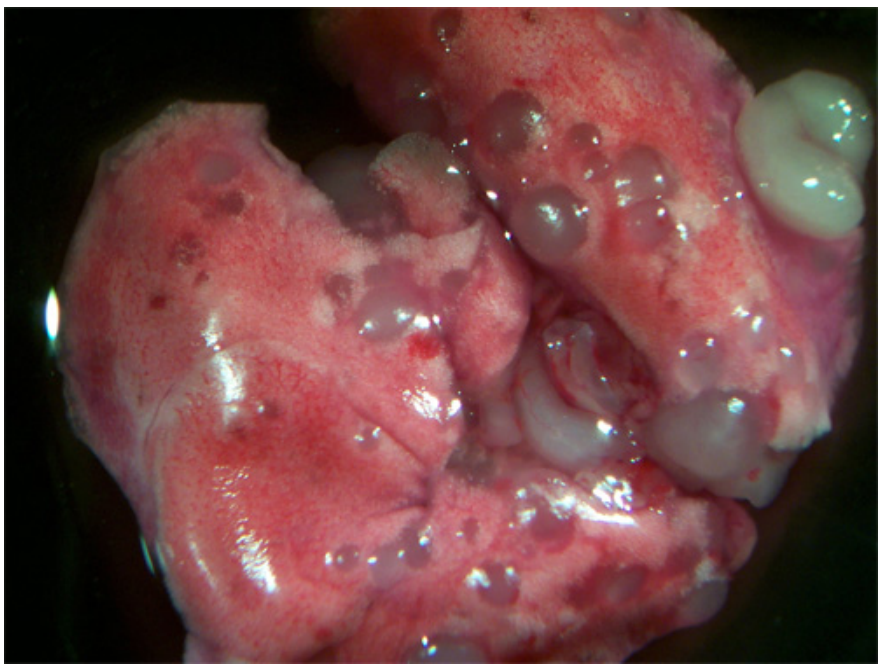

Figure 1. Representative images of mouse lungs after tail vein injections of cancer cells. 5 x 10^5 of the metastatic human melanoma cell line, SM cells,[4] were injected into the tail vein of immunodeficient mice. Two months later, the lungs were isolated and metastases were found dispersed among the normal lung tissue.

Discussion

Metastasis is the leading cause of death in cancer patients. It involves four major steps: detachment of cancer cells from their primary loci, their entry into circulation (intravasation), their exit from circulation (extravasation), and survival and growth in a distant organ. Metastasis in human is considered a slow process and often manifested after years of latency. To study its progression in a timely manner, the above relatively quick experimental assay was established in immunodeficient mice.[4] Since its establishment, it has been used effectively to isolate cells with different metastatic abilities, identify genes that are up- or down-regulated during metastasis [1,2,5], and test the causal roles of these genes during metastasis.[3]

The most challenging step in this assay is to inject cells into the tail vein. It takes practices to master the injecting skills. Even for an experienced researcher, several trials before achieving a successful injection are very common. Veins in mouse tails are extremely thin and insert needles right in the middle of them is not easy. Warming up the tail briefly using a lamp and gently massaging the tail before injections could induce dilation of the blood vessels and facilitate injections. A common mistake for most researchers is to assume that the veins are deep in the tail and therefore inject cells too deep into the skin. Veins are actually very close to the surface of the skin, therefore one should try to inject as shallowly as possible.

If the needle is successfully inserted inside the vein, the injection should go smoothly with no resistance. If the needle is not inserted in the vein but in the adjacent tissues, samples injected will build up fluid pressure quickly and then prevent the delivery of the rest of samples. Each of such failed trials will thus lose some samples. To minimize this loss, an experienced researcher tries to predict whether the needle is in the vein or not by injecting a minimal amount of sample and assessing the resistance from the fluid pressure. If he feels the resistance, the needle must not be in the vein and he will then pull the needle out and try again. If no resistance is felt, the needle must be in the vein and all of the sample will be injected at once. In the latter case, one can often see the sample "shoots" up the vein, displacing blood as it makes its way toward the body.

When to terminate the assay should be determined empirically for each cell line, since it varies significantly depending on the metastatic potentials of the cells. Mice will be dissected at different time points after injections and the extent of lung metastasis is determined. A length of time that results in a countable number of microscopically detectable lung metastases is usually chosen, since it gives a quantitative result and also leaves room for any increase or decrease of metastases caused by perturbation of candidate genes. For researchers who perform this assay for the first time, using a highly metastatic cell line (such as the human melanoma cell line, A375, or its highly metastatic derivatives) as a positive control is highly recommended. Furthermore, ages and genders of mice affect the results significantly, so it is advised to keep the ages and gender consistent when the metastatic abilities of different cell lines are compared. Due to the variations introduced by the factors mentioned above, at least five mice should be injected for each cell line to reach any statistically significant conclusion.

Acknowledgements

This protocol was tested and optimized initially in the laboratory of Dr. Richard Hynes (MIT). Funding is provided by the NYSTEM IDEA AWARD (to L.X.) and Ruth L. Kirschstein National Research Service Award (to L.X.).

References

1. Xu, L. *et al.*, Gene expression changes in an animal melanoma model correlate with aggressiveness of human melanoma metastases. *Mol Cancer Res* **6** (5), 760 (2008).
2. Clark, E. A., Golub, T. R., Lander, E. S., and Hynes, R. O., Genomic analysis of metastasis reveals an essential role for RhoC. *Nature* **406** (6795), 532 (2000).
3. Xu, L., Begum, S., Hearn, J. D., and Hynes, R. O., GPR56, an atypical G protein-coupled receptor, binds tissue transglutaminase, TG2, and inhibits melanoma tumor growth and metastasis. *Proc Natl Acad Sci U S A* **103** (24), 9023 (2006).
4. Kozlowski, J. M., Hart, I. R., Fidler, I. J., and Hanna, N., A human melanoma line heterogeneous with respect to metastatic capacity in athymic nude mice. *J Natl Cancer Inst* **72** (4), 913 (1984).
5. Kang, Y. *et al.*, A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* **3** (6), 537 (2003).

