Submission ID #: 61693
Scriptwriter Name: Anastasia Gomez
Project Page Link: https://www.jove.com/account/file-uploader?src=18816413

Title: In Vivo CRISPR/Cas9 Screening to Simultaneously Evaluate Gene Function in Mouse Skin and Oral Cavity

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interview Statements are read by JoVE's voiceover talent.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 14
Number of Shots: 40

Introduction

1. Introductory Interview Statements

Videographer: Skip the introduction and conclusion, authors have opted for VO talent to read these statements.

1.1. This protocol is significant because it allows researchers to simultaneously probe the tumor suppressive functions of hundreds of genes in head and neck cancer at a fraction of the cost of a conventional knock-out mouse model.

1.1.1. *2.8.1 – 2.8.3.*

1.2. This technique is flexible and can be adapted to over expression of genes to study their function in skin. The targeted CRISPR library protocol can also be applied for other cancer types.

1.2.1. *2.9.1 – 2.9.3.*

Ethics Title Card

1.3. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at the Toronto Centre for Phenogenomics (TCP).

Protocol

2. Ultra-sound Guided Surgery and Injection

2.1. Place the anesthetized animal ventral side up on the heated surgery stage **[1]** and use surgical tape to hold the paws in place **[2]**. Apply the nose-cone anesthesia tube for a constant supply of isoflurane and oxygen until the surgery is completed **[3]**.

2.1.1. WIDE: Establishing shot of talent placing the mouse on the stage.

2.1.2. Talent taping the paws in place.

2.1.3. Talent applying the nose-cone anesthesia tube.

2.2. To confirm E9.5 pregnancy, apply a small quantity of hair removal cream to the abdomen and spread it over a 3 by 3-centimeter squared area using a cotton-tipped applicator **[1]**. After 2 to 3 minutes, gently remove the cream with gauze or tissue **[2]** and wipe the area clean using PBS and 70% ethanol **[3]**.

2.2.1. Talent applying the hair removal cream.

2.2.2. Talent removing the cream.

2.2.3. Talent wiping the area clean, with the PBS and ethanol containers in the shot.

2.3. Using the ultrasound probe and gel, check the pregnant mouse for embryonic day E9.5. This can also be done the day before at E8.5 to save time on the day of surgery **[1]**. Remove the ultrasound gel and wipe the area clean using BPS and then 70% ethanol **[2]**. *Videographer: This step is important!*

2.3.1. Talent checking the mouse for embryonic development with the ultrasound.

2.3.2. Talent wiping the area clean.

2.4. Inject the mouse with subcutaneous analgesic **[1-TXT]**, then use sterile forceps and scissors to make a vertical incision in the skin **[2-TXT]**. Use blunt forceps to separate the skin around the incision from the peritoneum **[3]**, then use sterile forceps and scissors to cut through the peritoneum **[4]**.

2.4.1. Talent injecting analgesic. **TEXT: 0.02 cc Buprenorphine**

2.4.2. Talent making the incision. **TEXT: ~2 cm**

2.4.3. Talent separating the skin from the peritoneum.

2.4.4. Talent cutting through the peritoneum.

2.5. Using blunt forceps, gently pull out the left and right uterus horn and count the embryos **[1]**. Re-insert most of the uterine horns but leave the distal end of one uterine horn containing 3 embryos exposed **[2]**.

2.5.1. Talent pulling out the uterus horns.

2.5.2. Talent reinserting uterine horns but leaving the distal end of one exposed.

2.6. Using blunt forceps, gently push and pull the uterus with the 3 embryos through the opening in the silicone membrane of the modified Petri dish **[1]**. Stabilize the Petri dish using cubes of modeling clay **[2]**. *Videographer: This step is important!*

2.6.1. Talent pulling the uterus through the silicone membrane.

2.6.2. Talent stabilizing the dish.

2.7. Stabilize the uterus with the three embryos inside the Petri dish using a silicone mold **[1]**, then flatten the silicone plug at the side of the embryos using a sharp knife **[2]**. Fill the Petri dish with sterile PBS **[3]** and flush the silicon membrane on the bottom of the Petri dish with the pregnant dam's belly, thereby preventing any leakage **[4]**. *Videographer: This step is important!*

2.7.1. Talent stabilizing the uterus with the mold.

2.7.2. Talent flattening the silicone plug.

2.7.3. Talent filling the Petri dish with PBS.

2.7.4. Talent flushing the silicon membrane with the dam's belly.

2.8. Move the ultrasound head into the Petri dish approximately 0.5 centimeters above the top embryo **[1]** and adjust the stage **[2]** so that the amniotic cavity becomes clearly visible in the ultrasound view **[3]**. *Videographer: This step is important!*

2.8.1. Talent positioning the ultrasound head.

2.8.2. Talent adjusting the stage.

2.8.3. Amniotic cavity becoming visible on the display.

2.9. Align the injector needle carefully into the modified Petri dish **[1]**. Using the micromanipulator, place the tip of the needle within 5 millimeters of the top embryo **[2]**, then toggle the needle back and forth to move into the plane where its tip appears the brightest in the ultrasound image **[3]**. *Videographer: This step is difficult and important!*

2.9.1. Talent aligning the injector needle.

2.9.2. Talent using the micromanipulator to position the needle.

2.9.3. Needle coming into position in the ultrasound image.

2.10. Using the micromanipulator, push the needle through the uterine wall into the amniotic cavity **[1]**. Inject 62 nanoliters of the lentivirus with the sgRNA library at a slow injection speed **[2]**. Press the **Inject** button 8 times for a total of 496 nanoliters per embryo **[3-TXT]**. *Videographer: This step is important!*

2.10.1. Needle going into the amniotic cavity in the ultrasound image.

2.10.2. Embryo being injected in the ultrasound image.

2.10.3. Talent pushing inject. **TEXT: 1 x 10^8 pfu/mL and 496 nL injection volume will result in ~ 30% infectivity**

2.11. Repeat the same procedure for the other two embryos **[1]**. Then, lift the ultrasound head **[2]** and remove the silicone plug **[3]**. Gently push the first 2 of the 3 embryos into the mouse abdomen using a sterile cotton swap **[4]**.

2.11.1. Another embryo being injected.

2.11.2. Talent lifting the ultrasound head.

2.11.3. Talent removing the silicon plug.

2.11.4. Talent pushing the embryos into the mouse's abdomen.

2.12. Pull out the next 3 embryos and push in the previously injected third embryo. Repeat the injection procedure until the desired number of embryos are injected, making sure to not exceed 30 minutes of anesthesia **[1]**. *Videographer: This step is difficult!*

2.12.1. Talent pulling out the next 3 embryos and positioning them for injection.

2.13. When finished, lift the ultrasound head **[1]**, remove the micromanipulator with the needle **[2]**, aspirate the PBS, and remove the Petri dish **[3]**. Using sterile wipes, absorb any PBS that might have accumulated in the abdominal cavity **[4]**.

2.13.1. Talent lifting the ultrasound head.

2.13.2. Talent removing the micromanipulator with the needle.

2.13.3. Talent aspirating the PBS.

2.13.4. Talent absorbing PBS from the abdominal cavity.

2.14. Close the peritoneal incision using absorbable sutures **[1]**, then use two staples to close the incision in the abdominal skin **[2]**.

2.14.1. Talent closing the peritoneal incision.

2.14.2. Talent closing the abdominal skin.

Results

3. Results: *In vivo* CRISPR Screen for Tumor Suppressor Genes

3.1. Next-generation sequencing reads of PCR amplified DNA from plasmid and lentiviral library transduced cells should show a high correlation. If the sgRNA reads from plasmid DNA do not show equal representation of sgRNAs, then the viral preparation and concentration procedure must be repeated with care **[1]**.

3.1.1. LAB MEDIA: Figure 1 B.

3.2. The results of the ultra-sound guided injection of lentivirus carrying Cre-recombinase in E9.5 Lox-stop-Lox Confetti mouse pups at postnatal day P-zero are shown here. While high titer of virus would result in larger coverage of the mouse skin, it would also result in transduction of multiple sgRNAs into the same cell **[1]**.

3.2.1. LAB MEDIA: Figure 3.

3.3. This plot shows next-generation sequencing reads of PCR amplified DNA from plasmid and lentiviral library transduced cells as well as reads from 4 representative tumors **[1]**. sgRNA guides targeting tumor suppressors *Adam10 (pronounce 'adam-ten')* and *Ripk4 (pronounce 'rip-K-4')* are enriched in the tumor samples compared to the plasmid pool or infected cells **[2]**.

3.3.1. LAB MEDIA: Figure 4.

3.3.2. LAB MEDIA: Figure 4. *Video Editor: Emphasize the triangles and diamonds by the legend.*

Conclusion

4. Conclusion Interview Statements

4.1. Performing the surgery at E9.5 is critical. Remember to check for pregnancy with ultrasound 9 days after setting up the cross and identify the embryonic timepoint in each female mouse.

4.1.1. *2.3.1.*

4.2. Once the pups are born, the transduced cells can be isolated and extensively profiled using the latest gene profiling techniques such as single-cell RNA sequencing.

4.2.1. *2.10.2, 2.10.3.*