Chapter 8
In Vivo Studies of Breast Cancer Cells

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Keywords Xenograft model • 17 beta estradiol • HCG on malignant human breast epithelial cells • Labeling of cancer cells • Imaging procedure • TAIL vs. intraventricular injection • Metastatic foci in liver and lung

8.1 Introduction

The process of transplanting a living tissue, cells, or organs from one species to another is known as xenotransplantation and, the tissue, cells, or organs transplanted are known as xenografts. Xenotransplantation is possibly most well known for the transplant of organs originating from animals such as baboons and pigs into humans. The first and possibly most famous case of a xenotransplantation is that of Fae, an infant girl who successfully received a baboon heart in 1984. Despite the potential future use of this method to replace organs, cell xenotransplantation is a much more common procedure utilized in cancer research. This process most commonly involves implantation of human tumor cells into immunodeficient mice and is used to test the efficiency of compounds and their interactions with pathways within the body. Because human tumor cells can relatively easily be injected into mice and tumor growth can be routinely established, these models are commonly used for testing compounds. In a paper published by Baselga et al. xenografts were used to

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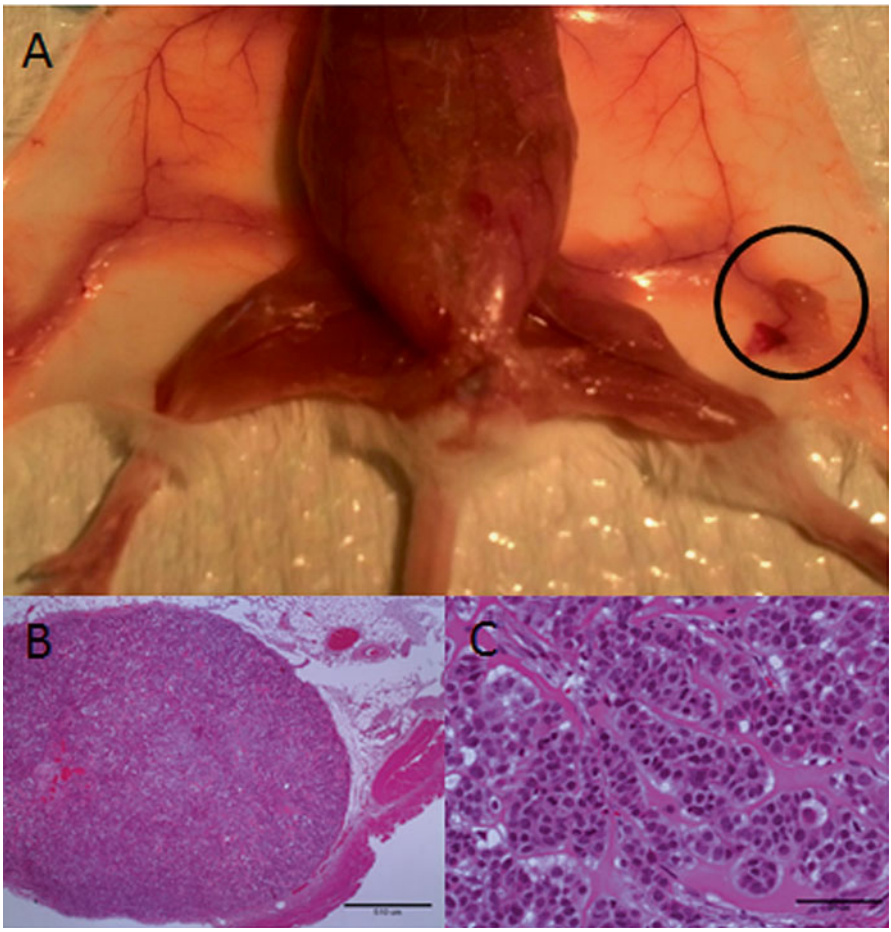

Fig. 8.1 (**a**) SUM149 xenograph-induced tumor on left fat pad of SCID mouse. Mice were administered subcutaneous injections of two million SUM149 cells suspended in 100 μL matrigel. Tumor was collected three weeks after implantation. (**b**) Histological section of xenographed-induced tumor 40×. Section was stained with Hemotoxylin and Eosin. (**c**) Poorly differentiated xenographed-induced tumor. Histological section of xenographed-induced tumor 400×. Section was stained with Hemotoxylin and Eosin

determine the effects of two anticancer drugs paclitaxel and doxorubicin in combination with an anti-HER2 antibody on breast cancer cell growth [1]. We have used xenografts to test the tumor formation of the SUM149 cell line in SCID mice. Mice were given subcutaneous injections of two million cells suspended in matrigel into the posterior quadrant of animals' abdomens. Tumors are observed 3 weeks after the implantation (Fig. 8.1a), and the histology reveals a well-defined poorly differentiated tumor (Fig. 8.1b, c).

8.2 The Use of a Xenograft Model for Testing the Tumorigenic Potential of MCF7 Cells

An important criterion of malignancy is the ability of transformed cells to grow in an adequate heterotransplantation system [2]. Immunologically depressed athymic mice (*nu/nu*) [3–6] have the striking capability of discriminating between normal and neoplastic cells. Normal cells do not induce tumors [5], whereas malignant cells do. MCF-7 cells, cultured as previously described [7], were removed from the culture vessel by trypsinization, suspended in phosphate buffer saline (PBS) (1×10^6 cells per 0.05 mL) and transplanted in 21-day-old Balb/c (*nu/nu*) mice into the mammary gland fat pad which was cleared according to the method of DeOme [8]. The first experiment demonstrated that MCF 7 cells were unable to grow neither in female nor in male athymic mice [9]. The gross examination of the area of cell inoculation and the histological study revealed a complete absence of the inoculated cells; only disorganization of the fat and some fibrosis were observed. Only those mice that have received a transplant of pituitary glands or ovaries from syngenic mice induced the growth of MCF-7 cells. Nine of the 11 (82 %) inoculated female mice that received pituitary grafts developed palpable tumors within 12–18 days after inoculation. The tumors adhered to the skin and underlying muscle. No macroscopic metastatic growths were observed. Eight of the 13 (61.5 %) inoculated female mice that received ovary grafts developed palpable tumors within 12–18 days. Tumors were attached to the skin and underlying muscles; no metastatic growths were observed. The tumors were small, oblong masses of 1.5–2.5 mm at their largest diameter. They adhered to the dermis of the skin and to the muscle of the abdominal wall. The tumors were firm, of a rubbery consistency, and presented resistance to sectioning. The tumor's vascular bed was well developed. The area of the tumor was easily distinguished from the scar produced by the cauterization and the incision made during the transplant procedure. The histological pattern of the 17 tumors studied was identical. The tumors were composed of nests of cells arranged in either clusters or single- or double-row strands. The inoculated epithelial cells were surrounded by a dense stroma formed by collagen fibers and fibroblasts. Blood vessels were scarce in the central portion of the tumor and more abundant in the periphery and in areas of invasion. The cells presented a considerable degree of pleomorphism and atypia. The nucleus was oval with few indentations. The nucleoplasm was pale, and a thin layer of heterochromatin was observed on the inner side of the nuclear envelope. More than two nucleoli per nucleus were frequently observed. Intracellular lumina with cellular detritus within were present in some cells. When stained with toluidine blue, the cytoplasm of most cells appeared strongly basophilic. A few cells with pale cytoplasm were also observed. Similar epithelial cells were also observed in the dermis of the skin overlying the inoculation site and among muscular fibers of the abdominal wall. The intense fibrous reaction observed at the inoculation site and in the dermis was not observed around cells invading skeletal muscle. Mitoses were frequently observed in areas of invasion. No metastases were found in any of the tissues studied; however, clusters of cells attached to the adventitia of blood

vessels or adjacent to the perineurium were observed in the periphery of the tumor. Invasion of blood vessels or nerves by neoplastic cells was not observed in serial sections. The tumors observed in mice isografted with pituitary glands or ovaries were indistinguishable.

The successful heterotransplantation of human tumors [6, 10] and cultured human malignant cells [3, 4] into nude mice has proven to be an excellent model for the study of neoplastic tissue and an effective diagnostic tool for differentiating malignant from benign cells [5]. The growth of MCF-7 cells as tumors in nude mice might be predicted by the malignant nature of the tumor of origin and by the demonstration of several transformation markers [9]. However, MCF-7 cells did not form tumors in all inoculated mice but only in those receiving pituitary or ovarian grafts, thus suggesting a hormone dependency for in vivo growth. The fact that more tumors were observed in mice receiving pituitary grafts (82 %) than in those receiving ovarian grafts (61.5 %) suggested that some pituitary hormone could be involved in the development of these tumors. The inoculation of MCF-7 cells into nude mice induces tumors morphologically similar to the tumor of origin. This property of malignant cells has been described for other cell lines maintained for almost 100 passages in vitro [5] and transplanted into nude mice, and for human tumors transplanted into the anterior chamber of the guinea pig eye [11]. MCF-7 cells develop a histological pattern in the nude mice similar to that observed in the tumor of origin. The tumor of origin was an infiltrative ductal carcinoma with productive fibrosis. This same pattern of epithelial cells surrounded by a dense stroma is observed in the mouse, suggesting that it is the neoplastic epithelial cell that elicits a stromal response in the host. This observation was also supported by results obtained in an experimental model developed for the study of scirrhous carcinoma [12].

The absence of tumors in untreated animals could be explained by an inadequate hormonal milieu for the growth of MCF-7 cells. The fact that the original tumor from which MCF-7 cells were derived was responsive to hormones and that MCF-7 cells still retain specific high-affinity estradiol and progesterone receptors after more than 160 passages in culture, supports this explanation. The utilization of hormonal supplementation in the growth of MCF-7 cells in 1976 [13], suggested the replacing the isografts by hormone pellets [14]. We found out that the use of castrated male, estrogen-supplemented, was also suitable for the growth of MCF 7 cells [9]. The removal of the uterus and supplementation with estradiol either as pellets or silastic tube containing 5 mg of 17-β-estradiol in female mice is also a standard procedure [9]. The removal of the uterus avoids the swelling and accumulation of fluid in this organ due to the estrogenic stimulation.

8.3 The Use of Xenografts for Testing the Tumorigenicity of Chemically Transformed Cells

In the experimental system of human breast epithelial cells (HBEC) transformed with chemical carcinogens the SCID mice as heterologous hosts for testing tumorigenicity has been used. SCID mice have an autosomal dominant recessive defect

that impairs the rearrangement of antigen receptor genes in both T and B lymphocyte progenitors. SCID mice also lack functional T and B cells, which make these animals a suitable host for heterotransplantation. Tumorigenesis in heterologous hosts is considered to be the final reliable criterion for assessing complete transformation of human cells [11, 15], even though the validity of this model has been questioned, since many human malignancies or cell lines derived from them are not tumorigenic in the nude mouse [16]. The adequacy of SCID mice for testing tumorigenicity has been validated by the observations that 100 % of the mice inoculated with either T24 or MCF-7 cells developed tumors with a short latency period. SCID mice proved to be more adequate as a host for MCF-7 cells than nude mice, since in our model they did not require estrogen supplementation [13]. The tumors developed in inoculated SCID mice were proven to be of human origin by determination of Alu sequences [17], which showed that they were derived from the cells inoculated and not a host reaction. T24 and BPI-E, the two cell lines expressing tumorigenesis in SCID mice [9], also exhibited the highest colony formation in agar–methocel. BP1-E cells also exhibited a higher colony efficiency (CE) and, larger colony size and higher colony number than the other non-tumorigenic clones, which indicated that these phenotypes need to be sequentially expressed prior to the manifestation of the tumorigenic phenotype. Anchorage independent growth is considered to be a predictor of tumorigenesis in other cell systems [11, 18]. Nonetheless, in the experimental model neither anchorage independence nor any of the other parameters served, when considered individually, as an indicator of cell tumorigenic potential. These observations are supported by results on HBEC obtained from milk, which have been reported to acquire anchorage independent growth and immortalization after SV40 infection, but do not elicit tumorigenesis in nude mice [11, 18–20] and by the observation that the extended lifespan induced in HBEC treated with B[*a*]P in vitro was not accompanied by the expression of anchorage independence or tumorigenicity [21]. Tumorigenesis in a heterologous host emerges in chemically treated immortal cells as a consequence of clonal expansion, in which phenotypes indicative of neoplastic transformation are cumulatively expressed through successive processes of selection over long periods of time. During the process of neoplastic transformation no chromosomal changes were detected. The molecular events that are operational in each phase of the transformation process indicate that each carcinogen induces different degrees of point mutations in codons 12 and 61 of the c-Ha-ras oncogene [22] as an event detectable by the 10th passage post-carcinogen treatment. Other genes such as p53, Rb, and erbB2 were differently expressed by the various clones derived from carcinogen-treated cells [23, 24].

8.4 Ha-ras Enhances the Tumorigenic Phenotype of Chemically Transformed Cells

As indicated above, the levels and localization of *ras* expression in normal and malignant breast tissues have been examined and quantitated by analyzing breast tissue samples for the expression of *ras*-related mRNA and p2l ras protein which

has been found to be expressed in biopsies of both normal and malignant breast tissues [25]. However, whether the *ras* oncogene is a causative agent of human breast cancer has not been proved as yet. Therefore, one way to evaluate the contribution of *ra*s genes in the development of the tumorigenic phenotype is to introduce this gene into suitable acceptor cells. Transfection of the non-tumorigenic cell lines, clones D3-1 and BP1, derived from the carcinogen-treated MCF-10F cell lines, and the MCF-10F cell line with the cHa-ras oncogene not only enhanced colony formation in agar–methocel and invasiveness but also induced tumorigenicity with a short latency period in SCID mice [9].

The MCF-10F cells, DMBA or BP-treated cells and the clones D3-1 and BPI did not exhibit tumorigenicity in SCID mice. We have already shown that the subclone BP1-E derived from BP1 expressed the tumorigenic phenotype after 101 days of inoculation whereas the MCF-10F-Tras had lower tumorigenicity because it took 99 days to induce tumors in 3/14 animals, the clones D3-1-Tras and BPI-Tras were highly tumorigenic and the tumors appeared between 47 and 60 days post-inoculation, in four out of four animals and 11 out of 14 animals, respectively [9]. All the tumors derived from D3-1-Tras and BP1-Tras cells were poorly differentiated adenocarcinomas. They were immunocytochemically positive for keratin whereas the human milk fat globule membrane antigen (HMFGMA) was frankly expressed only in tumors induced by BP1-E cells; tumors derived from *c-Ha-ras* transfected cells showed either a notably reduced expression of this antigen, as observed in D3-1-Tras-induced tumors, in which only 10 % of the tumor cells were positive, or complete abolishment of HMFGMA reactivity, as in the BP1-Tras-induced tumor cells. Tumor cell lines derived from the tumors thus originated have been an important resource for understanding the molecular basis of mammary carcinogenesis [9].

Altogether the data presented in this chapter summarizes an in vitro model of breast cancer in which the final malignant phenotype of tumorigenesis can be induced in HBEC by carcinogen treatment alone or in combination with *ra*s oncogene, provided that the treated cells are previously immortalized and enough time and clone selection are allowed for its expression. This model provided clones of cells expressing different stages of progression to malignant transformation, which are useful for determining whether specific phenotypes are the result of specific genotypic alterations.

8.5 Tumorigenicity of 17 Beta Estradiol Transformed Human Breast Epithelial Cells

The invasive bsMCF cells were plated at low density and cell colonies were isolated using cloning rings. The cells were cultured in Dulbecco's modified Eagle medium (DMEM): F12 medium containing 1.05 mM calcium, antimycotics, hormones, growth factors, and equine serum [26]. After trypsinization and plating, the clones obtained were identified as bcMCF-1, bcMCF-2, bcMCF-3, bcMCF-4, bcMCF-5,

Table 8.1 Histological and immunocytochemistry of the bcMCF clones

Clones	Tumor type	AE1	CAM5.2	EMA	Vimentin
bcMCF-1 bcMCF-4	Invasive poorly differentiated spindle cell type	–	–	–	+++
bcMCF-2 bcMCF-6 bcMCF-7	Invasive poorly differentiated epithelial cell type	±	±	±	++
bcMCF-3 bcMCF-5	Invasive poorly differentiated with mix features of spindle and epithelial type	–	±	±	++

The mouse monoclonal antibodies anti-human cytokeratin of low molecular weight (AE1, Biogenex, San Ramon, CA), cytokeratin peptides 7 and 8 (CAM5.2, Ventana, Tucson, AZ), epithelial membrane antigen (EMA) clone E29 and vimentin, clone V9, both from DakoCytomation Inc., Fort Collins, CO were used. Negative (–), weak (±), moderate (++), or strong (+++)

bcMCF-6, and bcMCF-7. The tumorigenic ability of the bcMCF subclones was tested by injecting them into the mammary fat pad of 45-day-old female SCID mice (Table 8.1).

MCF-10F cells that after treatment with 17β-estradiol (E_2) expressed high colony efficiency (CE) and loss of ductulogenic capacity in collagen-matrix represented the first level of in vitro transformation. Cells expressing these two parameters were classified as transformed (trMCF), which after further selection for invasiveness in a Matrigel invasion chamber originated the second level of transformation: the invasive (bsMCF) and the cloned (bcMCF) cells [27]. The bsMCF cells formed tumors in SCID mice from which four cell lines, caMCF, were derived. By ring cloning, seven subclones were isolated from the invasive bsMCF cells: bcMCF-1, bcMCF-2, bcMCF-3, bcMCF-4, bcMCF-5, bcMCF-6, and bcMCF-7. All the bcMCF subclones produced invasive poorly differentiated tumors in SCID mice with different morphological phenotypes: spindle cell type (bcMCF-1 and bcMCF-4), epithelial cell type (bcMCF-2, bcMCF-6, and bcMCF-7) and, with mix features of spindle and epithelial type (bcMCF-3 and bcMCF-5) (Table 8.1). As it was previously reported, MCF-10F cells were seeded on Boyden chamber as control; cells that passed through the membrane were selected, expanded, and injected in SCID mice; these cells did not produced tumors [26].

8.6 Tumoristatic Effect of HCG on Malignant Human Breast Epithelial Cells Transplanted in Heterologous Host

The observation that hCG had an inhibitory effect on chemically induced rat mammary carcinomas led us to test whether this hormone had an effect on the in vivo growth of malignant HBEC. For these purposes, MCF-7 cells, a cell line derived from a metastatic breast carcinoma, were injected to Balb/c nude mice (nu/nu). The animals were divided into five groups: the animals of four groups had implanted a silastic tube containing 5 mg 17-β-estradiol in the interscapular region 5 days after

castration, and one group was castrated but did not receive the estrogen supplementation. The cells were injected in the mammary fat pad of mice in all the groups at a concentration of 1×10^6 cells. HCG was administered to the group of animals that did not receive the estrogen at a dose of 1,000 IU/day, and to the three estrogen-supplemented groups at the doses of 10, 100, or 1,000 IU/day. The animals that received estradiol pellets alone had an incidence of 85 % tumor formation. The group of animals injected with hCG alone did not develop tumors [9]. Animals that received estradiol pellets and also hCG exhibited a reduction in both tumor incidence and tumor size which were dose-dependent. These studies led us to conclude that the treatment with hCG abrogates the effect of the estrogen growth dependency of MCF-7 cells in a heterologous hosts (For more details on MCF7 growth in athymic mice see Sect. 8.2).

8.7 The Labeling of Cancer Cells for an In Vivo Imaging System

Many times, it is necessary to follow the growth and migration of cancer cells while the animal is still living [28]. There is a plethora of reporter systems that are used to introduce and select for gene target in cells. These systems have been historically used to study protein expression, interaction between proteins, and function of the proteins in the cells. These reporter genes confer drug resistance, bioluminescence, or fluorescence properties into the cells they are introduced. Typical reporter studies link reporter genes directly to a promoter region of interest, the function of which can be monitored by the reporter activity. Tagging of fused proteins is used to detect intracellular localization, degradation, protein–protein interactions, etc. The most common fusion tags used in research are fluorescent proteins (e.g., eGFP) or small protein epitopes (e.g., FLAG, Myc HA) which can be detected by fluorescence FACS or western blots [29]. One method of in vivo imaging involves in vitro labeling of cancer cells prior to injection in animals. The labeling of cells can be done using a vector that encodes the luciferase reporter gene isolated from *Photinus pyralis*. This gene has been optimized for mammalian expression. These vectors often contain selection markers for prokaryotes and eukaryotes. Cells can be transfected with the plasmid through different techniques: electroporation, lipid-based transfection, calcium phosphate transfection, etc. Once transfected, cells that incorporated the vector are selected with the appropriate antibiotic. Because the gene is incorporated into the genome, as cells divide, the daughter cells will also contain the gene allowing for complete tracking of tumor and cancer cell growth (Fig. 8.2). Luciferase positive cells are then injected in animals. Noninvasive in vivo study of experimental tumor formation and/or metastasis with bioluminescent imaging allows the study of tumor distribution, growth, and regression in individual animals. This technology permits repeated measurements over an extended time period in living animals without the need to sacrifice animals at preset time points [30].

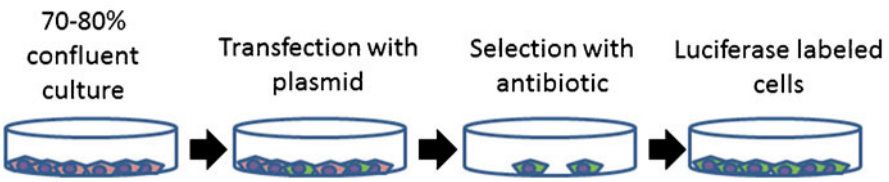

Fig. 8.2 Transfection of luciferase on breast cancer cells. Cells are plated to a 70–80 % confluence. Transfection is performed by the addition of pGL4.51 [luc2/CMV/Neo] plasmid. After transfection selection over the course of 10–14 days with Neomycin is necessary to select cells that are successfully transfected. After selection cells are expanded. *Pink cells* represent cells not transfected with plasmid, *green cells* represent transfected cells

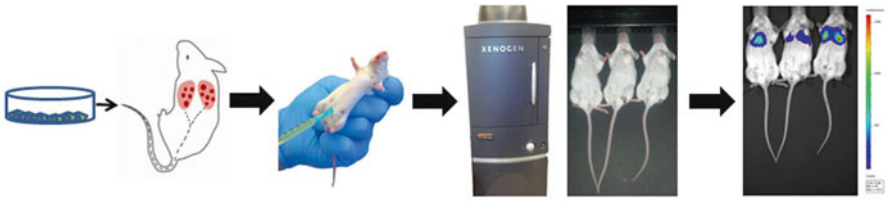

Fig. 8.3 Luciferase-labeled MDA-MB-231 cells are injected into the tail vein of SCID mice. Cells circulate throughout the body and metastasize in various organs. At the time of imaging, animals are injected with 4 mg of Luciferin dissolved in 200 μL PBS. Animals are anesthetized in an isoflurane chamber and are transferred to imaging station where isoflurane is delivered to animals during imaging. Imaging is conducted 10 min after Luciferin injections. Location of metastasis is confirmed by fluorescent cells. MDA-MB-231 cells metastasis occurred in the lungs of the animal. Animals were imaged weekly for 6 weeks at which sacrifice was performed

All imaging is performed using an imaging system, such as the "Perkin-Elmer IVIS Spectrum in vivo imaging system". This system is designed to image bioluminescent or fluorescent signals in animals. To visualize injected cancer cells in vivo, animals are anesthetized and then transferred to the imaging system, where the animals are imaged. For animals bearing tumors expressing firefly luciferase, an injection of the appropriate Luciferin substrate dissolved in phosphate-buffered saline at a physiological concentration is injected intraperitoneally. Then the animals are placed in the imaging system, and the light emitted by the tumors is measured. Animals bearing tumors that produce fluorescent markers (enhanced green fluorescent protein, red fluorescent protein, etc.) may be imaged, but injection of substrates is not needed. However, animals will be illuminated at the excitation frequency of the fluorophore for brief periods (generally a few seconds). Since the animals are under a general anesthetic they suffer no stress or discomfort due to the substrate injections or illumination (Fig. 8.3).

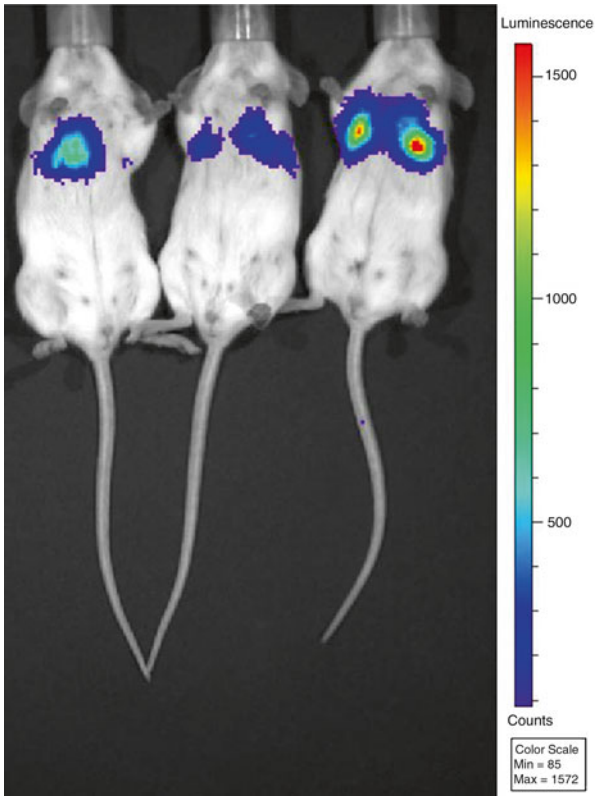

Fig. 8.4 Lung metastasis of SCID mice injected with Luciferase-labeled MDA-MB-231 cells. Image acquired with Perkin-Elmer IVIS Spectrum in vivo imaging system

We have used MDB.MA-231 cells transfected by Lipofectamine/Plus Reagent from Life Technologies. The plasmid used for the transfection was pGL4.51 [luc2/CMV/Neo] from Promega. Transfected cells were selected occurred over a period of 10–14 days using Neomycin (Invitrogen). After selection, cells were allowed to expand (Fig. 8.2). To ensure the presence of luciferase in the cells, a Luciferase Assay (Promega) was performed using the EnVision Workstation plate reader. Two million (2×10^6) cells suspended in PBS were injected into the lateral tail vein using a 26 gauge needle. The animals were followed over a period of 3 weeks to determine the location of the MDA-MB-231 cells (Fig. 8.4). Weekly inspection of the animals allows to follow up closely the process of nesting the metastatic cells as it is shown in Fig. 8.5.

8.7.1 Injection of Cells in the Vein Tail of Mice

For small animals, the procedure basically consists in injecting 2×10^6 cells suspended in 200 μL Dulbecco's PBS (calcium and magnesium free) per animal. The animals must be weighed and marked with an individual identifying mark. For

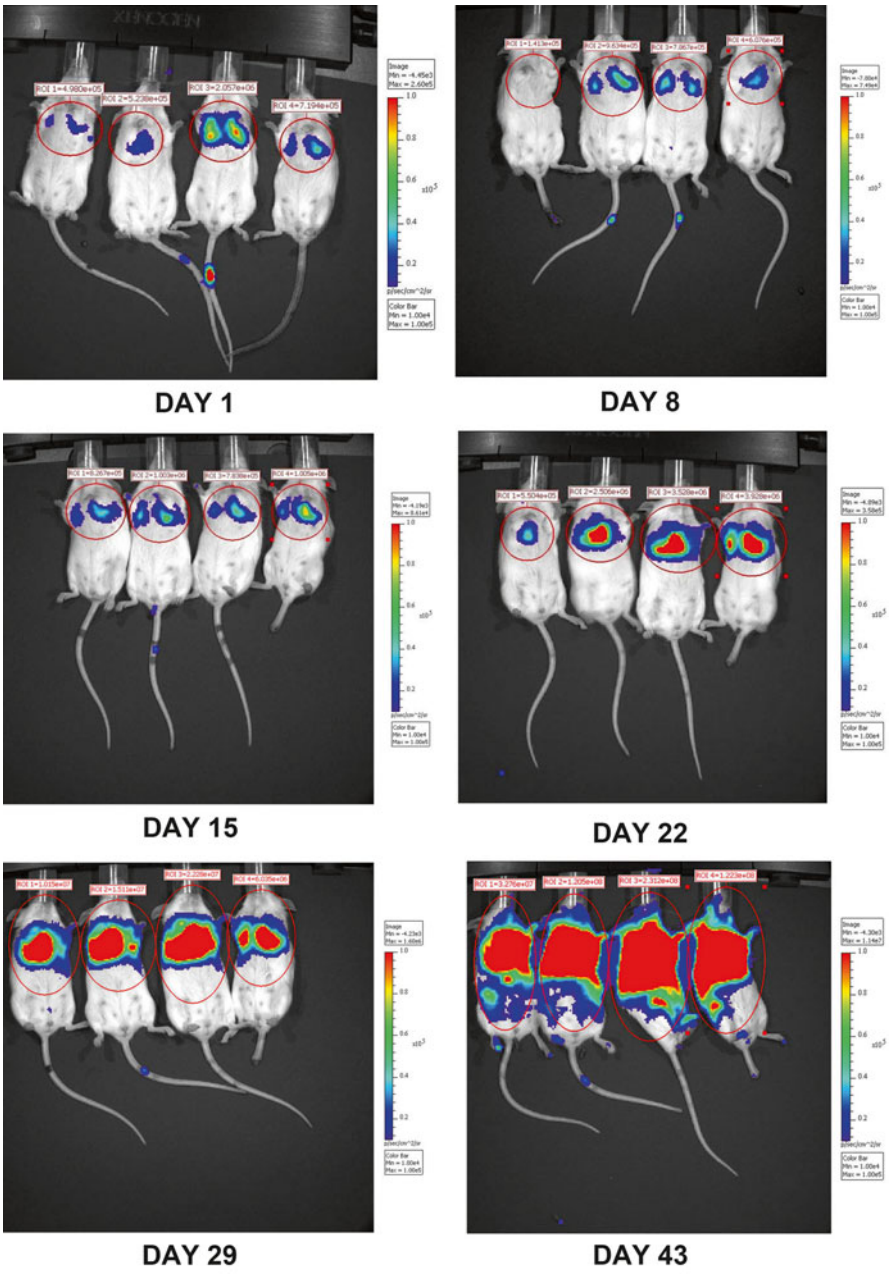

Fig. 8.5 Lung metastasis of SCID mice injected with Luciferase-labeled MDA-MB-231 cells. Image acquired with Perkin-Elmer IVIS Spectrum in vivo imaging system at different time up to 43 days post injection

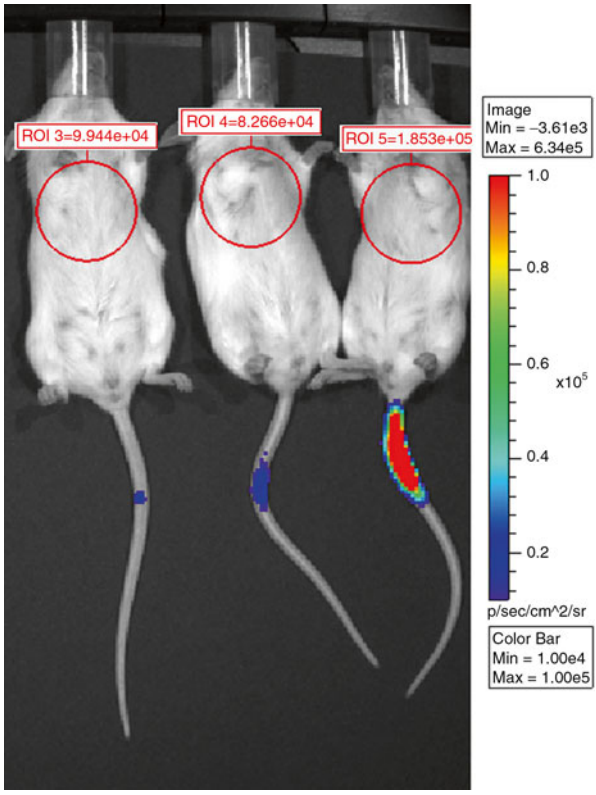

Fig. 8.6 On some occasion the operator does not inject the cells in the vein and they extravasate and grow in the tail

making the vein tail injection must be considered that the mouse tail is shaped like a cube, with a vein on each side. Use one of the lateral veins, using a 26-gauge needle. If the operator is in the vein the injected material will be seen going down the length of the vein. If the operator misses the vein it will get a "blib." In some cases, the cells remain there and the tumor is formed in the tail of the animals (Fig. 8.6). The largest volume that should be injected is 5 cm^3, but the mouse may go into shock.

8.7.2 Imaging Procedure Using the IVIS Spectrum Imaging System

The imaging procedure can be separated in several steps:

Step 1: Prepare Luciferin Solution. Luciferin is stored in the −80° freezer and the solution should be prepared in a 4 mg-200 μL PBS (per animal) concentration. Always prepare extra, you will lose some solution in the base of the syringe. You will waste less if you prepare the suspension in 1.7 mL Eppendorf tubes.

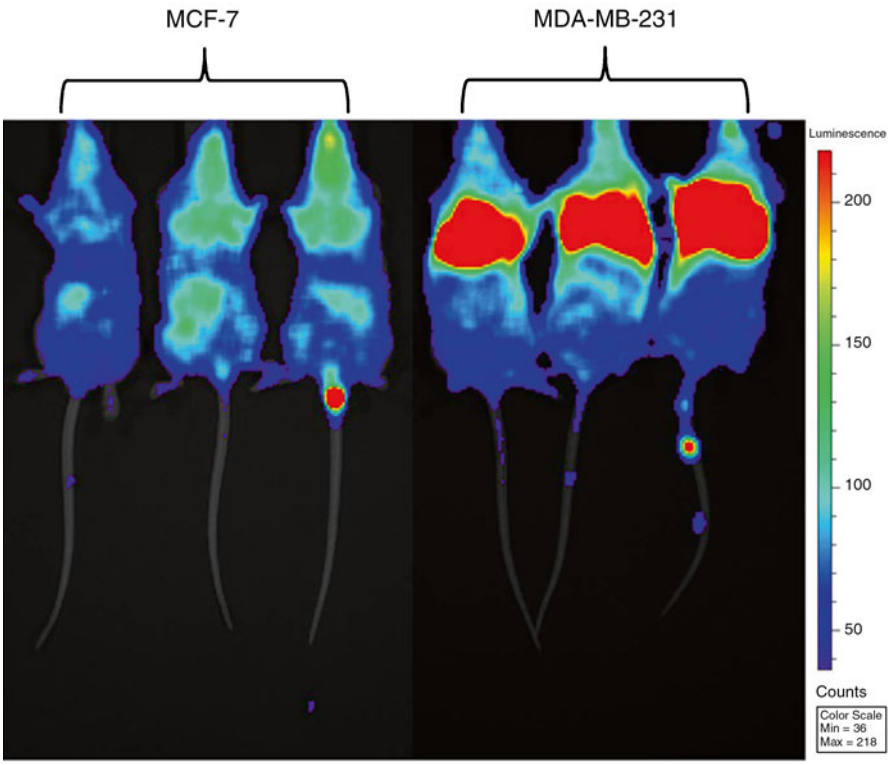

Fig. 8.7 SCID mice injected with Luciferase-labeled MCF-7 and MDA-MB-231 cells. Metastatic cells localize in the lungs of the MDA-MB-231 injected mice. Circulating cells can be seen in the head, neck, lungs, and liver of the MCF-7 injected mice. Image acquired with Perkin-Elmer IVIS Spectrum in vivo imaging system

Step 2: Animal Imaging. The operator needs to turn the IVIS flow and the oxygen "on" and at the same time adjusts the isoflurane dial. A piece of black art paper is placed under the area where the mice will be placed. The mice are placed into the anesthesia chamber. While the mice are "falling asleep," the operator prepares the syringes with 200 μL Luciferin. Once the mice are asleep the operator injects the Luciferin via IP injection. Once all mice are injected with Luciferin they place them in the IVIS chamber. Mice should be placed on their backs with their noses securely in the nose tubes (isoflurane will be released through the tube and it will keep the animal asleep).

Step 3: Living Image is done by following the software of the instrument. It is important to save the image and two have one bitmap image and one csv file for each group. To prepare the graph, take the average flux (p/s) for each group and plot the average flux per group. Include the standard deviation. In Fig. 8.7 is compared two different cells lines MCF7 and MBA-MD-231, whereas the latter one nest in the lung relatively fast as can be fully visualized at 43 days the MCF7 cells remain alive and circulating in the animal for longer period of time without nesting.

8.8 Tail vs. Intra-ventricular Injection Sites for Metastatic Assays

When using mice as a model for metastasis assays, the site of injection is important for proper absorption or distribution of additive. For intravenous (IV) injections, the Laboratory Animal Sciences Program of the National Cancer Institute recommends the use of the lateral tail vein. Although this injection can prove difficult because of the small size of the area of injection, it is readily used in metastasis assays and shown to be an effective means of cell administration, and this is the standard model for metastatic assays [31, 32]. Another option for injection site to observe metastasis is an intra-ventricular injection. This method is used less in literature due to the location of the injection site [32]. In order to inject drugs or molecules to be tested in the lateral ventricles of the brain of experimental animals, a cannula is implanted several days before the experiment, under anesthesia. In most of the protocols, the 3D location of the cannula is reported (anteroposterior, lateral, and depth). The drug or vehicle is injected at a preset schedule using a microinjector at a high rate or at a low rate. Special attention should be paid to the volume of fluid injected, as it will directly depend on the type of animal used and the age of the animal. For example, Passini et al. injected a volume of 2 μL into each lateral ventricle of neonate mice with a finely drawn glass micropipette [33]. When an experiment calls for multiple injections into the lateral ventricle/s of the brain, subcutaneously implanted osmotic minipump connected to an implanted indwelling lateral ventricle cannula similar to that used for the acute infusion studies can be used. At the end of the infusion, the cannula is closed, the infusion pump is removed under anesthesia, and the animals remain alive until the end of the experiment, time at which they are sacrificed and the tissue dissected and processed [34].

8.9 Evaluation of the Metastatic Foci in Liver and Lung

Evaluation of metastatic foci in the lungs and liver is a relatively simple, yet a laborious procedure. After dissection, the lungs and liver are washed in PBS and are placed in Bouin's fixative for a minimum of 24 h. Metastatic foci will appear white whereas the normal tissue will stain yellow (Fig. 8.8a). Quantification of surface metastasis can be done by counting the number of surface metastatic foci under a stereo microscope or by the naked eye. The number of foci can be expressed as a total sum, tumor volume, percentage, or metastatic score [35–39]. The presence of microscopic metastatic foci can be histologically examined by embedding the organ in a paraffin block and staining histological sections with Hematoxylin and Eosin (Fig. 8.8b, c) [35–37].

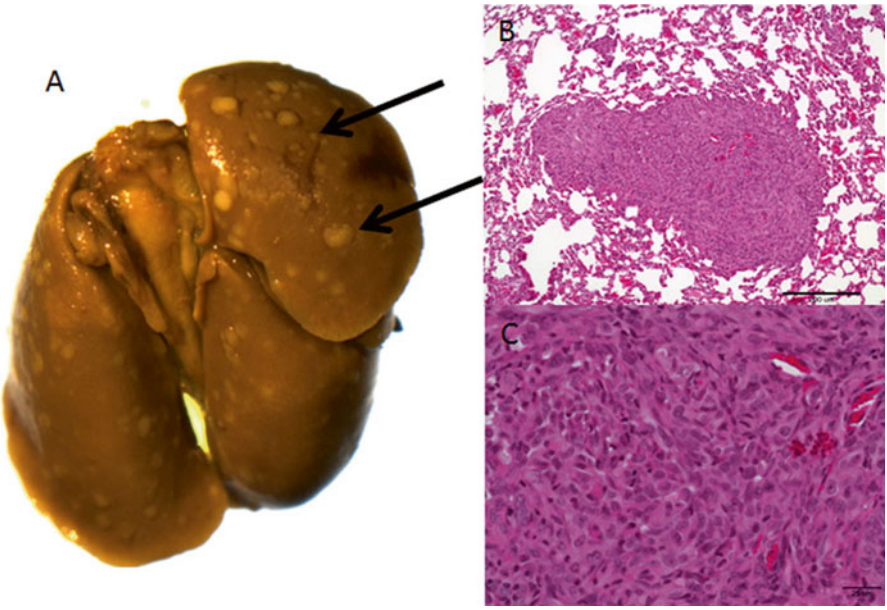

Fig. 8.8 (**a**) Lung metastasis of SCID mice injected with Luciferase-labeled MDA-MB-231 cells. Number of surface metastasis foci is counted under a stereo microscope or by the naked eye. *Arrows* indicate metastatic foci. (**b**) Histological section of metastatic tumor foci 10×. Section was stained with Hematoxylin and Eosin. (**c**) Histological section of metastatic tumor foci 100×. Section was stained with Hematoxylin and Eosin

8.10 Future Directions

Novel techniques for imaging biological processes in live animals are being offered by many companies and could eventually be further developed based on the demands of researchers that are pushing the limits of technology. These state-of-the-art procedures will definitely change our view and understanding of the development of diseases such as cancer. The possibilities are infinite, from the understanding of how cells work in the organism to the monitoring of physiological processes and the effects of drugs and small molecules.

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