Video Article

Initiation of Metastatic Breast Carcinoma by Targeting of the Ductal Epithelium with Adenovirus-Cre: A Novel Transgenic Mouse Model of Breast Cancer

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URL: https://www.jove.com/video/51171
DOI: doi:10.3791/51171

Keywords: Medicine, Issue 85, Transgenic mice, breast cancer, metastasis, intraductal injection, latent mutations, adenovirus-Cre

Date Published: 3/26/2014

Citation: Rutkowski, M.R., Allegrezza, M.J., Svoronos, N., Tesone, A.J., Stephen, T.L., Perales-Puchalt, A., Nguyen, J., Zhang, P.J., Fiering, S.N., Tchou, J., Conejo-Garcia, J.R. Initiation of Metastatic Breast Carcinoma by Targeting of the Ductal Epithelium with Adenovirus-Cre: A Novel Transgenic Mouse Model of Breast Cancer. *J. Vis. Exp.* (85), e51171, doi:10.3791/51171 (2014).

Abstract

Breast cancer is a heterogeneous disease involving complex cellular interactions between the developing tumor and immune system, eventually resulting in exponential tumor growth and metastasis to distal tissues and the collapse of anti-tumor immunity. Many useful animal models exist to study breast cancer, but none completely recapitulate the disease progression that occurs in humans. In order to gain a better understanding of the cellular interactions that result in the formation of latent metastasis and decreased survival, we have generated an inducible transgenic mouse model of YFP-expressing ductal carcinoma that develops after sexual maturity in immune-competent mice and is driven by consistent, endocrine-independent oncogene expression. Activation of YFP, ablation of p53, and expression of an oncogenic form of K-ras was achieved by the delivery of an adenovirus expressing Cre-recombinase into the mammary duct of sexually mature, virgin female mice. Tumors begin to appear 6 weeks after the initiation of oncogenic events. After tumors become apparent, they progress slowly for approximately two weeks before they begin to grow exponentially. After 7-8 weeks post-adenovirus injection, vasculature is observed connecting the tumor mass to distal lymph nodes, with eventual lymphovascular invasion of YFP+ tumor cells to the distal axillary lymph nodes. Infiltrating leukocyte populations are similar to those found in human breast carcinomas, including the presence of αβ and γδ T cells, macrophages and MDSCs. This unique model will facilitate the study of cellular and immunological mechanisms involved in latent metastasis and dormancy in addition to being useful for designing novel immunotherapeutic interventions to treat invasive breast cancer.

Video Link

The video component of this article can be found at https://www.jove.com/video/51171/

Introduction

Breast cancer is the most commonly occurring malignancy in women throughout the world[1,2] and the second leading cause of cancer-related deaths[2]. Complex genetic[3,4], histological[5], and clinical phenotypes[6] are used to characterize the various subtypes of breast cancer and often are used as a means to predict survival. Analysis of a large cohort of women with breast cancer indicated that most (approximately 80%) of the patients that died had recurred within 10 yr post removal of the primary tumor[7]. For a majority of invasive breast carcinomas, lymphovascular invasion has been shown to be strongly correlated to a poor outcome and more aggressive clinical course of disease[8].

Because of the genetic and phenotypic complexity of breast cancer, there is no animal model that recapitulates the entire course of disease. Human breast tumor cell lines have been frequently used as xenograft or orthotopic[9] models of invasive and metastatic breast cancer in immune deficient mice. Although informative, these models occur in the absence of immune pressure and because it is a cross species graft, distort the effects of the entire tumor microenvironment. Inducible genetic mutations driven by mammary specific promoters such as murine mammary tumor virus (MMTV) and whey acidic protein (WAP) have contributed a tremendous amount of knowledge about the genetic nature of breast cancer. However, the tissue specific expression of these promoters is compromised by their responsiveness to the endocrine system[10-16], resulting in the variable expression of induced genetic mutations that do not mirror the expression of oncogenes typically overexpressed in human breast cancer. To overcome endocrine control of MMTV driven expression of oncogenes, Moody *et al.* generated a conditional, doxycycline inducible model overexpressing Neu in the breast epithelium[17]. This model is useful for deinducing Neu after tumor formation

to study regression and recurrence, but requires constant doxycycline administration for consistent, long-term oncogene expression. A comprehensive discussion of the many relevant breast tumor models available can be found in the review by Vargo-Gogola *et al.*[10]

Our goal was to develop a mouse model of traceable breast cancer on a full C57BL/6 background that, after the permanent induction of mutational events, models the formation of a nascent tumor in the presence of immune pressure. We introduced an adenovirus expressing Cre-recombinase into the mammary ducts of transgenic mice containing floxed alleles of *Tp53,* and an oncogenic form of *K-ras,* and YFP. Cre expression ablates *Tp53*, a frequently mutated gene in many breast cancers[18] and induces an oncogenic allele of K-ras in addition to YFP expression specifically in the mammary ductal epithelium. Although mutations in K-ras are infrequent in breast cancer, occurring in only 6.5% of breast cancer patients[19,20], the overexpression of upstream kinases such as Her2/neu and EGFR result in constitutive activation of the Ras signaling pathway in human breast tumors[21-23]. Activation of the Ras signaling pathway in many breast tumor cell lines has also been reported[24,25]. We will describe the initiation of tumor formation and the technique of intraductal injection of an adenovirus expressing Cre-recombinase into sexually mature, virgin female mice. This model of breast cancer develops overt lesions that grow exponentially after about 8 weeks of slow tumor progression, with lymphovascular invasion and metastasis to the axillary lymph node by 7-8 weeks. Because these mice are on a full C57BL/6 background and YFP-expressing tumor cells are traceable in distal lymph nodes, this model provides a relevant tool to study the cellular and immunological mechanisms of latent metastasis and will help to develop novel therapeutic approaches for the treatment of metastatic ductal breast cancer.

Protocol

All animal experiments were approved by the Wistar Institute Animal Care and Use Committee.

1. Generation and Maintenance of Transgenic Mice

1. Breed LSL-K-ras$^{tm4Tyj\ 26}$ and Trp53$^{tm1Brn\ 27}$ (obtained from NCI mouse models of human cancer consortium on a mixed background) to a full C57BL/6 background[28] by backcrossing at least 10 generations with C57BL/6 mice. To track tumor metastasis, breed B6.129X1-*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$/J (LSL-EYFP, obtained from The Jackson Laboratory on a full C57BL/6 background) with double transgenic LSL-K-ras$^{G12D/+}$ p53$^{loxP/loxP}$ mice.
   Note: Transgenic LSL-K-ras$^{G12D/+}$ p53$^{loxP/loxP}$ mice have loxP sites flanking a transcriptionally silenced allele of oncogenic K-ras and the endogenous p53 locus, so that upon Cre-mediated excision, overexpression of an oncogenic K-ras mutant and ablation of p53 is achieved.
   Note: The LSL-EYFP mouse contains a stop codon flanking a gene for enhanced yellow fluorescent protein (YFP) that upon Cre-mediated excision results in the expression of YFP in the tissues where the YFP stop cassette is excised.
   1. Breed transgenic mice to obtain LSL-K-ras$^{G12D/+}$ p53$^{loxP/loxP}$ mice or LSL-K-ras$^{G12D/+}$ p53$^{loxP/loxP}$ LSL-EYFP mice for intraductal injections.
      Note: Mice are bred as homozygous for p53$^{loxP/loxP}$ and heterozygous for LSL-K-ras$^{G12D/+}$ because mice with a homozygous deletion of K-ras die *in utero*. Use naïve virgin females at least six-weeks old for intraductal injections.
      Note: The primers for genotyping homozygous floxed p53 allele are p53-T010-fwd (5'-AAGGGGTATGAGGGACAAGG-3') and p53-T011-rev (5'-GAAGACAGAAAAGGGGAGGG-3'). They produce a wild type allele at 391 bp and the p53 floxed allele at 461 bp[29,30].
      Note: The primers to detect the mutant form of K-ras are oIMR8273 (5'-CGCAGACTGTAGAGCAGCG-3') and oIMR8274 (5'-CCATGGCTTGAGTAAGTCTGC-3'). They produce the mutant band detected at 600bp.
      Note: For the YFP reporter triple transgenic mice, the primers to detect the ROSA cassette (5'-AAGACCGCGAAGAGTTTGTC-3'), the wild type allele (5'-GGAGCGGGAGAAATGGATATG-3'), and a shared allele (5'-AAAGTCGCTCTGAGTTGTTAT-3') result in the amplification of bands at 320 bp for floxed allele and 600 bp for the wild type allele.

2. Surgical Preparation

1. Clean surgical materials with 75% EtOH and autoclave them before and after all injections.
2. Perform surgery on a clean uncluttered laboratory bench in a sanitized room within an animal facility. Wipe down all surfaces including the stage and dials of the surgical microscope with a broad-spectrum disinfectant solution followed by 75% EtOH.
3. Weigh and anesthetize mice by intraperitoneal injection of a mix of ketamine (80-100 mg/kg) and xylazine (8-10 mg/kg) in sterile saline.
4. Gently place mice back into their cages undisturbed for 5 min while they go under anesthesia. During this time generate virus precipitates (see Protocol 3).
5. Verify lack of response to pain by toe pinching. Gently cover the eyes of anesthetized mice with veterinary ointment to prevent excessive corneal drying.
6. To prevent hypothermia, place anesthetized mice onto a heating pad set to **low heat** during the surgical procedure and until they begin to recover.
7. For the management of pain, administer mice meloxicam subcutaneously at 1 mg/kg before the surgery and 24 hr after.

3. Generation of Virus Precipitates

CAUTION: Adenovirus vectors, although they have been modified and are unable to replicate, pose the risk of infection. Handle adenovirus with caution. All personnel should be appropriately trained according to the institution's guidelines for handling BSL2 agents After intraductal injection, dispose of adenovirus in accordance with BSL2 guidelines.

1. Store adenovirus concentrated virus stocks at -80 °C frozen in aliquots of 4 x 10^8 pfu each, sufficient for injecting 16 animals with 3 µl of 2.5 x 10^7 pfu of adenovirus particles.
2. Store adenovirus aliquots on dry ice until approximately 15-20 min before beginning the injections.
   Note: Avoid repeated freeze thaw cycles, as virus titer drops significantly between each cycle.

Note: Adenovirus precipitates are formed by modifying a protocol described previously [31].

3. Reconstitute 504 mg of MEM powder with 50 ml of sterile molecular grade water, supplement with 244 mg of sodium bicarbonate and filter in sterile conditions and store at 4 °C.
    1. Prepare the calcium chloride solution by adding 1.5 g of calcium chloride to 50 ml of molecular grade water and filter in sterile conditions and store at 4 °C.
    2. Mix aliquots containing 4 x 10^8 pfu adenovirus-Cre with sufficient 3% sucrose in sterile water for a final volume of 10 µl. Add 34 µl of MEM to the virus and gently mix. Then add 4 µl of the $CaCl_2$ solution, gently mix, and incubate at room temperature for 15-20 min.
    3. Store adenovirus on dry ice until ready to form precipitates. Avoid thawing of the adenovirus and storing on ice or room temperature for extended times, unless precipitates are formed.
    Note: It is also possible to mix the sucrose, MEM and $CaCl_2$ prior to the surgeries if it is not possible to thaw the adenovirus aliquot and begin making precipitates immediately after removal from -80 °C. This aliquot of sucrose, MEM, and calcium can be saved on dry ice until ready to add the adenovirus.
    Note: Virus particles are stable for approximately 1 hr.
    4. Prior to each injection, gently flick the tube to make sure virus particles are mixed. Draw up 3 µl (2.5 x 10^7 pfu) of virus particles into the 10 µl syringe and prepare the mouse for the intraductal injection.

4. Intraductal Injection of Virus Particles

1. Gently place the mouse on its back onto the illuminated stage of a clean dissection microscope. Illuminate the abdominal side with an extra light source and locate the left 4th or right 9th inguinal mammary gland by the small white patches of fur (visible on C57BL/6 females) surrounding each nipple.
2. Rub the nipple gently with a sterile ethanol soaked cotton tipped applicator to clear hair away from the nipple and to sterilize the injection site. If they are difficult to locate, gently apply a thick layer of a depilatory cream or use shears to expose the nipples.
3. **Remove the keratin plug**, a layer of dense dead skin cells, which is covering the nipple. Once the nipple is exposed, the keratin plug should be easily visible under the dissection microscope.
4. Secure the nipple with fine surgical forceps and pull up with light force to remove the keratin plug.
5. Stabilize the nipple **between** the forceps.
6. Gently insert the needle between the forceps, cannulating the duct canal at 90°. Enter the nipple slightly past the bevel of the needle (not more than 2 mm) to prevent penetration through the mammary tissue and into the serous membranes of the ventral body cavity.
    1. **Do not insert the needle too deep.** To ensure proper depth of injection, gently pull the needle up after inserting it into the lumen of the duct, drawing the nipple up along the edges of the needle as it is pulled up.
    Note: Visualization of the injection is difficult; therefore practice for this step is recommended using trypan blue.
7. When the needle is appropriately placed into the mammary duct, release the 3 µl of virus precipitates (2.5 x 10^7 pfu of adenovirus-Cre) by gently plunging the syringe with the thumb of the hand holding the syringe. The nipple should slightly inflate as the liquid is added.

5. Recovery of Mice

1. Place the mouse back onto the heating pad after the injection until it begins to recover from the anesthesia.
2. Once the mouse is recovered, place it back into a clean cage and monitor for full recovery and movement.
3. 24 hr after the intraductal injection, subcutaneously administer meloxicam at 1 mg/kg.

6. Monitoring Tumor Progression

1. Palpate the injected mammary gland at day 30 for enlargement and swelling.
    1. Monitor tumor progression every 5-7 days once a swollen and enlarged mammary gland is observed.
2. Measure tumor volumes every 3-4 days for tumor growth kinetics once palpable tumors appear (approximately 50-60 days post adenoviral injection).
3. Euthanize mice when tumor volumes exceed 10% of the body weight of the mice.

Representative Results

Successful targeting of the mammary ductal tree can be visualized by preparing whole mounts of the mammary gland as previously described[32] after injection of trypan blue (to verify proper injection technique (**Figure 1A**) or an adenovirus expressing mCherry (to verify proper viral preparation and infection of ductal epithelial cells, **Figure 1B**).

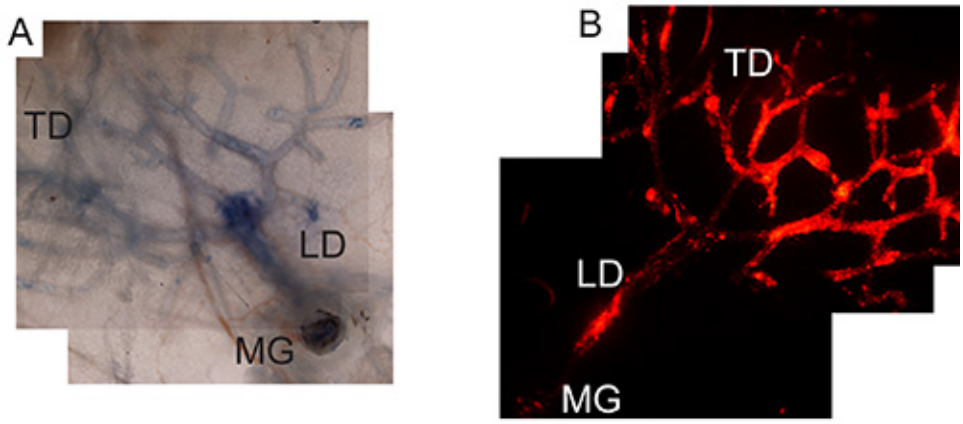

Figure 1. Intraductal targeting of mammary glands by injection with trypan blue or adenovirus-mCherry. A) A whole mount, as previously reported[32], of the mammary gland after injection of mammary gland #4 with trypan blue was prepared 3 hr post injection to visualize/confirm targeting of the entire ductal tree. Images are 4X magnification. **B**) Infection of the ductal epithelium with adenovirus by injecting 2.5 x 10^7 pfu of adenovirus expressing mCherry. Mice were injected intraductally, and 4 days post-injection, a whole mount of the mammary gland was prepared to confirm viral infection of the ductal tree. Image is 4X magnification. MG is mammary gland, LD is the lactiferous, or main duct, and TD is the terminal duct. Click here to view larger image.

When tumors are induced in $p53^{loxP/loxP}$ LSL-K-$ras^{G12D/+}$ transgenic mice, tumors will not be apparent until around day 40 when the mammary glands will become enlarged and swollen. It is necessary to begin palpations when this is observed, monitoring tumor growth every 5-7 days. In our hands, hardening of the mammary gland always precedes the onset of tumor development. Tumors will progress slowly for an additional 2 weeks. Beginning around day 56, tumors will begin to grow exponentially (**Figure 2B**). At this point, it is critical to measure tumor volumes every 3 days if kinetic studies are desired because there will be slight mouse to mouse variability in tumor progression, which is normal (**Figures 2A** and **3A**). Large abdominal masses will be apparent by day 80 (**Figure 2A, 2B,** and **3A**), after which mice should be euthanized if tumors exceed more than 10% of their body weight. cDNA analysis of three clones from a tumor-bearing mouse revealed expression of mesothelin, cytokeratin-8, Her2/neu, and estrogen receptor-α (**Figure 2C**).

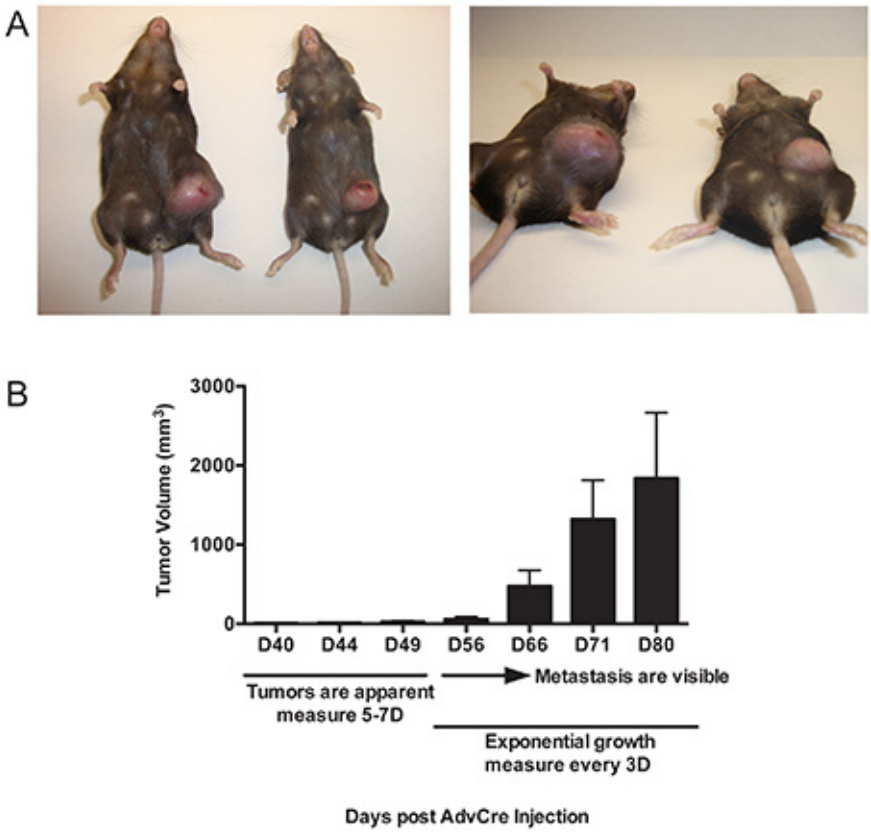

Figure 2. Tumor development in p53$^{loxP/loxP}$ LSL-K-ras$^{G12D/+}$ mice injected intraductally with adenovirus-Cre. A) Two examples of tumors 80 days after injection with adenovirus expressing Cre. Mice were given 2.5 x 10^7 PFU of adenovirus expressing Cre and 80 days post tumor-initiation, large palpable masses can be visualized protruding from the abdominal side of the animal. **B**) Typical tumor kinetics and palpation schedule for tumors induced in p53$^{loxP/loxP}$ LSL-K-ras$^{G12D/+}$ mice. **C**) Characterization of three tumor cell clones derived from the same homogenized tumor of a p53$^{loxP/loxP}$ LSL-K-ras$^{G12D/+}$ mouse. RNA was extracted and cDNA synthesized for RT-PCR analysis using primers specific to β-actin, mesothelin, cytokeratin-8, Her2/neu, progesterone receptor, estrogen receptor-α, and estrogen receptor-β. Click here to view larger image.

Similar to the cellular microenvironment in human breast cancer, we have observed infiltration of αβ and γδ T cells as well as myeloid derived suppressor cells and macrophages into the tumors (**Figure 4**). Vasculature draining to the axillary lymph node will begin to engorge before tumors have grown to encompass the entire mammary tissue where the injection was performed (**Figure 3A**). Lymphovascular invasion and metastasis of tumor cells can be tracked by crossing LSL-K-ras$^{G12D/+}$ p53$^{loxP/loxP}$ mice with LSL-EYFP mice. After Cre-mediated excision, tumor cells expressing YFP (both high and low) are detected in the tumor and can be traced metastasizing to the draining axillary lymph node (**Figure 3B**). Metastasis to the distal axillary lymph node was confirmed by successfully culturing a tumor cell line from this site in a tumor-bearing LSL-K-ras$^{G12D/+}$ p53$^{loxP/loxP}$ mouse (data not shown).

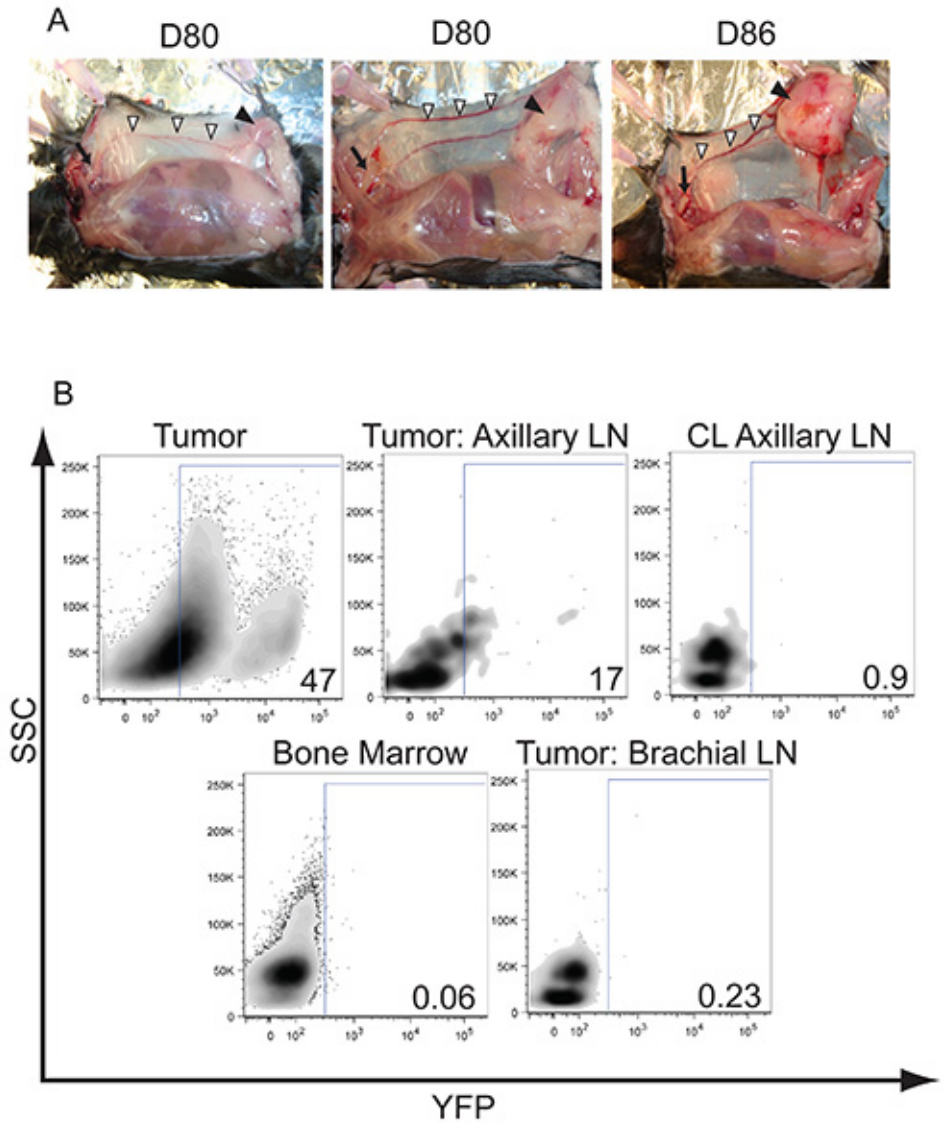

Figure 3. Formation of tumors and latent metastasis to the axillary lymph node. A) Example of three advanced breast tumors with different rates of tumor progression. A solid mass, indicated by the arrowhead, forms and eventually grows to the size of the entire abdominal mammary tissue. The tumor stays confined to the mammary tissue and is not observed to invade or attach to the muscle covering the peritoneal cavity. There is evident engorgement of the superficial epigastric vein between the inguinal and axillary lymph nodes, denoted by white arrowheads. After 7-8 weeks, the axillary lymph node begins to become enlarged due to lymphovascular invasion of the tumor cells, indicated by arrow. **B**) Metastasis of YFP positive tumor cells can be visualized in the axillary lymph node by flow cytometry. LSL-K-ras$^{G12D/+}$ p53$^{loxP/loxP}$ LSL-EYFP mice were used to induce tumors and activation of YFP by intraductal delivery of adenovirus-Cre. To verify lymphovascular invasion of tumor cells into the axillary lymph node, 80 days post adenoviral injection, the indicated lymph nodes and organs were harvested and stained for lymphocyte markers and examined for YFP expression. CL represents contralateral nontumor draining lymph node. Results represent gating on CD45 negative tumor cells, indicating the tumor cells are invading the distal axillary lymph node. Numbers represent percent YFP positive cells from total population. Click here to view larger image.

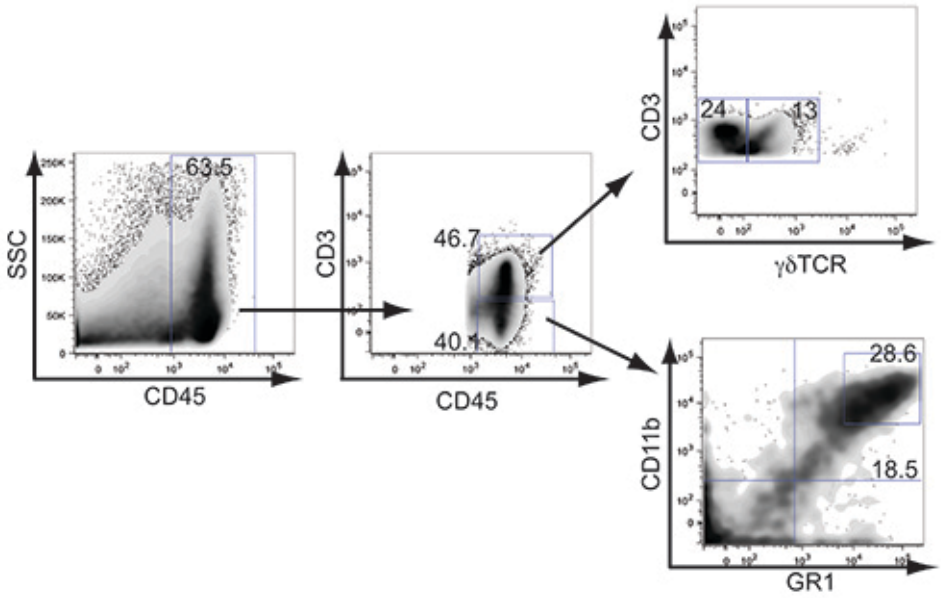

Figure 4. Immune infiltrates in mouse transgenic breast tumors. Mouse breast tumors were homogenized and stained for CD45, CD3, γδTCR, CD11b and GR1. Numbers represent percent of positive leukocytes in entire tumor (63.5), total $CD3^+$ (46.7), total CD3 negative (40.1), total $CD3^+$ $γδ^+$ (γδ T cells, 13), $CD3^+$ γδnegative (24), total GR1 high CD11b (MDSC, 28.6) and total CD11b GR1 low (macrophages, 18.5). Click here to view larger image.

Due to the anatomy of the mouse and technique of intraductal injections, we find targeting of mammary glands 4 and 9 (**Figure 5**) yields the most consistent results and reliable injections. However, any gland can be targeted depending upon the preference of the technician performing the surgery.

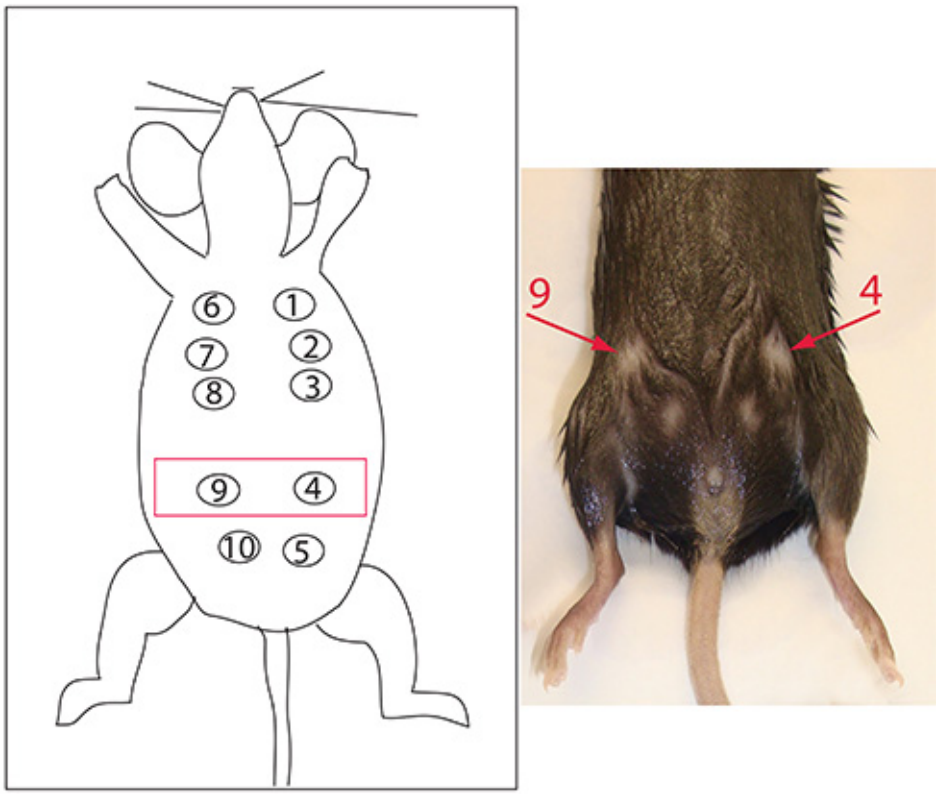

Figure 5. Numbering of mammary ducts. Mammary ducts 4 and 9 are highlighted in red on the diagram and indicated with arrows on the mouse. In our hands, we found that injections were easiest to perform on these mammary ducts, however all other mammary tissues that we targeted developed tumors with similar kinetics. Click here to view larger image.

Discussion

The success of this procedure hinges on proper technique during intraductal injections, which will be difficult for untrained experimenters. Experienced researchers in our laboratory typically obtain breast tumor masses 79% of the times they administer adenoviral injections. Problems with the injection can result in significantly delayed, variable, or absent tumor development. If the needle is inserted too deep or at an inappropriate angle, the ductal canal may be missed. It is important to enter the nipple slightly past the bevel of the needle (not more than 2 mm), to prevent penetration through the mammary tissue and into the serous membranes of the ventral body cavity. Also, too shallow placement of the needle or the injection of greater than 3 ml of virus precipitates can result in spillage of viral prep outside the mammary gland and the induction of unintentional tumors. One way to overcome these issues is to insert the needle into the nipple slightly deeper than 3 mm, and slowly draw the syringe back up out of the duct until 2 mm from the tip of the bevel. This will ensure that mammary tissue is targeted instead of the muscle surrounding the peritoneal cavity of mice (**Figure 1A**). This will also stretch the nipple along the edges of the syringe so that when the virus is expelled into the duct, there is no spillage and loss of viral precipitates.

Visualization of the injection is difficult and practice for this step is recommended. We have observed an increase in successful injections following practice, resulting in a higher penetrance of tumor development. Because this technique uses nonlactating virgin females, it is critical to remove the keratin plug covering the nipple to reveal the underlying duct canal. We recommend practicing this step by injecting trypan blue or some other sterile traceable dye inside of the duct and preparing whole mammary mounts to confirm targeting of the ductal tree. Additionally, other protocols describing intraductal injection of reagents have been published[33,34], which may be useful for developing proper technique. Issues with viral preparation or infection of the ductal lumen can also be investigated by using a mCherry expressing adenovirus. Nontransgenic mice can be used for each of these purposes until the injection technique is optimized.

Although any mammary gland can be used to initiate tumors, we have achieved the most consistent growth rates by targeting mammary gland 4 or 9, which we believe is because it is easier to perform proper injections on these glands, resulting in more efficient targeting of the duct. The proximity of the left 4th or right 9th inguinal mammary glands to the draining inguinal lymph node is also useful to examine anti-tumor immune responses during different temporal points of tumor progression. To model and track latent metastasis to distal sites, transgenic mice were crossed with LSL-EYFP mice. As the tumor progresses, vasculature connecting the tumor to the axillary lymph node will become slightly engorged at approximately 5 weeks, before the tumor begins to grow exponentially (**Figure 3A**). Eventually, after 7-8 weeks, lymphovascular invasion will result in tumor growth within the axillary lymph node (**Figures 3A** and **3B**). Using reporter mice and Cre-loxP technology, incorporation of YFP creates a platform to track tumor cells metastasizing to distal sites throughout tumor progression. This can facilitate studies aimed at elucidating the cellular and epigenetic mechanisms that promote latent metastasis. In our hands, breast tumor cell lines expressed cytokeratin-8, mesothelin, estrogen receptor-α, and Her2/neu, confirming targeting of the ductal epithelium. However, depending upon the mutations induced and due to the difficulty and the variability of injections, we recommend histological characterization of tumors once the model is well established in the laboratory.

Because breast cancer is such a deadly and pervasive disease[1,2], it is important to use animal models that accurately recapitulate the complex interplay between tumor and host. Here we describe a fully backcrossed C57BL/6 murine model of breast tumor. First, by inducing tumors from native cells, we allow the tumor to evolve naturally in a full immune microenvironment. The immune microenvironment in the advanced mouse breast tumors recapitulates the populations of αβ and γδ T cells, myeloid derived suppressor cells, and macrophages commonly observed in human breast cancer (**Figure 4**). As we have published previously using adenovirus-Cre to induce ovarian tumors, we found that the viral injection had negligible effect on the corresponding tumor infiltrates and tumor progression[28]. Second, endocrine independent expression of oncogenes ensures that tumor cells have persistently high levels of target gene expression. Third, by taking advantage of latent mutations, we can control the timing of tumorigenesis to facilitate precise temporal tracking of tumor evolution. Applications of this model include research on tumor cell biology, studies on factors in the tumor microenvironment, anti-tumor immune responses, and even efficacy evaluation of new therapeutics. Through the availability of the Cre-loxP system, this technique can be used as a platform for investigating a diverse array of additional mutations in the initiation and progression of breast tumors. We hope that the use of this model will enhance the understanding of breast cancer biology and eventually lead to new therapeutics aimed at treating metastatic breast cancer.

Disclosures

The authors declare no competing financial interests.

Acknowledgements

This work was supported by NCI Grants RO1CA157664 and RO1CA124515, and a Breast Cancer Alliance award. We would like to thank Jeffrey Faust, David Ambrose, and Scott Weiss from the Wistar Flow Cytometry core facility, James Hayden from the Wistar Imaging facility, and the entire staff of the Wistar Institute Animal Facility for their invaluable technical support.

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