Video Article

Identification and Characterization of Metastatic Factors by Gene Transfer into the Novel *RIP-Tag; RIP-tva* Murine Model

George Zhang[1], Yudan Chi[1], Yi-Chieh Nancy Du[1]

[1]Department of Pathology and Laboratory Medicine, Weill Cornell Medicine

Correspondence to: Yi-Chieh Nancy Du at nad2012@med.cornell.edu

URL: https://www.jove.com/video/55890
DOI: doi:10.3791/55890

Keywords: Mouse model, metastasis, RCAS, somatic gene transfer, RIP-Tag, RIP-tva

Date Published: 9/13/2017

Citation: Zhang, G., Chi, Y., Du, Y.C. Identification and Characterization of Metastatic Factors by Gene Transfer into the Novel *RIP-Tag; RIP-tva* Murine Model. *J. Vis. Exp.* (), e55890, doi:10.3791/55890 (2017).

Abstract

Metastatic cancer accounts for 90% of deaths in patients with solid tumors. There is an urgent need to better understand the drivers of cancer metastasis and to identify novel therapeutic targets. To investigate molecular events that drive the progression from primary cancer to metastasis, we have developed a bitransgenic mouse model, *RIP-Tag; RIP-tva*. In this mouse model, the rat insulin promoter (RIP) drives the expression of the SV40 T antigen (Tag) and the receptor for subgroup A avian leukosis virus (tva) in pancreatic β cells. The mice develop pancreatic neuroendocrine tumors with 100% penetrance through well-defined stages that are similar to human tumorigenesis, with stages including hyperplasia, angiogenesis, adenoma, and invasive carcinoma. Because *RIP-Tag; RIP-tva* mice do not develop metastatic disease, genetic alterations that promote metastasis can be identified easily. Somatic gene transfer into tva-expressing, proliferating pancreatic β premalignant lesions is achieved through intracardiac injection of avian retroviruses harboring the desired genetic alteration. A titer of >1 x 10^8 infectious units per ml is considered appropriate for *in vivo* infection. In addition, avian retroviruses can infect cell lines derived from tumors in *RIP-Tag; RIP-tva* mice with high efficiency. The cell lines can also be used to characterize the metastatic factors. Here we demonstrate how to utilize this mouse model and cell lines to assess the functions of candidate genes in tumor metastasis.

Video Link

The video component of this article can be found at https://www.jove.com/video/55890/

Introduction

Most cancers arise from somatic mutations [1]. Conventional genetically engineered mouse models (GEMM) have provided significant insights into the contribution of specific genetic alterations to tumorigenesis [2]. However, they have several limitations. The major drawback of these models is that they do not replicate the sporadic nature of tumor formation in humans, in which only some cells within a tissue acquire genetic alterations. The mutations in transgenic and knockout mice are also germline with potential to affect development. Moreover, generating these mouse models is expensive and time-consuming.

Metastasis is a significant issue in the field of cancer. Modeling metastasis has been difficult in GEMM. Spontaneous metastasis is rare in the mouse. Penetrance is variable and latency is long in GEMM of metastasis [3]. Experimental metastasis models employ direct injection of cells into the circulation of mice, so the early steps in the metastatic cascade are eliminated.

To overcome some of the above limitations in studying metastatic factors in mouse models, we have developed a bitransgenic mouse model, *RIP-Tag; RIP-tva*[4]. The strategy is based on combining the use of a highly-synchronized tumor progression mouse model, *RIP-Tag* [5], and the receptor for subgroup-A avian leukosis virus, tva [6,7]. This *RIP-Tag; RIP-tva*mouse model allows genes to be introduced somatically into a single bitransgenic mouse strain. With the SV40 T antigen suppressing the tumor suppressive functions of Rb and p53, mice develop pancreatic neuroendocrine tumors in a similar fashion to human tumorigenesis, with stages including hyperplasia, angiogenesis, adenoma, and invasive carcinoma.This *RIP-Tag* model has been very instructive for our understanding of hallmarks of cancer, not limited to pancreatic neuroendocrine tumors. It has also been used in preclinical trials [8].

We present a protocol for somatic gene transfer through injection of avian retroviruses intracardiacally into *RIP-Tag; RIP-tva* mice. Successful infection with RCASBP-derived avian retroviruses requires actively proliferating target cells. Therefore, we chose *RIP-Tag; RIP-tva* mice at 7 weeks of age, when hyperplasia develops in about 50% of the pancreatic islets. Left ventricular intracardiac injection of high titer viruses is required to achieve an infection efficiency of 10~20% [4]. This delivery method reduces the significant dilution of viral particles within circulation before viruses reach pancreatic islets.

Using this approach, we have previously demonstrated that Bcl-xL promotes cancer metastasis independent of its anti-apoptotic function [4,9]. This anti-apoptotic-independent metastatic function was not observed when Bcl-xL was expressed via a transgene in all pancreatic β cells throughout tumorigenic ontogeny in the *RIP-Tag; RIP-Bcl-xL* mouse model [10]. Therefore, our mouse model offers a unique opportunity to identify and characterize genes' functions when expressed at a later stage of tumorigenesis. Because 2~4% of islets develop into tumors in the *RIP-Tag; RIP-tva* bitransgenic mice without viral infection and not all the premalignant lesions are infected with the RCASBP-derived avian retroviruses,

Copyright © 2017 Journal of Visualized Experiments

only the factors that confer a selective advantage over the natural course of tumorigenesis can be identified. In particular, metastatic factors will be most easily recognized by this method, because metastasis to pancreatic lymph nodes or other organs does not normally occur in *RIP-Tag; RIP-tva* mice.

Protocol

Ethics Statement:

Experiments on animals were performed in accordance with the guidelines and regulations set forth by the Institute for Animal Care and Use Committee of Weill Cornell Medicine.

1. Choice of avian retroviral vectors (RCASBP(A)-based or RCANBP(A)-based).

- Vectors was derived from Rous sarcoma virus[11]. Avian retroviral vectors can deliver cDNAs (≤2.5 kb), short hairpin RNAs (shRNAs), microRNAs (miRNAs), and other noncoding RNAs. The commercially available vectors are listed in Materials and others need to be requested directly from the authors.
- **To overexpress genes of interest**, use one of the following vectors, RCASBP(A)[12] (**Figure 1A**), RCAS-X (**Figure 1B**), RCAS-Y[13] (**Figure 1C**), RCASBP-Y DV[14], or other ALV-A vectors (https://home.ncifcrf.gov/hivdrp/RCAS/mice.html).
- **To knock genes of interest by shRNA**, use one of the following vectors: RCAN-X DV[15] or RCAS-RNAi[16].
- **To overexpress miRNAs**, generate RCAS-miR vector as described in[17].
- **Transformation into *E. coli* and DNA requirement.**
- Preferably transform DNA into Stbl2 or Stbl3 competent cells, which have unique genotype to stabilize direct repeat and retroviral sequences.Purify plasmid DNA using a method that will yield pure, transfection grade DNA. 5 µg of DNA is needed with minimum concentration of 0.1 µg/µl.

2. Viral Propagation in chicken fibroblast DF1 cell line.

1. The use of filtered pipette tips for cell culture is required to prevent cross-contaminating viral stocks[18].
2. Maintain DF1 cells in 6-cm dish with growth medium (DMEM with high glucose, 10% fetal bovine serum, 6 mM (final concentration) L-glutamine, 10 units/ml penicillin, 10 µg/ml streptomycin) at 37°C and 5% CO_2. If DF1 cells are freshly thawed from frozen stocks, culture them for at least 3 days before transfection.
3. One day before transfection, pass DF1 cells to a 6-cm tissue culture dish so that they will be 30-50% confluent at the time of transfection.
4. Dilute 5 µg of pure viral DNA with DMEM (without serum, penicillin or streptomycin) to a total volume of 150 µl in a microcentrifuge tube inside a tissue culture hood.
5. Add 30 µl of Superfect directly into the diluted DNA tube; vortex this mixture for 10 seconds; leave the mixture at room temperature for 5 ~ 10 minutes in the tissue culture hood to allow transfection-complex formation.
6. While complex formation takes place, gently aspirate growth medium from the DF1 cell culture dish, and carefully wash cells with 4 ml $PBS^{-/-}$.
7. Pipet 5 ml of growth medium (containing serum and antibiotics); save 4 ml to a Falcon tube for the next step; and add the rest of 1 ml of growth medium to the transfection complexes. Mix by pipetting up and down twice, and immediately transfer the whole transfection complexes to the DF1 cells. Swirl to ensure that the cell layer is covered; incubate for 2-3 hours at 37°C.
8. Aspirate the transfection mixture; wash cells with 4 ml $PBS^{-/-}$; add 4 ml of fresh growth medium (containing serum and antibiotics); return cells to a 37°C incubator.
9. When cells reach confluency, passage cells (transient expression may be assayed 48 to 72 hours after transfection).
10. Continue to pass cells for about 7 days until all cells are presumably infected; test viral DNA integration by PCR and determine protein expression by Western blotting; and freeze cells in DMEM medium with 10% DMSO and 20% fetal bovine serum.

3. *in vivo* infection of *RIP-Tag; RIP-tva* mice.

1. **Virus collection and concentration**
   Use freshly concentrated viruses for *in vivo* infection. The titer of viruses kept in 4°C within a week is not significantly reduced. However, frozen aliquots of viral stock display 10-fold reduced titer upon thawing.
   1. Expand virus-producer DF1 cells into 15-cm dishes depending on the amount of virus needed. For *in vivo* mouse infection, prepare an average of one plate per mouse.
   2. Pre-chill the rotor (for example, SW28), the swing bucket, and the ultracentrifuge machine to 4°C.
   3. Collect supernatant from confluent 15-cm dishes. Remove cell debris by low-speed centrifugation at 1,650 x g for 10 min at 4°C.
   4. Transfer the viral supernatant to ultracentrifuge tubes (Polyallomer centrifuge tubes). Spin in an ultracentrifuge for 1.5 hours at 95,400 x g 4°C. For Beckman XL-100 ultracentrifuge machine, use Accel: 1; Decel: 1 settings.
   5. Remove supernatant from ultracentrifuge tubes as much as possible. Add PBS without calcium and magnesium ($PBS^{-/-}$) to the ultracentrifuge tube to make final 100 µl viral suspension from one 15-cm dish. Cover the tubes with Parafilm. Resuspend the invisible viral pellet by vortexing the ultracentrifuge tubes for 2 min at medium speed.
   6. Rock the ultracentrifuge tubes at 4°C for one hour to overnight.
   7. Transfer the viral suspension into a microcentrifuge tube. Warm up the viral suspension to room temperature before injection into mice.
2. **Viral Titer Determination.**
   For every new viral construct, viral titer needs to be determined before *in vivo* infection.
   1. Seed DF1 cells in all wells of a 12-well tissue culture plate (suggestion: 2 x 10^4 cells/well, 1 ml/well), so that they will be 30% confluent at the time of infection. One 12-well plate is needed for one viral titer determination.

2. Make a series of 10-fold dilutions of the viral supernatant in growth medium as following:
    1. Mix 5 µl viral supernatant with 495 µl growth medium for the 10^2 dilution in a microcentrifuge tube. Vortex briefly for a few seconds.
    2. Take 40 µl from the 10^2 dilution into 360 µl growth medium in a microcentrifuge tube for the 10^3 dilution. Vortex briefly for a few seconds.
    3. Follow step 3.2.2.2 for 10^4 and 10^5 dilution.
    4. Set up 5 of 6-ml polystyrene tubes. Aliquot 2.25 ml growth medium per tube, and label them 10^6, 10^7, 10^8, 10^9, 10^{10}, respectively.
    5. Mix 0.25 ml from the 10^5 dilution with 2.25 ml growth medium in the 6 ml Polystyrene tube for the 10^6 dilution. Vortex briefly for a few seconds.
    6. Follow step 3.2.2.5 for 10^7, 10^8, 10^9, and 10^{10} dilution.
    7. Remove the original culture medium from DF1 cells on the 12-well plate. Put 1 ml diluted viruses to each well in duplicated (uninfected, 10^{10} to 10^6 IU/ml).

3. Allow cells to grow for at least 7 days (perform trypsinzation when necessary). Collect cells to isolate DNA and check for the presence of viral DNA by PCR as described in "6. PCR protocols". If this viral titer is 10^8 infectious units per ml (IU/ml), a positive 398 bp PCR products from RCASBP will be observed using DNA from cells with 10^8 to 10^6 IU/ml viruses, but not from uninfected cells, or cells with 10^{10} to 10^9 IU/ml viruses. A titer of >1 x 10^8 infectious units per ml should be used for *in vivo* infection.

3. **Intracardiac injection**
Hemizygous *RIP-Tag* mice, not homozygous mice, are used in this study. Hemizygous *RIP-Tag* mice develop pancreatic neuroendocrine tumors around 10~12 weeks of age, while homozygous mice have a shorter tumor latency. Because cell proliferation is required for the incorporation of the viral cDNA into the host genome, 7-week-old *RIP-Tag; RIP-tva* mice with hyperplasic pancreatic β cells are used. Please note that most normal β cells are not proliferating in adult mice.
    1. Use 7-week-old *RIP-Tag; RIP-tva* mice in a pure C57BL/C background for the experiments.
    2. Anesthetize mouse using a ketamine/xylazine combination (150 mg/kg; 15 mg/kg). Use heat support to maintain the mouse body temperature throughout the entire period of anesthesia.
    3. Apply eye lubricant to both eyes to prevent corneal drying.
    4. Shave hair from the chest cavity of *RIP-Tag; RIP-tva* mice. Position mouse on its back with chest facing up. A toe-pinch is performed to observe the animal for non-responsiveness to confirm full anesthesia effect.
    5. Extend the front limb and secure them with tapes. Mark mouse's chest for injection. Mark a location midway between the sternal notch and top of xyphoid process, and slightly left (anatomical) of the sternum (**Figure 2**).
    6. Scrub the anterior chest wall with either a povidone-iodine scrub or a chlorhexidine scrub followed by a 70% isopropyl alcohol or 70% ethanol soaked gauze sponge.
    7. Draw 50 µl of air into a sterile 28 g ½ insulin syringe to create space between the plunger and meniscus of a 500-µl syringe, and then draw up 100 µl of viruses. The air space without any liquid on the wall is crucial for seeing cardiac pulse.
    8. Keep needle upright. Hold skin of mouse taut with one hand, and insert needle to the marked location. When a bright red pulse of blood appears in the syringe, stop advancing the needle and fix the depth of the needle in the mouse with one hand.
    9. Use the other hand to carefully and slowly push the plunger to deliver viral suspension (~100 µl volume) over a ~60 second period. Deliver 10~20 µl whenever seeing a bright red pulse of blood appear in the syringe. Continuous entrance of red oxygenated blood into the transparent needle hub indicates proper positioning of the needle into the left ventricle.
    10. After the last push of the plunger to deliver viral suspension and before seeing the next red pulse of blood, quickly retract the needle out of the chest cavity.
    11. Apply gentle pressure over the injection site to reduce bleeding for at least 1 min.
    12. Carefully move the mouse onto a heating pad or under a heat lamp until fully conscious. Mice will be watched until anesthesia wears off so that they can walk away from stimuli, a period usually lasting approximately one hour. Mice will be monitored daily for even and intact coat and any changes in behavior.

4. Histopathological analysis of tumors.

1. Nine weeks after injection, euthanize 16-week-old *RIP-Tag; RIP-tva* mice. Record sizes and numbers of primary pancreatic tumors and any gross metastases.
2. Fix pancreas, liver, and/or other organs in 10% buffered formalin overnight at room temperature in a rocking platform.
3. Transfer the fixed tissues into 70% ethanol on the next day. Process tissues into paraffin-embedded sections.
4. Perform immunohistochemical staining for either synaptophysin (a neuroendocrine marker) or insulin (a β cell-specific marker) following manufacturer's instructions to identify metastases of pancreatic β cells in pancreatic lymph nodes or livers. However, de-differentiated tumor cells or poorly differentiated tumor cells may lose the expression of insulin and only can be detected by synaptophysin immunostaining.

5. *in vitro* infection of tva-expressing cells.

It is not necessary to use concentrated viruses for *in vitro* infection. The protocol described below is for two rounds of infection. Further rounds of infection can be performed by repeating the following protocol.

1. Collect viral supernatant from confluent DF1 cells. Keep the viral supernatant at 4°C for infection within a week. Before infection, pass the viral supernatant through a 0.45 µm filter to obtain cell-free viruses.
2. Briefly rinse tva-expressing target cells (i.e. N134 pancreatic β cell tumor cell line from a *RIP-Tag; RIP-tva* mouse) with $PBS^{-/-}$ to remove all traces of serum, which contain trypsin inhibitor. Briefly rinse tva-expressing target cells with trypsin-EDTA (such as 0.25% Trypsin-2.21 mM EDTA) solution. Add Trypsin-EDTA solution and incubate for 2 minutes. Gently tap the plate. Observe cells under an inverted microscope. Cells usually detach in 2 to 3 minutes. Seed target cells into a 6-cm plate. Use 3~4 ml filtered viral supernatant as growth medium.

NOTE: The nutrition in the viral supernatant is consumed by DF1 cells, so change the medium the next day.

3. On the next day, warm up enough amount of viral supernatant to room temperature. Remove the medium from target cells, and replace with 3~4 ml viral supernatant.
4. On the third day, passage cells as needed. If cells are not dense enough to be passaged, warm up ~4 ml of regular growth medium (DMEM with high glucose, 10% fetal bovine serum, 6 mM (final concentration) L-glutamine, 10 units/ml penicillin, 10 µg/ml streptomycin) to replace the viral supernatant.
5. Expand infected cells and examine the candidate protein expression by Western blotting [19]. The effects of the candidate genes on migration and invasion can be analyzed in transwell assays. These cell lines can also be tested for their metastatic potential to the liver in a tail vein assay for metastasis.

6. PCR protocols.

Solutions should be assembled as quickly as possible on ice. A mastermix without the template can be made for a large number of samples and aliquoted into PCR tubes. Multiply the amount of each reagent needed by the number of samples. Mix the reagents by flicking prior and swirl with pipet tip before taking some to add to the mastermix. After adding each reagent to the mastermix tube, pipet up and down to deliver any residuals inside the tips.

1. **To detect the presence of RCASBP in the infected or transfected cells.**
   The following protocol calls for 11.5 µl of the mastermix and 1 µl of genomic DNA for each sample. Make sure to swirl before adding each reagent.
   1. Prepare a mastermix of 7.68 µl nuclease-free water, 0.75 µl DMSO, 1.25 µl 10x buffer II for AmpliTaq DNA Polymerase, 1.375 µl of 25 mM $MgCl_2$, and 0.125 µl of 25 mM dNTP.
   2. Add 0.125 µl of 100 µM forward primer RCAS5626F: (5'-ACCGGGGGATGCGTAGGCTTCA-3') and 0.125 µl of 100 µM reverse primer RCAS6023R: (5'-CCGCAACACCCACTGGCATTACC-3') to the mastermix.
   3. Add 0.075 µl AmpliTaq DNA Polymerase to the mastermix.
   4. Mix the mastermix by flicking the tube with fingers. Spin down the matermix in a centrifuge machine briefly for 3~5 seconds.
   5. Aliquot 11.5 µl from the mastermix to each individual PCR tubes. Swirl every time before aliquoting to a new tube.
   6. Swirl and add 1 µl genomic DNA template to each PCR tube. To prepare genomic DNA:
      1. Lyse cell pellet in 200 µl of 0.05 M NaOH. Pipet up and down to dissolve the pellet.
      2. Heat the cell lysate to 98°C for 20 minutes in a PCR machine, then cooled down to 25°C.
      3. Add 20 µl 1.0 M Tris-HCl (pH 7.5) to the tubes, and store the DNA samples at 4°C.
   7. Mix the samples by flicking and centrifuge briefly for 3~5 seconds. Put tubes into a PCR machine.
   8. The PCR condition is set for 2 minutes at 92°C for initial denaturation, followed by 40 cycles of 30 seconds at 94°C for denaturation, 30 seconds at 67°C for annealing, 30 seconds at 72°C for extension, and 10 minutes at 72°C for final extension.
   9. After the PCR is finished, add 3 µl of 6x DNA loading dye to each sample. Mix by flicking and centrifuge briefly for 3~5 seconds.
   10. PCR products are examined by electrophoresis in a 2% (w/v) agarose gel in 1X TAE buffer. The size of its PCR product is 398 bp.

2. **Tag genotyping.**
   The following protocol calls for 10.5 µl of the mastermix and 2 µl of genomic DNA for each sample. Make sure to swirl before adding each reagent.
   1. Prepare a mastermix of 4.5 µl nuclease-free water and 4.00 µl 5x MyTaq Reaction Buffer for MyTaq DNA Polymerase.
   2. Add 0.4 µl of 100 µM forward primer TagF2: (5'-GGACAAACCACAACTAGAATGCAGTG-3') and 0.4 µl of 100 µM reverse primer TagR2: (5'-CAGAGCAGAATTGTGGAGTGG-3') to the master mix.
   3. Add 0.8 µl of 10 µM beta-2-microglobulin precursor (B2m) primer mix for the internal control. PCR primers for B2m the internal control, beta-2-microglobulin precursor (B2m), are B2 F (5'-CACCGGAGAATGGGAAGCCGAA-3') and B2 R (5'-TCCACACAGATGGAGCGTCCAG-3').
   4. Add 0.4 µl MyTaq DNA Polymerase to the mastermix.
   5. Mix the mastermix by flicking the tube with fingers. Spin down the mastermix in centrifuge machine for 3~5 seconds.
   6. Aliquot 10.5 µl from the mastermix to each individual PCR tubes. Swirl every time before aliquoting to a new tube.
   7. Swirl and add 2 µl genomic DNA template to each PCR tube. Genomic DNA is prepared as described above.
   8. Mix the solution by flicking and centrifuge briefly for 3~5 seconds. Put tubes into PCR machine.
   9. The PCR condition is set for 3 minutes at 95°C for initial denaturation, followed by 35 cycles of 15 seconds at 95°C for denaturation, 15 seconds at 65°C for annealing, 30 seconds at 72°C for extension, and 10 minutes at 72°C for final extension.
   10. After the PCR is finished, add 3 µl of 6x loading dye to each sample. Mix by flicking and centrifuge briefly for 3~5 seconds.
   11. PCR products are examined by electrophoresis in 2% (w/v) agarose gel in 1X TAE buffer. The sizes of Tag and B2m PCR products are ~450 bp and 300 bp, respectively.

3. **tva genotyping.**
   The following protocol calls for 10.5 µl of the reagent mix for each sample. Make sure to swirl before adding each reagent.
   1. Prepare a mastermix of 5.6 µl nuclease free water, 0.5 µl DMSO, 1.25 µl 10x Buffer II for AmpliTaq DNA Polymerase, and 1.375 µl of 25 mM $MgCl_2$, and 0.125 µl of 25 mM dNTP.
   2. Add 0.125 µl of 100 µM tva-3 (5'-GCCCTGGGGAAGGTCCTGCCC-3') and 0.125 µl of 100 µM tva-5 (5'-CTGCTGCCCGGTAACGTGACCGG-3') to the mastermix.
   3. Add 1.275 µl of 10 µM B2m primer mix for the internal control to the mastermix.
   4. Add 0.125 µl AmpliTaq DNA Polymerase to the mastermix.
   5. Mix the mastermix by flicking the tube with fingers. Spin down the mastermix in centrifuge machine briefly for 3~5 seconds.
   6. Aliquot 10.5 µl from the mastermix to each individual PCR tubes. Swirl every time before aliquoting to a new tube.
   7. Swirl and add 2 µl genomic DNA template to each PCR tube. Genomic DNA is prepared as described above.
   8. Mix the solution by flicking and centrifuge briefly for 3~5 seconds. Put tubes into PCR machine.

9. The PCR condition is set for 2 minutes at 92°C for initial denaturation, followed by 35 cycles of 30 seconds at 94°C for denaturation, 30 seconds at 60°C for annealing, 30 seconds at 72°C for extension, and 10 minutes at 72°C for final extension.
10. After the PCR is finished, add 3 µl of 6x loading dye to each sample. Mix by flicking and centrifuge briefly for 3~5 seconds.
11. PCR products are examined by electrophoresis in a 2% (w/v) agarose gel in 1X TAE buffer. The sizes of tva and B2m PCR products are 500 bp and 300 bp, respectively.

Representative Results

The *in vivo* and *in vitro* infection rate of *RIP-Tag; RIP-tva* tumor cells by RCASBP-based viruses are ~20% and ~80% respectively [20]. In the *RIP-Tag; RIP-tva* mouse model, approximately 4% of the 400 pancreatic islets in each mouse will naturally develop into tumors [20]; therefore there is sufficient amount of tumor cells in each mouse for histological and phenotypic analysis of the potential effect of the genes delivered by the viruses. Using this system, a novel nuclear function of Bcl-xL in metastasis was identified [9]. *RIP-Tag; RIP-tva* mice infected with RCASBP-*Bcl-xL* exhibited a higher incidence of invasive carcinomas than mice infected with control viruses, RCASBP-*ALPP* (96% vs. 74%). Furthermore, 47% of RCASBP-*Bcl-xL*-infected *RIP-Tag; RIP-tva* mice developed metastases in pancreatic lymph nodes when euthanized at 16 weeks of age (**Figure 3A**), while no metastasis was found in control mice [20].

Moreover, we screened a library of cancer genes in *RIP-Tag; RIP-tva* mice, and identified the first gene that promotes metastasis to pancreatic lymph nodes and the liver [21] (**Figure 3B** and **3C**). This gene encodes the Receptor for hyaluronan-mediated motility isoform B (RHAMM^{B}) protein and activates EGFR signaling [21]. We demonstrated that liver-specific metastasis can be recapitulated in a tail vein assay of experimental metastasis in which N134 tumor cells initially circulated through the lung capillary beds of the recipient immunodeficient mice [21].

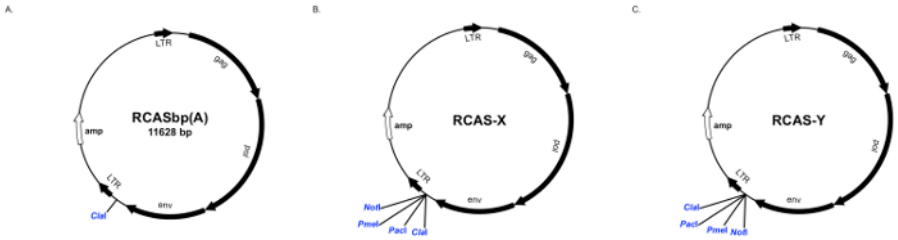

Figure 1: Schematic of RCASBP(A), RCAS-X, and RCAS-Y constructs. Cloning sites are indicated in blue, bold font. Please click here to view a larger version of this figure.

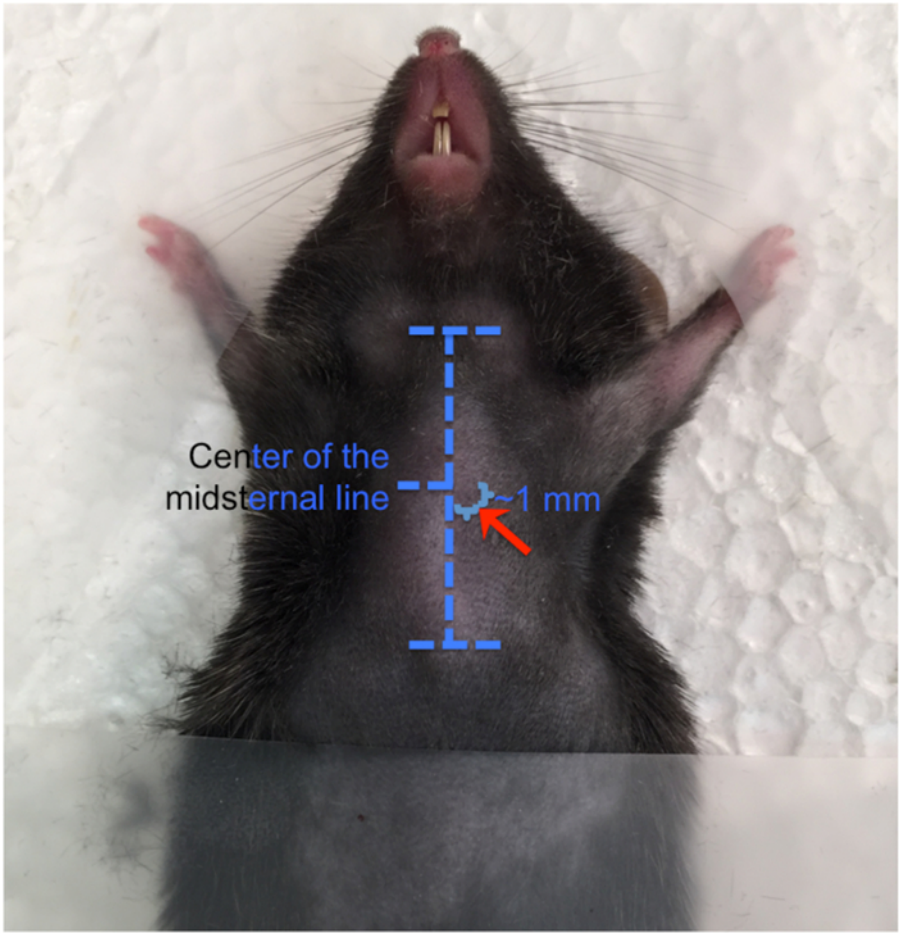

Figure 2: Placement of intracardiac injection. Anatomical landmarks are shown with horizontal dashed lines on the sternum (outlined in white). On the mouse's skin, the sternal notch and xyphoid process serve as landmarks, and the needle is inserted 1 mm away from the mid-sternum and slightly left (anatomical) of the sternum. Please click here to view a larger version of this figure.

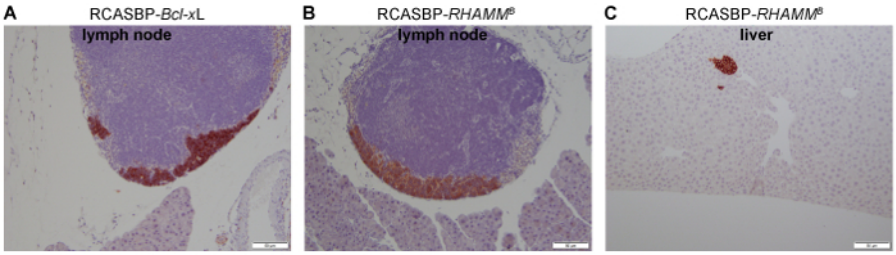

Figure 3: Detection of metastatic pancreatic β cells by immunostaining. Photographs show representative synaptophysin staining of metastatic pancreatic neuroendocrine tumors in pancreatic lymph nodes (A, B) or in the liver (C). *RIP-Tag; RIP-tva* mice were infected with the indicated RCASBP retroviruses at 7 weeks of age and euthanized at 16 weeks of age. Scale bar, 50 µm. Original magnification, x 20. Please click here to view a larger version of this figure.

Discussion

In this study, we described a powerful mouse model, *RIP-Tag; RIP-tva,* to achieve somatic gene delivery via avian retroviruses for the identification and characterization of metastatic factors. Although *RIP-Tag; RIP-tva* mice develop pancreatic neuroendocrine tumors, metastatic factors identified in this mouse model may also promote metastasis of other cancer types.

Our approach has the advantage of introducing somatic genetic changes specifically into premalignant lesions of pancreatic β cells in a time-control manner, thus more faithfully mimicking sporadic human tumor development. This approach avoids any potential perturbation of normal tissue formation, which is often observed in conventional transgenic models due to the ectopic expression of the gene of interest during development. Furthermore, it is much faster to generate avian retroviral vectors carrying genes of interest than to generate transgenic mice. RCASBP-derived avian retroviral vector can deliver cDNAs (≤2.5 kb), shRNAs, miRNAs, and other noncoding RNAs to tva-expressing cells *in vitro* and *in vivo*. The efficiency of infection (and multiple infection) is dependent on the proliferation rate of target cells and the accessibility of the

cells. A titer of >1 x 10^8 infectious units per ml is required for *in vivo* infection. More efficient viral delivery can be achieved *in vitro* due to induced cell proliferation and the possibility of repetitive exposure of all cells to viruses.

Precise intracardiac injection technique is critical for the viability of mice. First, a 50 µl of air space in an insulin syringe before drawing up viral suspension is crucial for seeing cardiac pulse. Second, it is important to fix the position of syringe once a red pulse of blood appears, and to slowly deliver 10~20 µl viral suspension whenever seeing a bright red pulse of blood appear in the syringe. If bright red pulses of blood stop after the delivery of 10~20 µl viral suspension, slightly repositioning the needle will help. Third, after the last push of the plunger to deliver viral suspension and before seeing the blood pulse, the needle needs to be quickly retracted out of the chest cavity and a gentle pressure applied over the injection site will help to reduce internal bleeding. Last but not least, do not re-use the insulin syringe on another mouse.

For future applications, this *RIP-Tag; RIP-tva* mouse model can be combined with other transgenic, knock-in, and knockout mouse models. Moreover, we envision combining this *RIP-Tag; RIP-tva* mouse model with CRISPR-Cas9 genome-editing tool to generate single point mutations, deletion, genomic rearrangements such as inversions and translocations [22].

Disclosures

The authors have nothing to disclose.

Acknowledgements

We thank Harold Varmus, Brian C. Lewis, Douglas Hanahan, Danny Huang, Sharon Pang, Megan Wong, and Manasi M. Godbole. Y.C.N.D. is supported by DOD grant W81XWH-16-1-0619 and NIH grant 1R01CA204916.

References

1. Vogelstein, B., Kinzler, K. W. The multistep nature of cancer. *Trends Genet.* **9** (4), 138-141 (1993).
2. Walrath, J. C., Hawes, J. J., Van Dyke, T., Reilly, K. M. Genetically engineered mouse models in cancer research. *Adv Cancer Res.* **106**, 113-164 (2010).
3. Khanna, C., Hunter, K. Modeling metastasis in vivo. *Carcinogenesis.* **26** (3), 513-523 (2005).
4. Du, Y. C., Lewis, B. C., Hanahan, D., Varmus, H. Assessing tumor progression factors by somatic gene transfer into a mouse model: Bcl-xL promotes islet tumor cell invasion. *PLoS biology.* **5** (10), e276 (2007).
5. Hanahan, D. Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. *Nature.* **315** (6015), 115-122 (1985).
6. Fisher, G. H. *et al.* Development of a flexible and specific gene delivery system for production of murine tumor models. *Oncogene.* **18** (38), 5253-5260 (1999).
7. Orsulic, S. An RCAS-TVA-based approach to designer mouse models. *Mamm Genome.* **13** (10), 543-547 (2002).
8. Tuveson, D., Hanahan, D. Translational medicine: Cancer lessons from mice to humans. *Nature.* **471** (7338), 316-317 (2011).
9. Choi, S. *et al.* Bcl-xL promotes metastasis independent of its anti-apoptotic activity. *Nat Commun.* **7**, 10384 (2016).
10. Naik, P., Karrim, J., Hanahan, D. The rise and fall of apoptosis during multistage tumorigenesis: down-modulation contributes to tumor progression from angiogenic progenitors. *Genes Dev.* **10** (17), 2105-2116 (1996).
11. Hughes, S. H., Greenhouse, J. J., Petropoulos, C. J., Sutrave, P. Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. *J Virol.* **61** (10), 3004-3012 (1987).
12. Petropoulos, C. J., Payne, W., Salter, D. W., Hughes, S. H. Appropriate in vivo expression of a muscle-specific promoter by using avian retroviral vectors for gene transfer [corrected]. *J Virol.* **66** (6), 3391-3397 (1992).
13. Dunn, K. J., Williams, B. O., Li, Y., Pavan, W. J. Neural crest-directed gene transfer demonstrates Wnt1 role in melanocyte expansion and differentiation during mouse development. *Proc Natl Acad Sci USA.* **97** (18), 10050-10055 (2000).
14. Loftus, S. K., Larson, D. M., Watkins-Chow, D., Church, D. M., Pavan, W. J. Generation of RCAS vectors useful for functional genomic analyses. *DNA Res.* **8** (5), 221-226 (2001).
15. Bromberg-White, J. L. *et al.* Delivery of short hairpin RNA sequences by using a replication-competent avian retroviral vector. *J Virol.* **78** (9), 4914-4916 (2004).
16. Harpavat, S., Cepko, C. L. RCAS-RNAi: a loss-of-function method for the developing chick retina. *BMC Dev Biol.* **6** 2 (2006).
17. Huse, J. T. *et al.* The PTEN-regulating microRNA miR-26a is amplified in high-grade glioma and facilitates gliomagenesis in vivo. *Genes Dev.* **23** (11), 1327-1337 (2009).
18. Ahronian, L. G., Lewis, B. C. Generation of high-titer RCAS virus from DF1 chicken fibroblasts. *Cold Spring Harb Protoc.* **2014** (11), 1161-1166 (2014).
19. Green, M. R., Sambrook, J., Sambrook, J. *Molecular cloning: a laboratory manual.* 4th edn. Cold Spring Harbor Laboratory Press. (2012).
20. Du, Y. C., Lewis, B. C., Hanahan, D., Varmus, H. Assessing tumor progression factors by somatic gene transfer into a mouse model: Bcl-xL promotes islet tumor cell invasion. *PLoS Biol.* **5** (10), 2255-2269 (2007).
21. Du, Y. C., Chou, C. K., Klimstra, D. S., Varmus, H. Receptor for hyaluronan-mediated motility isoform B promotes liver metastasis in a mouse model of multistep tumorigenesis and a tail vein assay for metastasis. *Proc Natl Acad Sci USA.* **108** (40), 16753-16758 (2011).
22. Guernet, A., Grumolato, L. CRISPR/Cas9 editing of the genome for cancer modeling. *Methods.* (2017).

Copyright © 2017 Journal of Visualized Experiments