Screening for melanoma modifiers using a zebrafish autochthonous tumor model

Sharanya Iyengar  
*University of Massachusetts Medical School*

*Et al.*

---

**Let us know how access to this document benefits you.**

Follow this and additional works at: [https://escholarship.umassmed.edu/gsbs_sp](https://escholarship.umassmed.edu/gsbs_sp)

Part of the Cancer Biology Commons, Cell Biology Commons, Molecular Biology Commons, and the Research Methods in Life Sciences Commons

---

**Repository Citation**

Iyengar S, Houvras Y, Ceol CJ. (2012). Screening for melanoma modifiers using a zebrafish autochthonous tumor model. GSBS Student Publications. [https://doi.org/10.3791/50086](https://doi.org/10.3791/50086). Retrieved from [https://escholarship.umassmed.edu/gsbs_sp/2039](https://escholarship.umassmed.edu/gsbs_sp/2039)

---

This material is brought to you by eScholarship@UMMS. It has been accepted for inclusion in GSBS Student Publications by an authorized administrator of eScholarship@UMMS. For more information, please contact Lisa.Palmer@umassmed.edu.
Screening for Melanoma Modifiers using a Zebrafish Autochthonous Tumor Model

Sharanya Iyengar1, Yariv Houvras2,3, Craig J. Ceol1

1Program in Molecular Medicine and Department of Cancer Biology, University of Massachusetts Medical School
2Departments of Surgery and Medicine, Weill Cornell Medical College
3Departments of Surgery and Medicine, New York Presbyterian Hospital

Correspondence to: Craig J. Ceol at craig.ceol@umassmed.edu

URL: http://www.jove.com/video/50086
DOI: doi:10.3791/50086

Keywords: Cancer Biology, Issue 69, Medicine, Genetics, Molecular Biology, Melanoma, zebrafish, Danio rerio, mitfa, melanocytes, tumor model, miniCoopR

Date Published: 11/13/2012


Abstract

Genomic studies of human cancers have yielded a wealth of information about genes that are altered in tumors1,2,3. A challenge arising from these studies is that many genes are altered, and it can be difficult to distinguish genetic alterations that drove tumorigenesis from those that arose incidentally during transformation. To draw this distinction it is beneficial to have an assay that can quantitatively measure the effect of an altered gene on tumor initiation and other processes that enable tumors to persist and disseminate. Here we present a rapid means to screen large numbers of candidate melanoma modifiers in zebrafish using an autochthonous tumor model4 that encompasses steps required for melanoma initiation and maintenance. A key reagent in this assay is the miniCoopR vector, which couples a wild-type copy of the mitfa melanocyte specification factor to a Gateway recombination cassette into which candidate melanoma genes can be recombined5. The miniCoopR vector has a mitfa rescuing minigene which contains the promoter, open reading frame and 3'-untranslated region of the wild-type mitfa gene. It allows us to make constructs using full-length open reading frames of candidate melanoma modifiers. These individual clones can then be injected into single cell Tg(mitfa:BRAFV600E);p53(lf);mitfa(lf) triply homozygous zebrafish embryos. Incubate the injected embryos at 28.5 °C. Remove any dead embryos at 24 hpf.

Protocol

1. Screening for Melanoma Onset Modifiers

1. Create Gateway middle entry clones by PCR amplifying the full-length open reading frame of genes of interest (GOI) and recombining into pDONR 221 using BP clonase II (Invitrogen). Use Multisite Gateway technology (Invitrogen) to recombine p5E_mitfa, pME_GOI, Tol2kit #302 p3E_SV40polyA5 and miniCoopR5 to place genes of interest under the mitfa promoter in the miniCoopR vector (Figure 1A).
2. Inject 25 picograms of each clone along with 25 picograms Tol2 transposase mRNA into one-cell Tg(mitfa:BRAFV600E);p53(lf);mitfa(lf) zebrafish embryos. The miniCoopR vector gets integrated by Tol2-mediated transgenesis5 and rescues melanocytes. Because they are physically coupled to the mitfa rescuing minigene, candidate genes are expressed in rescued melanocytes, some of which will transform and develop into tumors. The effect of a candidate gene on melanoma initiation and melanoma cell properties can be measured using melanoma-free survival curves, invasion assays, antibody staining and transplantation assays.
3. Select transgenic animals with rescued melanocytes at 72 hpf by placing the Petri dish containing injected embryos on a dissecting microscope under incident light against a white background (Figure 1A).
4. Transfer animals to 3 L tanks in the nursery of the zebrafish facility at 4 dpf as previously described8.
5. At 2 months, select the animals with at least one area of melanocyte rescue greater than 4 mm² (Figure 1A). There is a strong correlation between the degree of melanocyte rescue at 2 hpf and the degree of melanocyte rescue at 2 months. When embryos with melanocyte rescue are picked at 27 hpf, the majority of them (~80%) will have at least one area of melanocyte rescue greater than 4 mm² at 2 months.
6. Screen the selected animals weekly for the presence of tumors (Figure 1B). There is a strong correlation between histopathologic and morphologic changes. Transition from benign to malignant is recognized as a morphologic change when lesions become raised off the surface of the animal. Isolate tumor bearing animals for study.
7. Draw melanoma-free survival curves with age in weeks on the abscissa and percent melanoma-free survival on the ordinate. Animals with melanocytes that express a gene of interest are compared to control animals that express EGFP (enhanced green fluorescent protein)
2. Tumor Invasion Assay

1. Select isolated zebrafish that develop tumors dorsally, in a region between the posterior boundary of the hindbrain and anterior border of the dorsal fin (Figure 2A).

2. Two weeks after melanoma onset euthanize the fish according to guidelines specified by the American Veterinary Medical Association Panel on Euthanasia and approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee (IACUC). Specifically, a fish is placed into a dish with 0.6 mg/ml tricaine until gill movement stops.

3. Make an incision in the peritoneal cavity of the fish with a scalpel and then place the fish in 4% paraformaldehyde (PFA) overnight at 4 °C for fixation.

4. Treat with 0.5 M EDTA overnight at 4 °C to decalcify.

5. Fix with formalin in cassette overnight, dehydrate for 1 hr, clear with xylene 3 times for 5 min at room temperature, while turning, for ≥ 2 hr. Transfer the fish into fish water and monitor the fish to confirm successful recovery.

6. To bleach pigmented melanocytes wash fixed scales in PBST (1x PBS pH 7.4, 0.1% Triton X-100) 2 times for 5 min at room temperature, while turning, for ≥ 2 hr. Transfer the fish into fish water and monitor the fish to confirm successful recovery.

7. Anesthetize the fish in 0.17 mg/ml tricaine.

8. Make 5 μm sections, one every 50 μm. The sections should be transverse and through the entire lesion so that the point of deepest invasion can be identified. Follow by hematoxylin and eosin staining.

9. Wash the scales 3 times for 30 min in PBST at room temperature.

10. Transfer the zebrafish to a dish with 10% KOH and 10% HCl and 15% ethanol for 20 min. This will bleach the pigmented melanocytes.

11. Stain for 10 min with PBST+ 0.1 μM DAPI.

12. Wash 3 times for 5 min with PBST.

13. Mount the scales on a glass slide in a drop of vectashield so that the concave side of the scales faces down towards the slide and place a glass cover slip over them. Seal the edges of the slide with clear nail polish and observe the slides under a fluorescence microscope (Figure 3B,C). Determine statistical difference between the two sets of samples.

3. Antibody Staining of Scale Melanocytes

1. Make up PO4 buffer with 80 ml 0.1 M Na2HPO4 and 20 ml 0.1M NaH2PO4. Make sure the pH is 7.3. Make up 2x fix buffer containing 8.0 g sucrose, 0.15 ml 0.2M CaCl2 and 90 ml 0.2M PO4 buffer, pH 7.3. If necessary adjust the pH to 7.3 with NaOH or HCl then make up to 100 ml with PO4 buffer.

2. Weigh up to 300 mg solid PFA and add to a microcentrifuge tube. Add 5 mM NaOH to a volume equal to 4.5 x mass, in mg, of PFA (e.g. 450 ul 5 mM NaOH to 100 mg PFA). Heat at 60-70 °C with occasional shaking until the PFA is dissolved. Spin down remaining particulates and recover 20% PFA supernatant. Make fresh every time.

3. Prepare fixation solution containing 1x fix buffer and 4% PFA.

4. Incubate with primary antibody in block solution overnight at room temperature. You need to have at least 400 μl of solution over the samples to keep them submerged.

5. Wash in PBST four times for 5 min at room temperature.

6. Incubate with secondary antibody from 2 hr to overnight in block solution at room temperature.

7. Incubate with primary antibody in block solution overnight at room temperature. You need to have at least 400 μl of solution over the samples to keep them submerged.

8. Wash the scales 3 times for 30 min in PBST at room temperature.

9. Wash in PBST two times for 5 min at room temperature.

10. Wash in PBST one time for 5 min at room temperature.

11. Stain for 10 min with PBST+ 0.1 μM DAPI.

12. Wash 3 times for 5 min with PBST.

13. Mount the scales on a glass slide in a drop of vectashield so that the concave side of the scales faces down towards the slide and place a glass cover slip over them. Seal the edges of the slide with clear nail polish and observe the slides under a fluorescence microscope (Figure 3D,E,F,G).

4. Transplantation Assay

1. Prepare recipient 2-3 month old casper® fish by irradiating with 25 Gy of gamma irradiation one day prior to transplantation. Allow the fish to recover in fish water.

2. Euthanize a tumor-bearing fish as previously described (Section 2.2).

3. Two weeks after melanoma onset euthanize the fish according to guidelines specified by the American Veterinary Medical Association Panel on Euthanasia and approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee (IACUC). Specifically, a fish is placed into a dish with 0.6 mg/ml tricaine until gill movement stops.

4. Make an incision in the peritoneal cavity of the fish with a scalpel and then place the fish in 4% paraformaldehyde (PFA) overnight at 4 °C for fixation.

5. Treat with 0.5 M EDTA overnight at 4 °C to decalcify.

6. Fix with formalin in cassette overnight, dehydrate for 30 min to 1 hr.

7. Embed the tissue in paraffin in a mould and allow it to solidify for 30 min to 1 hr.

8. Spin in an Eppendorf 5810R tabletop centrifuge at 1,500 rpm (453 rcf) for 10 min.

9. Filter through 40 μM mesh filter.

10. Working at room temperature, triturate with a P1000 pipette to get single cells. Make up the volume to 25 ml with filter sterilized 0.9x PBS with solution.

11. Make up PO4 buffer with 80 ml 0.1 M Na2HPO4 and 20 ml 0.1M NaH2PO4. Make sure the pH is 7.3. Make up 2x fix buffer containing 8.0 g sucrose, 0.15 ml 0.2M CaCl2 and 90 ml 0.2M PO4 buffer, pH 7.3. If necessary adjust the pH to 7.3 with NaOH or HCl then make up to 100 ml with PO4 buffer.

12. Weigh up to 300 mg solid PFA and add to a microcentrifuge tube. Add 5 mM NaOH to a volume equal to 4.5 x mass, in mg, of PFA (e.g. 450 ul 5 mM NaOH to 100 mg PFA). Heat at 60-70 °C with occasional shaking until the PFA is dissolved. Spin down remaining particulates and recover 20% PFA supernatant. Make fresh every time.

13. Prepare fixation solution containing 1x fix buffer and 4% PFA.

14. Incubate with primary antibody in block solution overnight at room temperature. You need to have at least 400 μl of solution over the samples to keep them submerged.

15. Wash the scales 3 times for 30 min in PBST at room temperature.

16. Incubate with secondary antibody from 2 hr to overnight in block solution at room temperature.

17. Incubate with primary antibody in block solution overnight at room temperature. You need to have at least 400 μl of solution over the samples to keep them submerged.

18. Wash in PBST two times for 5 min at room temperature.

19. Wash in PBST one time for 5 min at room temperature.

20. Wash 3 times for 5 min with PBST.

21. Mount the scales on a glass slide in a drop of vectashield so that the concave side of the scales faces down towards the slide and place a glass cover slip over them. Seal the edges of the slide with clear nail polish and observe the slides under a fluorescence microscope (Figure 3D,E,F,G).

22. Load the syringe with 5 μl of the cell suspension.

23. Wash a 26s gauge (bevel tip) 701 N 10 μl Hamilton syringe 2-3 times with 100% ethanol and 0.9x PBS. Do not use the syringe with 5 μl of the cell suspension.
10. Anesthetize the irradiated recipient fish in 0.17 mg/ml tricaine. Place fish on its side on a damp Kimwipe (Figure 4A).
11. Stabilize the fish with one hand and insert the needle with the bevel facing up at a 45° angle into the flank of the fish above the peritoneal cavity about halfway between the posterior boundary of the hindbrain and anterior border of the dorsal fin. Gently depress the plunger (Figure 4B).
12. Allow the fish to recover in fresh fish water and observe the fish daily for tumor engraftment (Figure 4C). If engraftment has occurred, continued growth and disease development can also be observed.

**Representative Results**

One-cell Tg(mitfa:BRAFV600E);p53(lf);mitfa(lf) zebrafish embryos were injected with the miniCoopR vector containing the melanoma oncogene SETDB1 or EGFP, each under the control of the mitfa promoter. Embryos with melanocyte rescue were selected and allowed to mature. At 2 months of age animals with melanocyte rescue greater than 4 mm$^2$ were selected. The animals were screened weekly for melanomas. Tumor incidence curves for the adults showed that the SETDB1 oncogene significantly accelerated melanoma onset as compared to the EGFP control (Figure 1). Animals which developed tumors between the posterior boundary of the hindbrain and the anterior border of the dorsal fin (Figure 2A) were isolated. Two weeks after melanoma onset they were fixed in 4% paraformaldehyde, sectioned and stained with hematoxylin and eosin to assess melanoma invasion into underlying tissues. Melanomas expressing SETDB1 were more locally invasive than EGFP control melanomas (Figure 2C). In order to look for expression of a candidate gene, dorsal scales were plucked from a wild-type zebrafish and stained using a 1:100 dilution of a primary antibody that recognizes the Mitfa transcription factor followed by a 1:1,000 dilution of FITC goat anti-rabbit IgG antibody (Figure 3). To assess transplantability of the tumor, melanoma cells were isolated from a Tg(mitfa:BRAFV600E);p53(lf);mitfa(lf) fish injected with miniCoopR-EGFP. 50,000 cells were subcutaneously injected into a recipient casper mutant that had been irradiated the day before with 25 Gy. By 2 weeks of age, pigmented donor-derived cells were easily recognized (Figure 4).

---

**Figure 1.** Screening for melanoma onset modifiers using the miniCoopR assay. A) Schematic of the miniCoopR assay. Embryo with rescued melanocytes (arrowhead) containing the miniCoopR vector and the gene of interest. Scale bar = 250 μM. Adult with greater than 4 mm$^2$ melanocyte rescue (arrowhead). Scale bar = 500 μM B) MiniCoopR-EGFP rescued zebrafish with an amelanotic and a pigmented tumor (arrowheads). C) Representative melanoma-free survival curve comparing tumors expressing oncogene SETDB1 and a control EGFP gene (p = 9.4×10$^{-7}$, logrank $\chi^2$). Click here to view larger figure.
Figure 2. Tumor invasion assay. A) MiniCoopR-rescued zebrafish with a dorsal tumor (arrowhead) between the posterior boundary of the hindbrain and the anterior border of the dorsal fin. B) Transverse section showing stratum compactum (SC), scales, scale-associated melanocyte (SAM) (inset, scale bar = 50 μM), muscle (M) and spinal column (SpC). Scale bar = 200 μM. C) Transverse sections showing a non-invasive miniCoopR-EGFP tumor (T) (left) and a miniCoopR-SETDB1 tumor (right) that has invaded through the stratum compactum into muscle (M) and the spinal column. Scale bar = 200 μM.
Figure 3. Antibody staining of scale melanocytes. A) Scales being plucked from an anesthetized miniCoopR-EGFP zebrafish. B) Unbleached scale with pigmented melanocytes and C) bleached scale from miniCoopR-EGFP zebrafish. Scale bar = 100 μM. Unbleached scale stained with D) Mitfa antibody and E) DAPI. Bleached scale stained with F) Mitfa antibody and G) DAPI. Scale bar = 40 μM.
Figure 4. Transplantation of melanoma cells. A) Uninjected casper zebrafish. Scale bar = 500 μM. B) Subcutaneous transplantation site (arrowhead) on an irradiated casper recipient immediately after injection with 50,000 melanoma cells. Scale bar = 200 μM. C) Irradiated casper recipient showing tumor engraftment (arrowhead) two weeks after injection with 50,000 melanoma cells.

Discussion

The miniCoopR method enables expression of genes of interest in zebrafish melanocytes. This approach takes advantage of the fact that the zebrafish mitta gene acts cell-autonomously. For this reason, melanocytes rescued by the miniCoopR vector are certain to contain the minigene and any gene of interest to which it is physically coupled. Rescued melanocytes are clearly visible and can be obtained in animals that were injected as single-cell embryos. A specified degree of chimerism is selected, and animals surpassing this cutoff can be scored for tumor onset or other characteristics. A major benefit of the miniCoopR method is that animals with sufficient chimerism can be readily identified and scored without having to obtain stable transgenic lines corresponding to every gene of interest tested. Time, effort and expense are saved in proportion to the number of genes of interest to be surveyed.

As reported previously, miniCoopR-based methodology allowed us to screen many candidate oncogenes present in regions of chromosome 1 that is recurrently amplified in human melanomas. Other chromosomal intervals can be screened in a similar fashion. In addition, this approach can be used to express any gene in melanocytes, and any characteristic of these cells can be monitored. Even more broadly, the principle of coupling a gene of interest to a rescuing minigene can be applied to other tissue types. For such applications, a tissue-specific genetic mutant and a corresponding cell-autonomously acting rescuing gene are required. Mutant and gene pairs for other tissue types exist in zebrafish and other species, opening the possibility of rapid functional assays in a broader spectrum of tissue types.

Disclosures

No conflicts of interest declared.

Acknowledgements

We thank Dr. Leonard I. Zon in whose laboratory these techniques were initially developed; Kristen Kwan and the late Chi-Bin Chien for gifts of plasmids used in this work; James Lister and David Raible for assistance with antibody staining; and James Neiswender for the microinjection video. This work was funded by NIH grant R00AR056899-03 to C.J.C.

References


