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Modeling breast cancer via intraductal injection of Cre-expressing adenovirus to mouse mammary gland

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Assistant Professor of Medicine

January 30, 2019

Vineeta Bajaj, Ph.D.
Review Editor
JoVE

Dear Dr. Bajaj,

We resubmit our further revised manuscript, "*Modeling breast cancer via intraductal injection of Cre-expressing adenovirus to mouse mammary gland*" (JoVE59502), for consideration of publication in *JoVE*.

In the revision, we addressed all the specific comments marked in the manuscript. We also highlighted the steps corresponding to the intraductal injection procedure of this protocol. For a detailed point-to-point response, a rebuttal letter is enclosed.

We thank you again for considering our manuscript.

Yours sincerely,

Zhe Li, Ph.D.

TITLE:

Modeling Breast Cancer via an Intraductal Injection of Cre-Expressing Adenovirus into the Mouse Mammary Gland

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KEYWORDS:

Breast cancer, mammary tumor, mouse modeling, intraductal injection, mammary gland, cellular origin, mammary epithelial cell, Cre/loxP recombination, adenovirus

SUMMARY:

The goal of this protocol is to describe a new breast cancer modeling approach based on the intraductal injection of Cre-expressing adenovirus into mouse mammary glands. This approach allows both cell-type- and organ-specific manipulation of oncogenic events in a temporally controlled manner.

ABSTRACT:

Breast cancer is a heterogeneous disease, possibly due to complex interactions between different cells of origins and oncogenic events. Mouse models are instrumental in gaining insights into these complex processes. Although many mouse models have been developed to study contributions of various oncogenic events and cells of origin to breast tumorigenesis, these models are often not cell-type or organ specific or cannot induce the initiation of mammary tumorigenesis in a temporally controlled manner. Here we describe a protocol to generate a new type of breast cancer mouse models based on the intraductal injection of Cre-expressing adenovirus (Ad-Cre) into mouse mammary glands (MGs). Due to the direct injection of Ad-Cre into mammary ducts, this approach is MG specific, without any unwanted cancer induction in other organs. The intraductal injection procedure can be performed in mice at different stages of their MG development (thus, it permits temporal control of cancer induction, starting from ~3–4 weeks of age). The cell-type specificity can be achieved by using different cell-type-specific promoters to drive Cre expression in the adenoviral vector. We show that luminal and basal mammary epithelial cells (MECs) can be tightly targeted for Cre/loxP-based genetic manipulation via an intraductal injection of Ad-Cre under the control of the Keratin 8 or Keratin 5 promoter, respectively. By incorporating a conditional Cre reporter (e.g., Cre/loxP-inducible *Rosa26-YFP*

reporter), we show that MECs targeted by Ad-Cre, and tumor cells derived from them, can be traced by following the reporter-positive cells after intraductal injection.

INTRODUCTION:

The overall goal of this method is to develop a new breast cancer modeling approach based on an intraductal injection of Ad-Cre into the mouse MG. The Cre/loxP recombination-based genetic approach has been widely used to model human breast cancer in mice. The first generation of Cre/loxP-based breast cancer mouse models are generated by using Cre-expressing transgenic mice under the control of MEC-specific promoters (e.g., *MMTV-Cre* for luminal MECs and a portion of basal MECs, *Wap-Cre* and *Blg-Cre* for luminal progenitors and alveolar luminal MECs, *K14-Cre* for basal and a portion of luminal MECs[1–5])[6–9]. However, while these Cre transgenic lines enable spatial control of Cre expression (i.e., in different subsets of MECs), they do not allow temporal control of Cre expression and Cre/loxP-mediated genetic manipulation. The second generation of Cre/loxP-based breast cancer mouse models utilize inducible Cre activity/expression approaches (e.g., use of Cre-estrogen receptor fusion [CreER], which can only induce Cre/loxP recombination upon administration of tamoxifen), and as a result, these genetic tools permit both spatial and temporal controls of the activation of oncogenic events in MECs (e.g., *K8-CreER*- and *K5-CreER*-based models)[10–12]. In both generations of breast cancer mouse models, as promoters used to drive Cre or CreER expression (e.g., *Krt8*, *Krt5*) may also be active in epithelial cells of other organs (i.e., they are cell-type-specific but not organ-specific) or have a leaky expression in cell types other than epithelial cells (e.g., *MMTV*, which has leaky activity in bone marrow hematopoietic cells), these approaches may lead to the development of unwanted cancer(s) in other organ(s). If these unexpected cancers cause lethality in the affected mice, the original purpose of modeling breast cancer in these mice may be prohibited (e.g., *MMTV-Cre*-driven oncogenic events may lead to hematopoietic malignancies and early death of the mice, due to leakiness of the *MMTV* promoter in hematopoietic cells)[4].

Here we report a breast cancer modeling approach in mice that allows both cell-type- and organ-specific manipulation of oncogenic events in a temporally controlled manner. This approach is based on an intraductal injection of Ad-Cre into mouse MGs (and is, thus, organ-specific). Cre expression can be controlled by using different MEC subpopulation-specific promoters embedded in the adenoviral vector (e.g., *Krt8* for luminal MECs, *Krt5* for basal MECs, thus achieving cell-type specificity). Cancer induction in MGs can be temporally controlled by an injection of Ad-Cre into mice at different ages, starting from 3–4 weeks of age (pubertal) to the adult stage.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Brigham and Women's Hospital.

1. Generation and maintenance of floxed mice

1.1. Obtain breast cancer-relevant floxed conditional knockout (e.g., *Trp53*^{tm1Brn} [referred to as *Trp53*$^{L/L}$], *Brca1*^{tm1Aash} [*Brca1*$^{L/L}$]) or conditional knock-in mouse lines (e.g.,

Gt(ROSA)26Sor$^{tm1(Pik3ca*H1047R)Egan}$) from The Jackson Laboratory (JAX) or NCI Mouse Models of Human Cancer Consortium (MMHCC) repository. In addition, to facilitate the chasing of MECs that undergo Cre-mediated recombination, a conditional Cre-reporter line can also be obtained from JAX (e.g., *Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$ [referred to as *R26Y*]).

1.2. Breed *Trp53*$^{L/L}$ homozygous mice with *R26Y* homozygous reporter mice or with homozygous mice carrying the *R26Y* reporter alleles and any additional floxed conditional knockout or knock-in alleles for different mouse models, to obtain heterozygous F1 male and female progeny.

1.3. Intercross heterozygous F1 male and female mice to obtain F2 compound female mice that are homozygous for each allele (as experimental mice), as well as *R26Y*-only homozygous females (as control mice). Genotype F2 mice based on the PCR primers and cycling conditions listed below, by setting up two standard 20 µL PCR reactions (using Taq 5X Master Mix) in two different PCR tubes, one with the *R26Y* primers and the other with the *Trp53*^{L} primers. Use adult mice (typically around 2–4 months of age) for all breeding.

1.3.1. For *R26Y*, perform PCR at 94 °C for 3 min, then at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min for 35 cycles, followed by 72 °C for 3 min, and maintaining at 14 °C. Use primers (i) R26YFP-1: AAA GTC GCT CTG AGT TGT TAT; (ii) R26YFP-2: GCG AAG AGT TTG TCC TCA ACC; (iii) R26YFP-3: GGA GCG GGA GAA ATG GAT ATG.

NOTE: A single PCR band of 250 bp indicates an *R26Y* homozygote, a single PCR band of 500 bp indicates a wild-type (WT), and two PCR bands (*R26Y*: 250 bp, WT: 500 bp) indicate an *R26Y* heterozygote (**Figure 1A**).

For *Trp53*^{L}, perform PCR at 94 °C for 3 min, then at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min for 35 cycles, followed by 72 °C for 3 min, and maintaining at 14 °C. Use primers (i) p53F2-10 1F: CAC AAA AAC AGG TTA AAC CCA G; (ii) p53F2-10 1R: AGC ACA TAG GAG GCA GAG AC.

NOTE: A single PCR band of 370 bp indicates a *Trp53*$^{L/L}$ homozygote, a single PCR band of 288 bp indicates a *Trp53*$^{+/+}$ WT, and two PCR bands (WT: 288 bp, *Trp53*^{L}: 370 bp) indicate a *Trp53*$^{L/+}$ heterozygote (**Figure 1B**).

2. Preoperative preparation

2.1. Autoclave all surgical tools 1 day before the surgery.

2.2. Prepare 0.1% bromophenol blue in phosphate-buffered saline (PBS) and store it at 4 °C. Dilute the Ad-Cre that will be used for the intraductal injection in DMEM medium with 0.01 M $CaCl_2$ and bromophenol blue at a ratio of 1:10 (i.e., the injection mixture).

NOTE: The Ad-Cre used here was obtained from the University of Iowa Viral Vector Core, with a stock viral titer of ~10^{10}–10^{11} pfu/mL.

2.3. Anesthetize the female mouse (F2 generation as described in step 1.2, age ranging from 3–4 weeks of age to adult) using an isoflurane chamber and apply eye ointment. During the procedure, anesthetize the mouse continuously by ensuring it inhales 1%–2.5% isoflurane in oxygen. Check the depth of anesthesia at least every 15 min by performing a toe pinch. Carefully monitor the mouse for any change in respiratory rate, adjusting the level of isoflurane accordingly, if needed.

2.4. Inject meloxicam as analgesia subcutaneously at a dose of 5 mg/kg, prior to the surgical procedure.

2.5. Expose the nipple surgical site by applying several drops of hair removal cream; remove excessive cream and loose hair using soft paper towels.

NOTE: Perform this step in an area separate from where the surgery is to be performed. Shaving is not recommended in order to avoid damage to the nipples.

2.6. Disinfect the surgical site with iodophors first, followed by 70% alcohol, and end with a final application of scrub iodophors. Do this in a circular motion from the center of the work area toward the periphery using a gauze sponge or cotton-tipped applicator. Repeat the cycle 3x–4x.

3. Intraductal injection

3.1. Use aseptic techniques throughout the surgical procedure.

3.2. Make an incision site on the skin at a length of ~1 cm between the two fourth inguinal MGs (**Figure 2**). Carefully separate the skin flap (with the MG) from the parietal peritoneum so as to visualize the mammary ductal tree.

3.3. Carefully hold the nipple with Watchmaker's forceps and remove the exterior nipple without cutting any nearby skin, using a micro-dissection scissor.

3.4. Load ~3–5 µL of Ad-Cre injection mixture into a 25 µL Hamilton syringe with a 33 G metal hub needle affixed. Estimate the volume of the injection mixture in the syringe based on the blue dye included in the mixture.

NOTE: Use a smaller volume (e.g., 3 µL) when injecting into MGs of 3–4 weeks old females and a larger volume (e.g., 10 µL) when injecting into MGs of lactating females.

3.5. Gently hold the edge of the skin flap with a fine curved tweezer and inject the Ad-Cre injection mixture slowly into the nipple, meanwhile monitoring the spreading of blue dye into the mammary ductal tree. Maintain the injection rate as low as possible to avoid damage to the ductal lumen.

NOTE: Injected fluid (as illustrated by the included bromophenol blue dye) spreading throughout

the entire ductal tree without leaking into the stromal compartment indicates a successful intraductal injection.

3.6. Gently withdraw the needle from the nipple to avoid any leakage of the injected fluid.

3.7. Examine the distal side (i.e., away from the nipple) of the MG or the surrounding area of the injected nipple. Note that swelling blue dye (i.e., dye diffusing into the nearby stroma) indicates a mammary fat pad injection rather than a successful intraductal injection.

3.8. Close the surgical wounds (from step 3.2) in the skin with wound clips.

4. Postoperative care

4.1. Remove the mouse from the anesthesia and place it on a heating pad inside a clean cage for recovery.

4.2. Administer meloxicam subcutaneously at 5 mg/kg again, 24 h after the surgery.

4.3. Monitor the general conditions of the animal and look for signs of infection at the incision site for 5 days.

5. Monitoring the development of the mammary tumor

5.1. Monitor the injected mice weekly by palpation for any sign of mammary tumor development.

5.2. Once the tumor is palpable, monitor the mouse 2x a week until it reaches the experimental endpoint, as determined by the size (e.g., reaching 10%–15% of the mouse's body weight) or condition (e.g., ulcerated or necrotic) of the tumor, or by the general health condition of the mouse (e.g., comatose, moribund).

5.3. Euthanize the mouse by carbon dioxide asphyxiation.

5.4. Isolate the mammary tumor tissues and analyze them by flow cytometry, immunofluorescence, or expression profiling (e.g., by RNA sequencing [RNAseq] or microarray), as described previously[12].

5.5. Perform flow cytometric analysis by gating for lineage-negative cells (Lin^-: negative for lineage markers CD45 [leukocyte marker], CD31 [endothelial cell marker], and TER119 [erythrocyte marker]), and analyze the cells in the tumor based on their expression of YFP, CD24, and CD29.

REPRESENTATIVE RESULTS:

Representative PCR genotyping results for the *R26Y* and *Trp53*^{L} alleles are shown in **Figure 1**.

Although, in principle, all 10 MGs can be subjected to the intraductal injection procedure, practically, the two fourth inguinal MGs are typically selected for injection, due to their easier accessibility and larger MG sizes (**Figure 2**). During the surgery, it is important to maintain a disinfected and uncluttered working area and perform the procedure with sterile tools (**Figure 3**). During the intraductal injection, the inclusion of a blue dye (e.g., bromophenol blue) in the injection mixture helps the visualization of a successful injection of Ad-Cre into the entire ductal tree (**Figure 4**). The youngest female mice in which intraductal injection (with a smaller volume of the injection mixture) can be performed successfully are those at ~3 weeks of age (**Figure 4A**), although for most mammary tumor induction experiments, young adult female mice (e.g., 2 months of age) are typically used (**Figure 4B**). In addition, intraductal injection (with a larger volume of the injection mixture) can also be performed in female mice during early/mid-gestation to target alveolar cells (**Figure 4C**).

In our experience, in mice with the *R26Y* reporter and *Trp53*$^{L/L}$ (with or without any additional conditional alleles), Cre-mediated recombination disrupted the *Trp53* conditional knockout alleles (and any additional conditional knockout alleles, if used) and, meanwhile, turned on the YFP reporter (from the *R26Y* allele, as well as from any additional conditional knock-in allele, if used). To target different MEC subpopulations for mammary tumor induction, Ad-Cre viruses under the control of different MEC subset-specific promoters were used for injection (**Figure 5**). For instance, Ad-Cre under the control of Keratin 8 (*Krt8*) promoter (*Ad-K8-Cre*) was used to target luminal MECs. Previously, we reported the use of Ad-Cre under the control of the Keratin 14 (*Krt14*) promoter (*Ad-K14-Cre*) to target basal MECs[13]. However, as we reported, intraductal injection of *Ad-K14-Cre* not only targeted basal MECs but also a portion of luminal MECs[13]. We recently tested another Ad-Cre under the control of Keratin 5 (*Krt5*) promoter (*Ad-K5-Cre*)[14] and found that it can more tightly target the basal lineage, leading to genetic marking of only basal MECs (**Figure 5**). The typical percentages of YFP-marked MECs from either *Ad-K8-Cre* or *Ad-K5-Cre* injection are about 0.1%–1%.

For *Trp53*$^{L/L}$*; R26Y* female mice under the FVB genetic background, the intraductal injection of *Ad-K8-Cre*, which targets their luminal MECs, led to the development of mammary tumors several months after the injection (**Figure 6A**). Mice with a different genetic background (e.g., C57/B6) may exhibit a longer latency of developing mammary tumors after injection. Due to the inclusion of the conditional *R26Y* reporter, tumor epithelial cells were typically marked by YFP and could be detected by flow cytometry (**Figure 6B**); they could be enriched by the flow-sorting of YFP^{+} cells for further analysis.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative PCR genotyping results for the *R26Y* and *Trp53*^{L} alleles. WT = wild-type; Homo = homozygote; Het = heterozygote.

Figure 2: Schematic diagram of the intraductal injection of Ad-Cre virus into an MG. (**A**) Incision site in the midline between the two fourth MGs. (**B**) Intraductal injection of Ad-Cre with a blue

dye (for better visualization) into one of the fourth MGs. (**C**) Closing of the incision in the skin by wound clips.

Figure 3: Overview of the aseptic setup for rodent surgery.

Figure 4: Visualization of a successful intraductal injection into the entire mammary ductal tree. (**A**) An example of the intraductal injection of 3 µL of injection mixture (with bromophenol blue) into an MG of a 3-week-old female mouse. (**B**) Intraductal injection of 5 µL of injection mixture into an MG of a young adult female mouse. (**C**) Intraductal injection of 10 µL of injection mixture into an MG of a female mouse at early/mid-gestation.

Figure 5: Representative plots of the flow cytometric analysis of YFP-marked cells upon intraductal injection. YFP^+ populations from MGs of *R26Y* virgin females 3 days after an intraductal injection of *Ad-K8-Cre* (left, injection at a titer of 7 x 10^9 pfu/mL) or *Ad-K5-Cre* (right, injection at a titer of 7.86 x 10^9 pfu/mL) viruses. Plots are based on an analysis of CD24 and CD29 staining in lineage-negative (Lin^-; i.e., negative for CD45, CD31, and TER119 expression) YFP^+ cells. Lu = $Lin^-CD24^{high}CD29^{low}$ luminal MEC gate; Ba = $Lin^-CD24^{low}CD29^{high}$ basal MEC gate; the gating strategy for luminal and basal MECs is based on Shackleton et al.[15].

Figure 6: Tumor development in *$Trp53^{L/L}$; R26Y* female mice intraductally injected with *Ad-K8-Cre*. (**A**) One representative mouse showing tumor growth (arrows) several months after an injection with *Ad-K8-Cre*. (**B**) About 8.8% of live cells (based on DAPI staining) from a representative tumor were positive for YFP expression, based on flow cytometric analysis.

DISCUSSION:

The success of this approach for inducing mammary tumors from different subpopulations of MECs relies not only on choosing appropriate cell-type-specific promoters (to drive Cre expression) but also on the intraductal injection procedure itself. The idea behind this approach is that the injected Ad-Cre viruses are retained in the ductal tree, which is a concealed structure with lumen, and therefore, only MECs are exposed to the viruses and are infected by Ad-Cre. Due to the limited lumen space within the mammary ducts, it is important to only inject a small volume of the injection mixture to each MG (i.e., ~3–5 µL). The injected volume should also be adjusted based on the age of the mice (i.e., a smaller volume should be used when it is injected into 3- to 4-week-old mice). When the volume of the injected fluid is excessive due to the pressure from the injection and the limited ductal lumen space, fluid may be "pushed out" through the epithelial layers into the stroma, leading to an unwanted viral infection in stromal cells.

Since the cell-type specificity is achieved by the promoter used in the adenoviral vector to drive Cre expression, a limitation of this approach is the potential lack of an appropriate promoter to target Cre expression to a specific MEC subpopulation. We previously reported the use of pan-luminal *Ad-K8-Cre* virus to target luminal MECs[12,13] and the use of *Ad-Wap-Cre* virus to target alveolar luminal progenitors[5]. In this study, we showed the use of *Ad-K5-Cre* virus to target basal MECs (**Figure 5**). We still lack the ability to use this approach to target the estrogen receptor-

positive luminal MEC subpopulation. The adenoviral vector we used here could accommodate an insert of up to 8 kb. Thus, to develop MEC-subset-specific Ad-Cre, the promoter used to drive Cre expression could only be less than 7 kb. Practically, a large promoter fragment, even if less than 7 kb in size, may be difficult to subclone. In order to fit into the adenoviral vector, although a truncated promoter may be used, it may not faithfully recapitulate the expression pattern of its corresponding gene when under the control of the endogenous, full promoter.

The *R26Y* conditional reporter included in the mouse model here provided a way to mark the cells of origin and trace their progression to cancer cells. Of note, the percentage of YFP-marked cancer cells in the resulting tumor appeared to be fairly low (**Figure 6B**). This could be due to a possibility that, in addition to the YFP-marked tumor epithelial cells, the tumor also included many immune cells and stromal cells, which constituted the bulk of the tumor mass.

Compared to other mouse models of breast cancer, this approach leads to the mammary tumor initiation from a small number of MECs only (e.g., luminal MECs when *Ad-K8-Cre* is injected), often at a clonal level[12]. As the initiation of human tumorigenesis is likely to be clonal, this approach recapitulates that aspect of human cancer development more faithfully. In addition, even when p53 is disrupted in only a small number of MECs, loss of p53 leads to their clonal expansion, leading to the production of a larger pool of mutated MECs; this would permit further clonal evolution from the p53-deficient MECs (upon acquisition of additional somatic mutations)[12]. As *TP53* is the most commonly mutated gene in human breast cancer[16] and as *TP53* mutation is an early event in human breast tumorigenesis[17,18], by combining the *Trp53* floxed mouse model with mouse models for other oncogenic events, we can study how these oncogenic events cooperate with p53 loss and how they jointly contribute to mammary tumor development from a defined cellular origin. In addition, as less breeding is needed to put multiple alleles together, this approach would minimize breeding cost and time, which should facilitate breast cancer modeling studies on a larger scale, in a shorter period.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

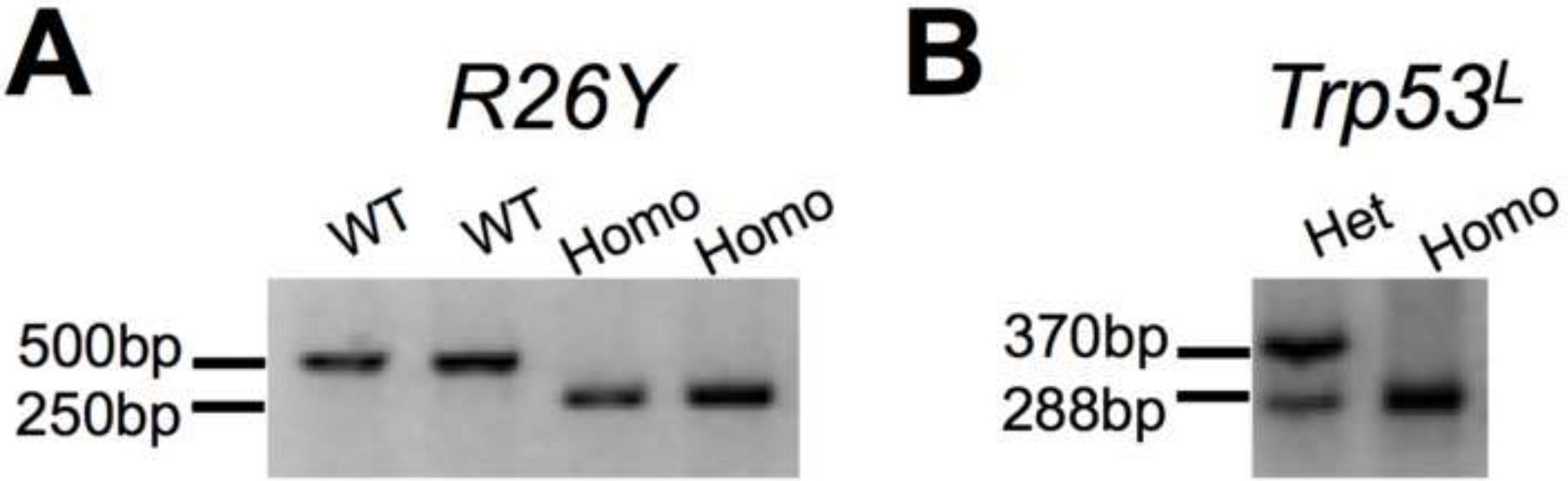

Figure 2

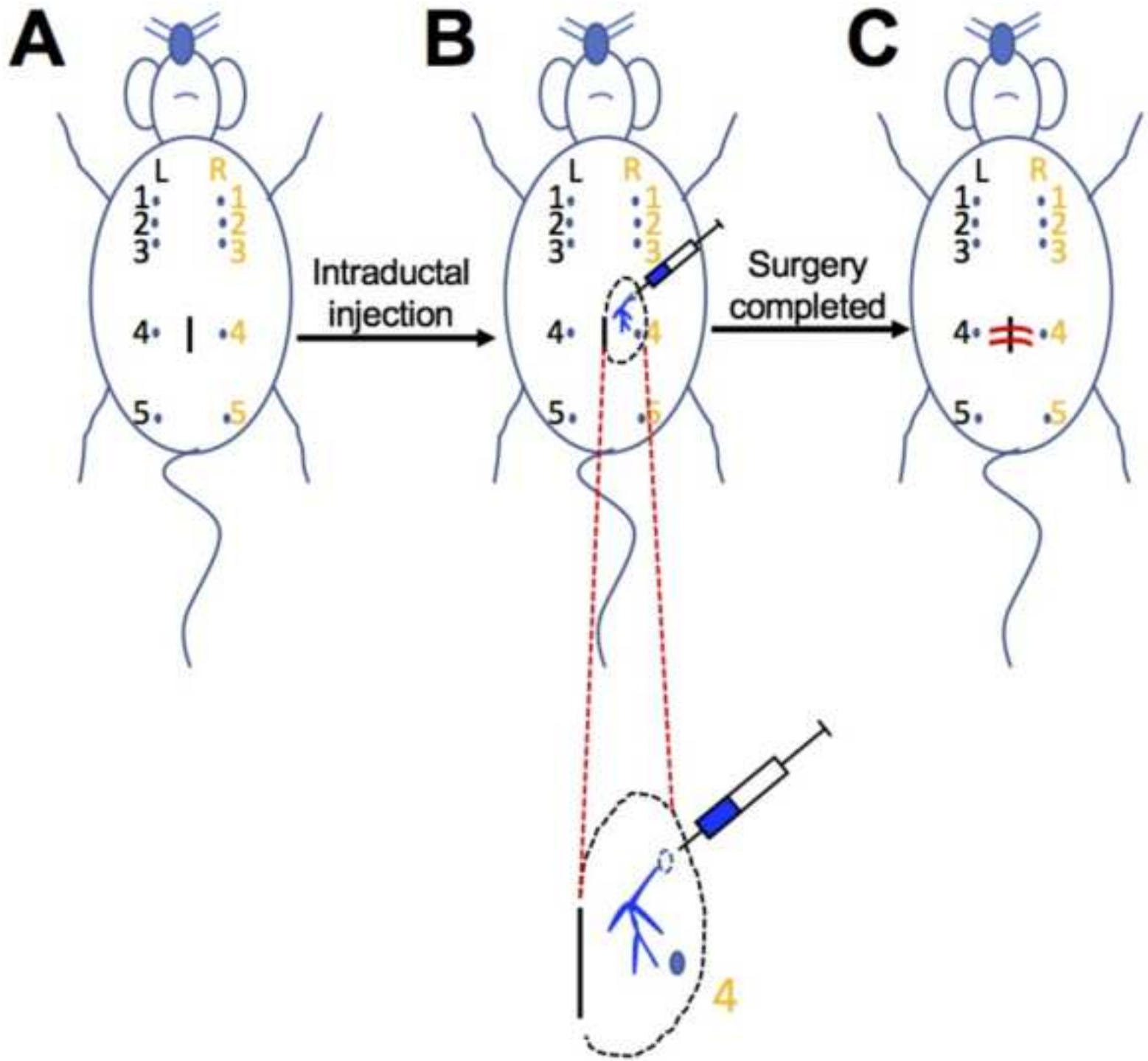

Figure 3

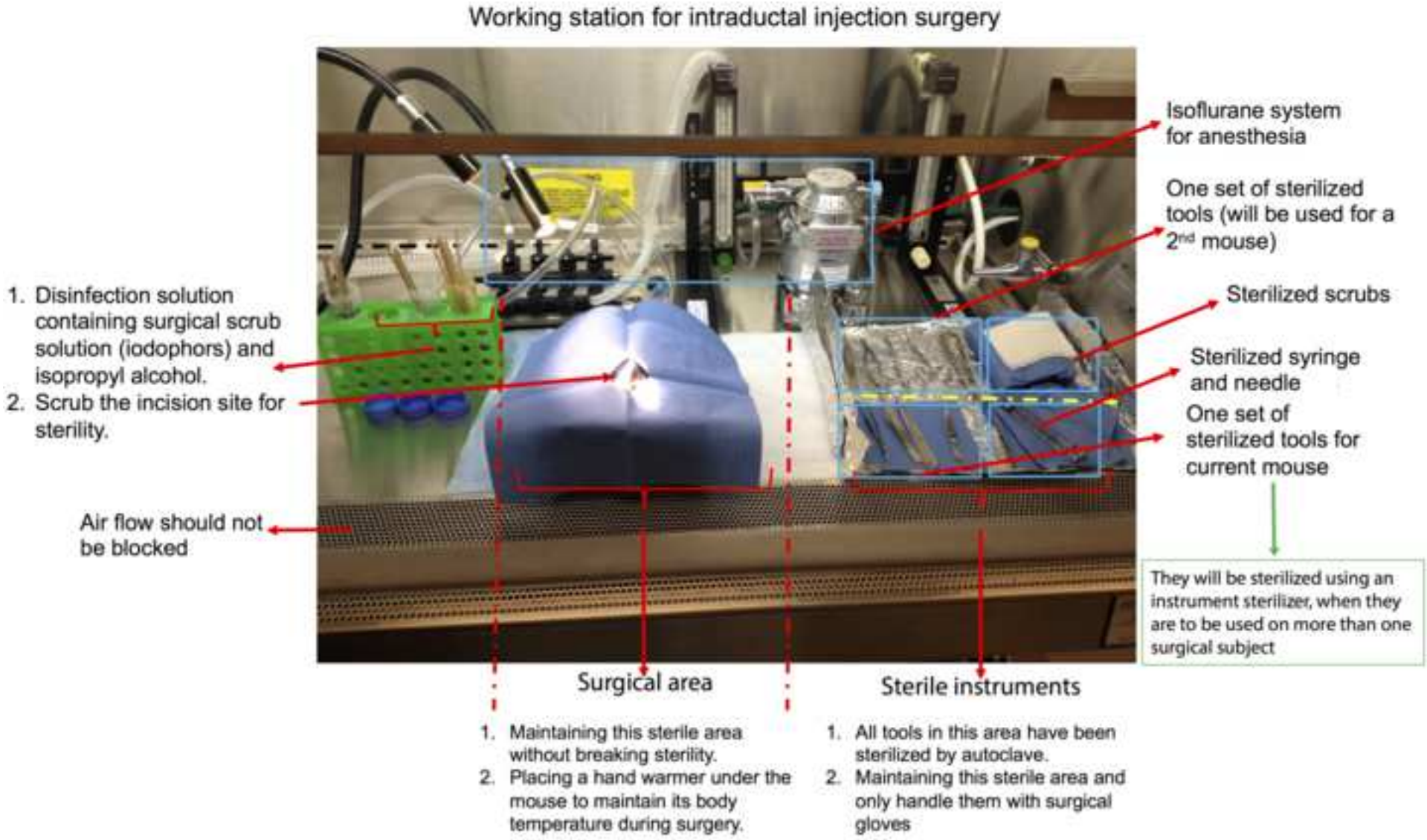

Figure 4

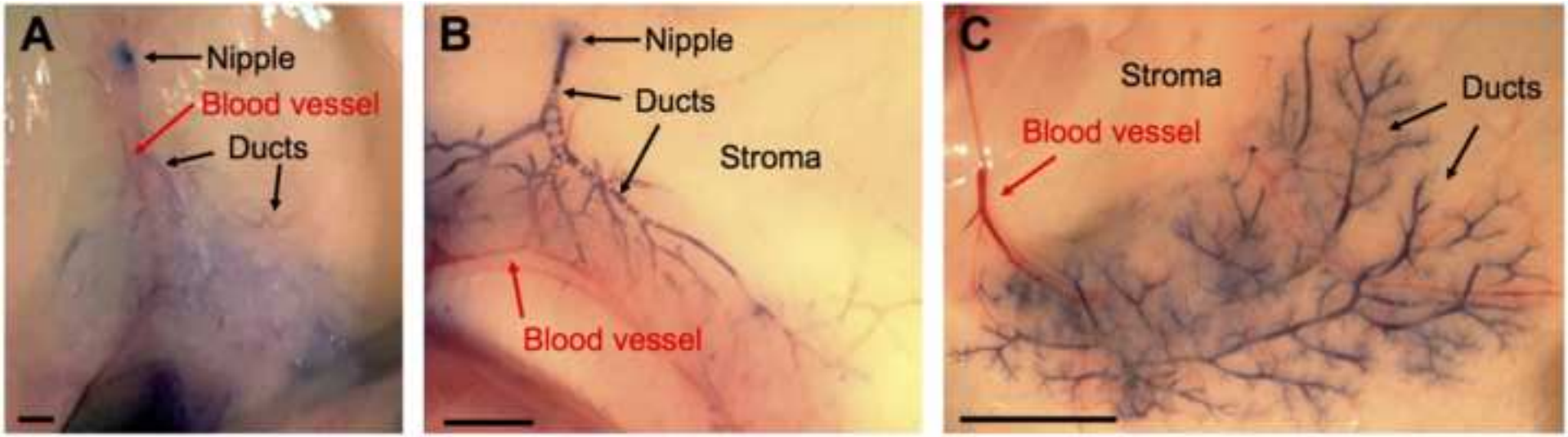

Figure 5

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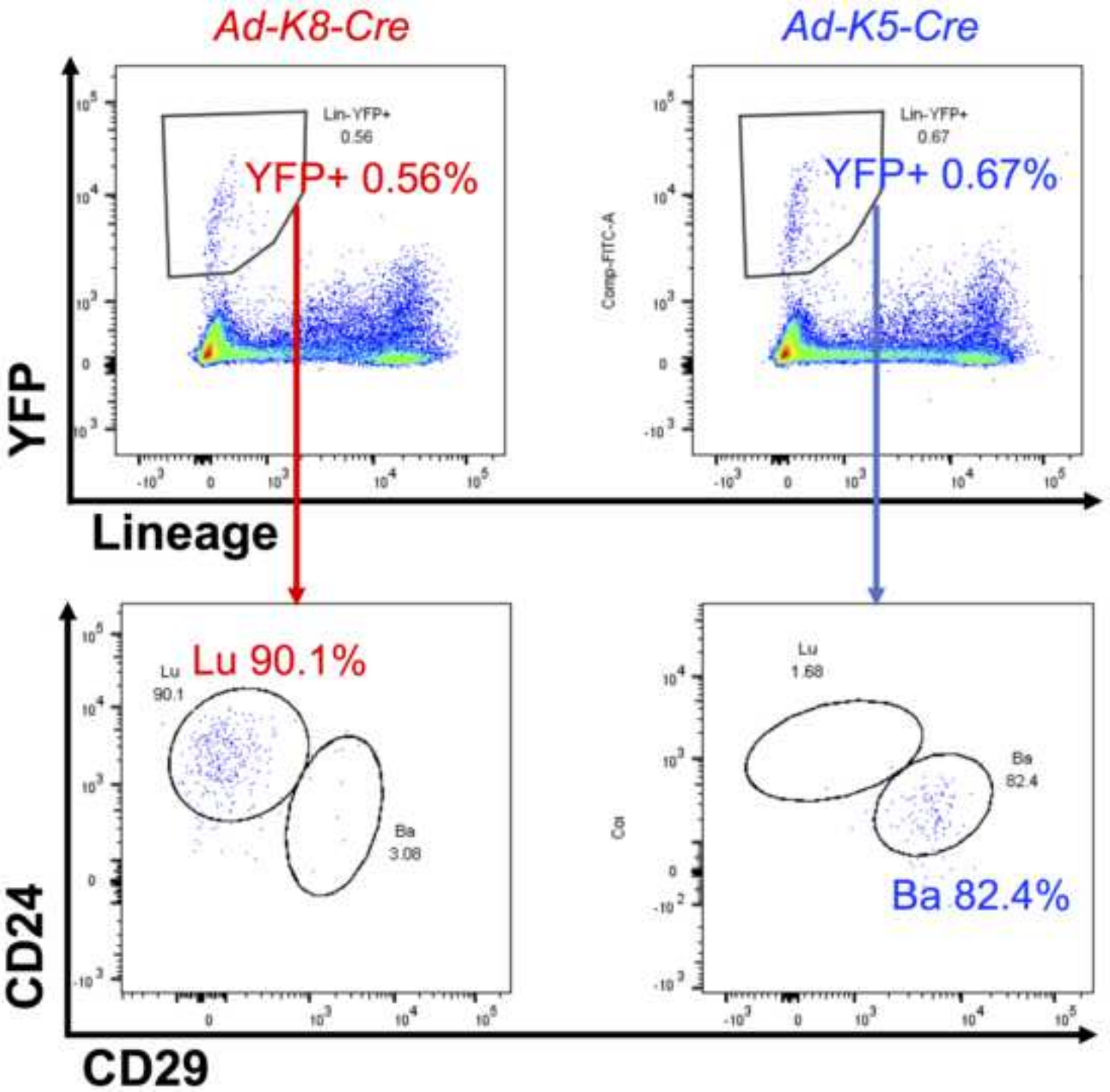

Figure 6

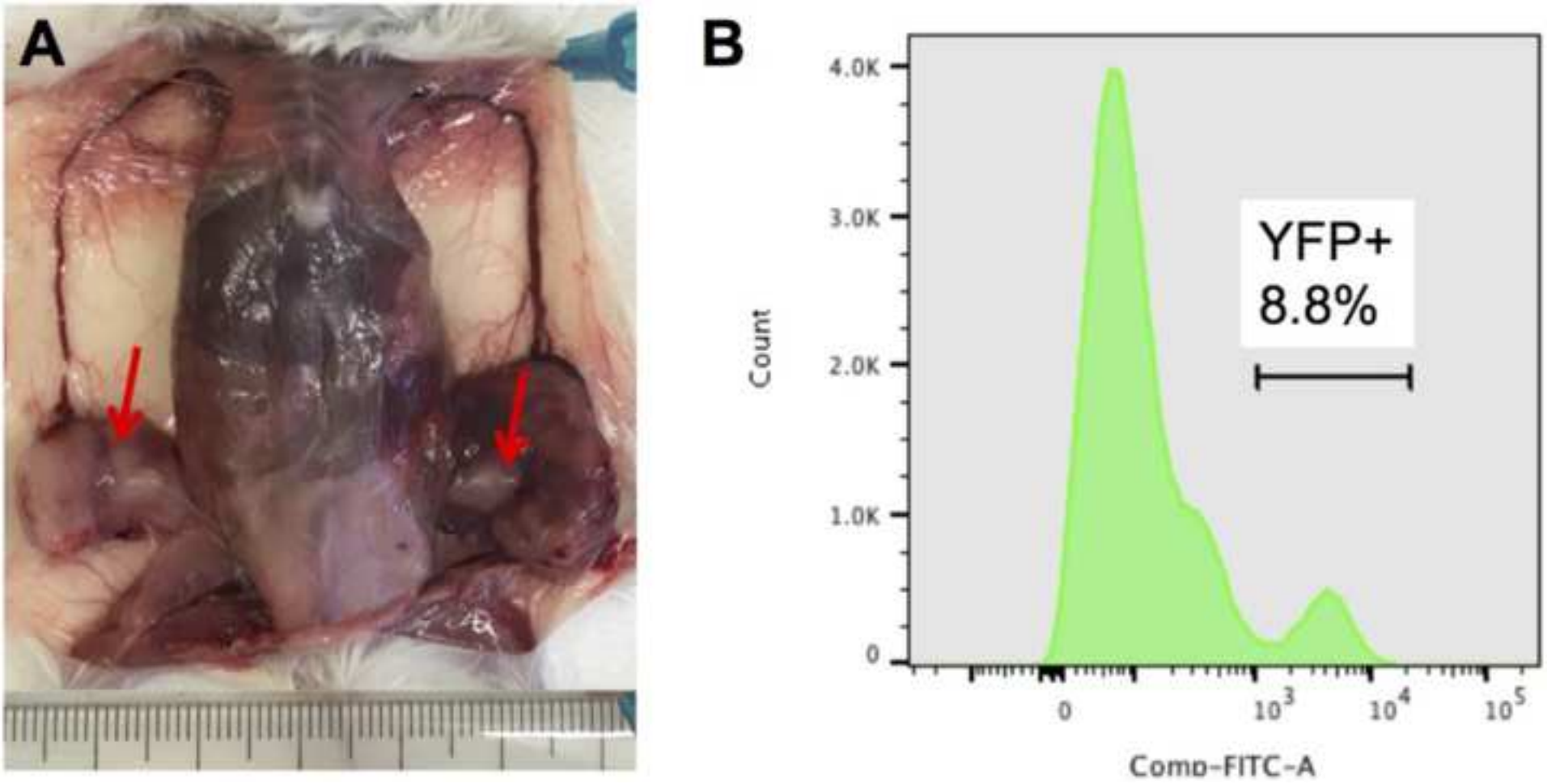

Table of Materials

Click here to access/download;Table of Materials;Intraductal_AdCre_JoVE_Materials_R2.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
33-gauge needle	Hamilton	7803-05	point style 3 blunt
7mm Reflex Clip	Braintree Scientific	RF7 CS	
Adenovirus, Ad-K5-Cre	University of Iowa Viral Vector Core	Ad5-bk5-Cre (VVC-Berns-1547)	
Adenovirus, Ad-K8-Cre	University of Iowa Viral Vector Core	Ad5mK8-nlsCre	
Alcohol	Fisher	HC800-1GAL	Prepare to 70% in use
biotinylated CD31	eBiosciences	13-0311-85	
biotinylated CD45	eBiosciences	13-0451-85	
biotinylated TER119	eBiosciences	13-5921-85	
Bromophenol Blue	Sigma-Aldrich	B0126-25G	
CD24-AF-700	BD Pharmingen	564237	
CD24-PE	eBiosciences	12-0242-83	
CD29-APC	eBiosciences	17-0291-82	
CD29-PE	eBiosciences	12-0291-82	
Hair Remover Lotion	Nair		9 Oz
Hamilton syringe	Hamilton	7636-01	0.025 mL
Iodophors	Betadine		10% Povidone-iodine
Isoflurane	Baxter	NDC 10019-360-40	1-2.5%
Loxicam	Norbrook	NDC 55529-040-10	5 mg/ml
Lubricant Eye Ointment	Akorn	NDC 17478-062-35	
Micro-dissecting scissors	Pentair	9M	Watchmaker's Forceps
Micro-dissecting tweezers	Dumont	M5	

Taq 5X Master Mix	New England Biolab:	M0285L

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Author(s):	Dongxi Xiang, Luwei Tao, Zhe Li

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Editorial comments:

1. Step 1.3 “Genotyping needs clarity. How do you prepare the PCR mix…? Do you add all 5 primers in each individual tube or prepare two different PCR tube one with R26Y primers and other with Trp 53 primers? What is the PCR condition? What are the sizes of band observed? Please rewrite this part”.

Response: More details for PCR genotyping are provided now, including how the PCR reactions are set up, the PCR cycling conditions, as well as the sizes of PCR bands and their corresponding genotypes.

“Also please provide representative result (a genotype gel picture) to understand the band size and which genotype is selected, etc.”

Response: A new Figure (Figure 1) with representative PCR results is now included. The numbering for all the other five figures is adjusted accordingly.

2. Step 2.2 “You haven’t used the Ad-Cre for intraductal injection till now. Please reword”.

Response: Reworded to “that will be used for intraductal injection”.

“How was Ad-Cre obtained? Citation if any? If obtained commercially please include commercially obtained Ad-Cre adenovirus. What is the stock concentration and what is the diluted concentration? Volume?”.

Response: Additional information provided.

3. Step 3.1 “Reworded to make it crisp and bring out clarity”.

Response: **Reworded (if this is what you mean).**

4. Step 3.5 Note “???? Please proofread the manuscript well”.

Response: **Reworded to increase clarity.**

5. Step 3.6 “Isnt the injected liquid 3 microlitres?”.

Response: **This originally referred to the possible amount of fluid that may leak out, we removed it to avoid any confusion.**

6. Step 3.8 “The wounds on the external nipple site?”.

Response: **No, the wounds are NOT on the nipple site; please refer to step 3.2 and the schematic diagram in Fig. 1A (now Fig. 2A).**

7. Step 4.1 “??? isoflurane chamber is for anesthetization; nose cone isoflurane is for maintenance? Something seem to be incorrect here”.

Response: **We removed “the nose cone in” to avoid any confusion here.**

8. Step 5.2 “How? visually? Lumps will be visible?”.

Response: **“detectable” is changed to “palpable”.**

9. Figure 4 legends “The observed YFP tumor cells are fairly less as expected. Please provide a discussion on the same. It need not be too long but some perspective on this is needed. Also, please do not cite unpublished result (as cited in the reviewer’s comment) as we cannot have the same in the manuscript. ”.

<u>Response:</u> A new paragraph is provided in the Discussion section to discuss this. The data referred to here is actually in Figure 5B (now Figure 6B) instead of Figure 4 (now Figure 5).

10. Figure 5 legends “Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account.”.

<u>Response:</u> The reference here is for the gating strategy; the actual flow cytometric data is from our own work, NOT from this publication.