Somatic Genome-Engineered Mouse Models Using *In Vivo* Microinjection and Electroporation

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Abstract

Germline genetically engineered mouse models (G-GEMMs) have provided valuable insight into *in vivo* gene function in development, homeostasis, and disease. However, the time and cost associated with colony creation and maintenance are high. Recent advances in CRISPR-mediated genome editing have allowed the generation of somatic GEMMs (S-GEMMs) by directly targeting the cell/tissue/organ of interest.

The oviduct, or fallopian tube in humans, is considered the tissue-of-origin of the most common ovarian cancer, high-grade serous ovarian carcinomas (HGSCs). HGSCs initiate in the region of the fallopian tube distal to the uterus, located adjacent to the ovary, but not the proximal fallopian tube. However, traditional mouse models of HGSC target the entire oviduct, and thus do not recapitulate the human condition. We present a method of DNA, RNA, or ribonucleoprotein (RNP) solution microinjection into the oviduct lumen and *in vivo* electroporation to target mucosal epithelial cells in restricted regions along the oviduct. There are several advantages of this method for cancer modeling, such as 1) high adaptability in targeting the area/tissue/organ and region of electroporation, 2) high flexibility in targeted cell types (cellular pliancy) when used in combination with specific promoters for Cas9 expression, 3) high flexibility in the number of electroporated cells (relatively low frequency), 4) no specific mouse line is required (immunocompetent disease modeling), 5) high flexibility in gene mutation combination, and 6) possibility of tracking electroporated cells when used in combination with a Cre reporter line. Thus, this cost-effective method recapitulates human cancer initiation.

Introduction

The fallopian tube, called the oviduct in mice, is a tubular structure that connects the uterus to the ovary. It plays an essential role in mammalian reproduction, providing the environment for internal fertilization and preimplantation

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development[1,2]. Despite its importance, little is known about its function and homeostasis, partly due to the development of *in vitro* fertilization techniques circumventing any infertility issue related to this organ[3]. However, it has been recognized that precancerous lesions of high-grade serous ovarian carcinoma (HGSC), an aggressive histotype of ovarian cancer that accounts for around 75% of ovarian carcinomas and 85% of related deaths[4], are restricted to the distal fallopian tube epithelium[5,6,7,8]. This indicates that not all cells in our body are equally susceptible to oncogenic insults, but rather only unique/susceptible cells in each tissue/organ become the cell-of-origin in cancer — termed cellular pliancy[9]. Along these lines, it has been shown that the epithelial cells of the distal fallopian tube, located adjacent to the ovary, are distinct from the rest of the tube[10,11]. Thus, traditional mouse models of HGSC, that target all cells in the oviduct, do not recapitulate the human condition. In a recent study, we used a combination of CRISPR-mediated genome editing, *in vivo* oviduct electroporation, and Cre-based lineage tracing to successfully induce HGSC by mutating four tumor suppressor genes in the distal mouse oviduct[12,13]. This manuscript presents a step-by-step protocol describing this procedure of microinjection and *in vivo* electroporation to target the mouse oviduct mucosal epithelium.

This method has several advantages. It can be adapted to target other tissues/organs, including organ parenchyma[14]. Although other *in vivo* gene delivery approaches like lentiviral and adenoviral systems can be used to achieve similar tissue/organ-specific targeting, the area of targeting is more easily adjusted using different sizes of tweezer-type electrodes for electroporation-based delivery. Depending on the concentration of DNA/RNA/ribonucleoproteins (RNPs), electroporation parameters, and size of electrodes, the number of electroporated cells can be altered. Further, specific cell types can be targeted when used in combination with promotors for Cas9 expression, without the absolute need for Germline genetically engineered mouse models (G-GEMMs). In addition, unlike viral delivery systems, electroporation allows for multiple plasmid delivery into single cells and less constraint in the insert DNA size[15]. *In vivo* screening of gene mutations can also be performed with relative ease due to this high flexibility. Further, electroporated cells can be tracked or traced when this method is used in combination with Cre reporter lines such as Tdtomato or Confetti[16,17].

Protocol

Animals were housed in static microisolation cages with filter tops, located in a dedicated room containing a type II biosafety cabinet. All animal work was performed in accordance with institutional guidelines and was approved by McGill University's Faculty of Medicine and Health Sciences Animal Care Committee (AUP #7843).

1. Microinjection needle preparation

1. Pull a glass capillary tube into a sharp point using a micropipette puller with the following program: P = 500, Heat = 576, PULL = 50, VEL = 80, DEL = 70.
2. Using a pair of tapered ultrafine tip forceps, snip the pointed end of the pulled capillary tube under a dissecting microscope to create an opening, as shown in **Figure 1A,B**. Make sure that the opening is not too large, as this will damage the oviduct and prevent slow injection.

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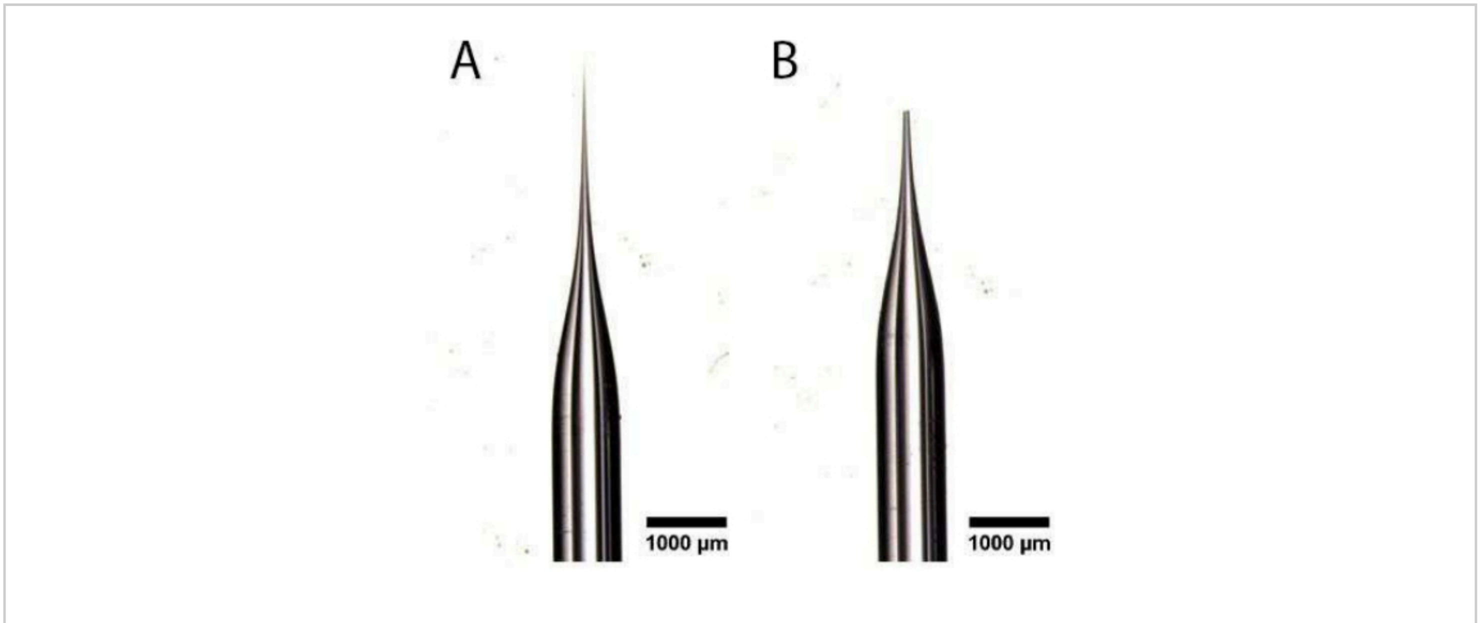

Figure 1: Microinjection needle preparation. (**A,B**) Pulled capillary tube with a pointed end (**A**) that was snipped to create an opening (**B**). Please click here to view a larger version of this figure.

2. Preparing the anesthetic for intraperitoneal injection

1. Prepare filtered, diluted avertin on the day of surgery. Avertin (2,2,2-tribromoethanol) stock solution is prepared at a concentration of 1.6 g/mL in tert-amyl alcohol by vigorous stirring and stored in the dark.
2. Dilute the avertin stock solution with 1.25% v/v isotonic saline to a final concentration of 20 mg/mL inside a fume hood. Vortex the diluted avertin and filter through a 0.22 µm sterile filter. Keep in the dark.

3. Preparing the surgery area

1. The surgery area should be a dedicated sterile environment, preferably a clean bench or biosafety cabinet. Arrange the sterile surgery equipment, microscope, micromanipulator, and electroporator as needed. An example of this is shown in **Figure 2**.
2. Heat up the heating pad/disc and place it underneath a clean, fully equipped cage. This cage will be used to house the mice following the surgery.
3. Pour sterile 1x phosphate buffered saline (PBS) into a sterile petri dish. Use a pair of clean scissors to cut absorbent paper into squares, with approximate dimensions of 1 cm x 1 cm. Drop these pieces of absorbent paper into the 1x PBS to soak them.

4. Preparing the solution and needle for injection

1. Prepare the injection solution on the day of the surgery. Mix the DNA/RNA/RNPs with filtered trypan blue to a minimum final concentration of 200 ng/µL each and 5%, respectively. Store at room temperature prior to injection.
NOTE: Use a 1 mL syringe to take up 100% trypan blue and filter through a 0.22 µm sterile filter prior to use.

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2. Attach the pulled capillary needle, henceforth called a microinjection needle, to the clamp mount micromanipulator stabilized by a magnetic mount (setup shown in **Figure 2B**).

3. Pipette 2 µL of the injection solution onto a sterile Petri dish. While observing under the microscope, slowly take up 1-2 µL of this solution into the microinjection needle using the air pressured syringe attached to the micromanipulator. Avoid bubbles/taking up bubbles into the microinjection needle.

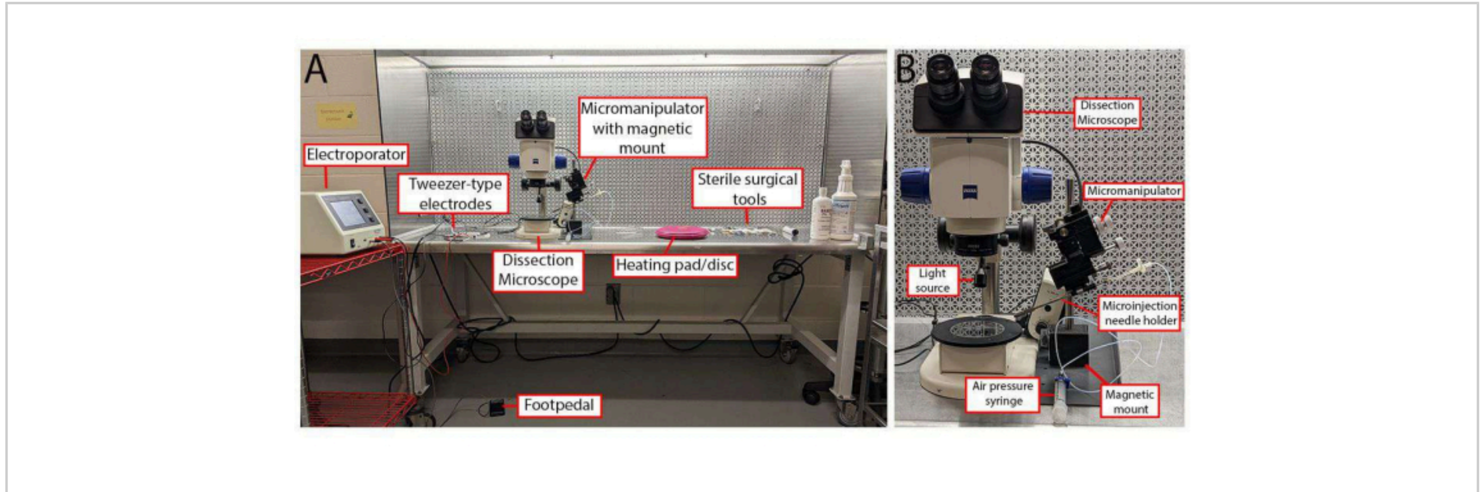

Figure 2: Surgery area preparation. (**A**) Surgery area setup inside a clean bench. (**B**) Dissection microscope and micromanipulator setup for a right-handed individual. Please click here to view a larger version of this figure.

5. Exposing the female reproductive tract

1. Anesthetize a 6-8-week-old female mouse by administering filtered, diluted avertin (anesthetic) intraperitoneally at a dose of 0.25-0.5 mg/g. Following this, administer carprofen (preemptive analgesic) subcutaneously at a dose of 5 mg/kg.

   NOTE: Other alternatives that can be used as an anesthetic are isoflurane or ketamine/xylazine.

2. Place absorbent paper on a clean, heated pad/disc. Then, place the anesthetized mouse on this heated absorbent paper with the dorsal side facing up. Apply eye lubricant to each eye to prevent drying during the surgery.

   NOTE: Confirm that the heating pad/disc is slightly warm to the touch by testing with the back of the hand.

3. Test for deep anesthetic arrest by pinching the toe of the anesthetized mouse with a pair of forceps. Remove dorsal fur around the prospective incision site (location shown in **Figure 3A**) and wipe the bare skin with antiseptic.

4. Use a pair of straight blunt forceps to pinch the bare skin and create a 1 cm long incision along the body midline using a pair of sterile sharp blunt scissors. Clean the area around the incision with antiseptic.

5. Pinch and hold up one side of the cut site using a pair of sterile Adson forceps. Then, using a pair of sterile curved

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serrated forceps, gently separate the skin from the body wall, starting at the midline incision and moving laterally.

6. Locate the fat pad below the kidney. Using a pair of sterile straight blunt forceps, pinch the body wall directly above the fat pad. Create a small incision in the body wall using a pair of sterile sharp-pointed dissecting scissors, taking care to avoid blood vessels.
7. While still pinching the body wall with straight blunt forceps, insert a pair of sterile blunt curved forceps into the incision and widen the incision created in the body wall.
8. Grab the visible fat pad with the blunt curved forceps and pull it out of the hole to expose the ovary, oviduct, and uterus.
9. To keep the reproductive tract exposed, clamp the fat pad with a sterile bulldog clamp, as shown in **Figure 3B**.

6. *In vivo* oviduct injection and electroporation

1. Carefully place the heated absorbent paper and anesthetized mouse with the reproductive tract exposed on the stage of a dissecting microscope, such that the tract can be observed. An example of the view as observed under the microscope is shown in **Figure 3B**.
2. Adjust the micromanipulator so that the injection needle with solution can also be observed under the microscope.
3. Using a pair of sterile tapered ultrafine tip forceps, hold the region of the oviduct that is to be injected steady. The region to be injected must be in line with the direction of the microinjection needle.
   NOTE: Use the bulldog clamp anchoring the fat pad and reproductive tract to gently turn/move the tract to an optimal position, if needed.
4. Adjust the micromanipulator to puncture the oviduct with the microinjection needle, while simultaneously feeding the oviduct onto the needle using the tapered ultrafine tip forceps. Gently move the microinjection needle to confirm that it has been inserted into the oviduct.
   NOTE: Insert the microinjection needle into a straight segment instead of into a turning point of the coiled oviduct to prevent multiple punctures and leakage of injection solution.
5. Slowly inject up to 1 µL of solution into the oviduct, while observing the movement of the blue solution and expansion of the oviduct lumen under the microscope. Take care not to introduce any bubbles into the lumen. Representative images of oviducts injected with 100% trypan blue are shown in **Figure 3C,D**.
6. Remove the needle from the oviduct. Cover the area to be targeted with a piece of absorbent paper presoaked in sterile 1x PBS (from step 3.3).
7. Grasp the presoaked paper and the area/region to be targeted with a pair of electrode tweezers (1 mm, 3 mm, or 5 mm, depending on the size of the region to be targeted) and pull away from the body, before electroporating using these settings on the pulse generator/electroporator: 30 V, three pulses, 1 s interval, 50 ms pulse length, unipolar.
   NOTE: The distance between the electrodes should be set such that the electrodes can clamp onto the targeted region without deforming the oviduct. The recommended distance is around 1 mm.
8. After electroporation, remove the absorbent paper and place the mouse (with the heated absorbent paper underneath) back onto a heat pad. Unclamp the bulldog clamp and carefully push the exposed reproductive tract

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back under the body wall. Repeat section 5 to expose the reproductive tract on the other side.

NOTE: Electroporation is successful regardless of the mouse estrous stage as long as the lumen is filled with the solution.

9. Cover the exposed tract with absorbent paper presoaked in sterile 1x PBS to prevent it from drying out, then prepare the microinjection needle for the second injection by repeating steps 4.2 and 4.3.

   NOTE: Replace the microinjection needle as needed. Prevent drying of the exposed tissue by applying sterile 1x PBS when required.

10. Repeat the steps above to inject and electroporate the oviduct on the opposing side.

    NOTE: Reapply the eye lubricant as needed during and after the surgery.

11. After both oviducts have been electroporated and placed back under the body wall, suture or staple the dorsal incision site. For suturing, use a hemostat to grasp the needle of the silk braided sutures (3/8 circle; gauge: 5-0; needle size: 18 mm; thread length: 75 cm). Pinch and hold up one side of the cut site using a pair of sterile curved serrated forceps, then use the hemostat to manipulate the suture to close the wound.

12. Move the mouse into a clean cage that is placed on top of a heated pad/disc (from step 3.2) and monitor until the mouse is active.

13. Administer 5 mg/kg carprofen subcutaneously every 24 h for the next 3 days and monitor over the next 10 days.

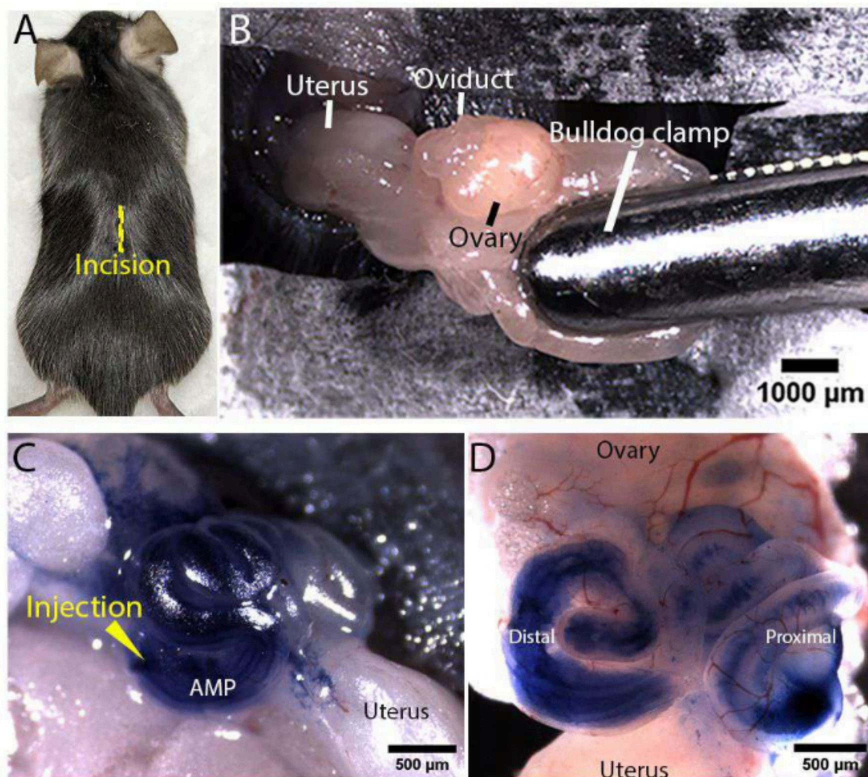

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Figure 3: Female reproductive tract exposure and microinjection. (**A**) Location of midline incision (shown as a yellow line) on the dorsal side of a Rosa-LSLtdTomato mouse. (**B**) Female reproductive tract exposure. The fat pad was clamped using a sterile bulldog clamp to anchor the tract and keep it exposed. (**C,D**) Representative images of *in vivo* oviduct injection. A microinjection needle was inserted into the distal ampulla (labelled AMP), and filtered 100% trypan blue was injected into the oviduct lumen while the tract was exposed (**C**). Representative image of a dissected oviduct, demonstrating that injected trypan blue solution distributes throughout the distal and proximal oviduct lumen (**D**). Please click here to view a larger version of this figure.

Representative Results

Figure 1 and **Figure 2** depict the microinjection needle preparation and surgery area setup, respectively, for *in vivo* microinjection and electroporation of the mouse oviduct. During the surgery, the female reproductive tract was exposed through incisions made in the dorsal skin (**Figure 3A**) and body wall of an anesthetized Rosa-LSLtdTomato (Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze/J}$)[17] mouse. A bulldog clamp was clamped onto the fat pad above the tract to keep it exposed and anchored (**Figure 3B**). Injection solution containing PCS2 CreNLS plasmids[18] at 400 ng/µL concentration was injected into the ampulla (labelled AMP) of the coiled oviduct and allowed to disperse throughout the oviduct lumen (**Figure 3C,D**). Although PCS2 CreNLS plasmids were used to generate representative results in this manuscript, the described method can be used with easily interchangeable DNA/RNA/RNPs in the injection solution for CRISPR/Cas-mediated gene editing in the mouse oviduct. Then, 1 mm or 3 mm tweezer type electrodes were positioned, such that the distal oviduct was in between the electrodes for electroporation-based delivery.

Oviducts were harvested 4 days following the surgery and stretched out by removing the mesosalpinx to visualize electroporation specificity. Using either 1 mm or 3 mm tweezer-type electrodes, the targeted area, labelled with TdTomato, was restricted to the distal oviduct epithelium (**Figure 4A,D**). The size of this targeted area was further controlled by using electrodes of different sizes. Using 3 mm tweezer-type electrodes, we targeted a much larger area of the AMP (**Figure 4B,C**), as compared to targeting only the distal-most tip, the INF (**Figure 4E,F**), using 1 mm tweezer-type electrodes. These harvested oviducts were fixed, treated with a sucrose gradient, and sectioned using a cryostat for detailed analyses. Sections were counterstained with Hoescht 33342 and Phalloidin to stain the nuclei and cytoskeleton respectively, then they were imaged using a point-scanning confocal microscope. In the targeted region, electroporated cells, marked by TdTomato, were randomly distributed among non-electroporated (TdTomato-ve) cells (**Figure 4G**). Additionally, electroporated cells were restricted to the mucosal epithelium and not found in the underlying stromal or muscle layer (**Figure 4G,H**). Finally, to confirm CRISPR-Cas-mediated gene editing, TdTomato+ve cells can be isolated by fluorescence-activated cell sorting (FACS) for DNA isolation and MiSeq sequencing[12].

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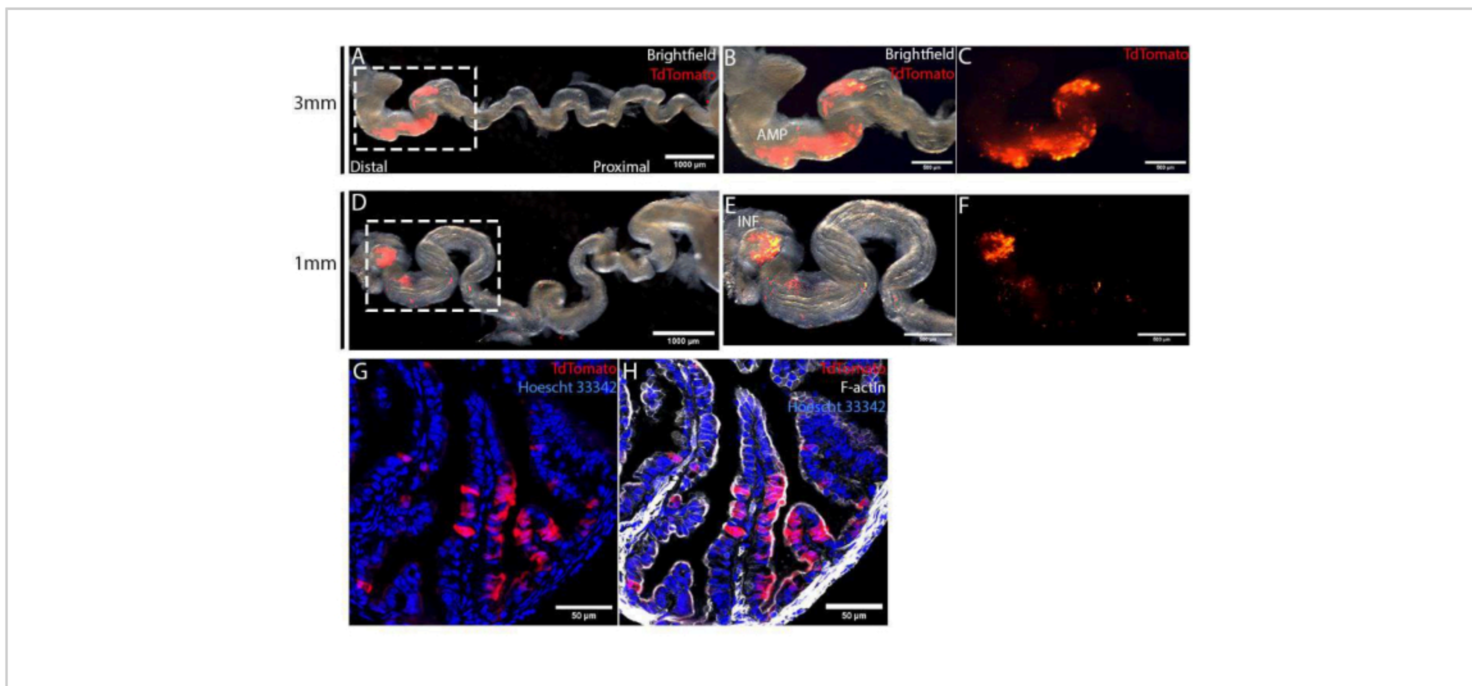

Figure 4: Validation of successful *in vivo* injection and electroporation of oviduct epithelial cells, 4 days post-surgery. (**A-C**) Electroporation using 3 mm sized tweezer-type electrodes. The oviduct was uncoiled by removing the mesosalpinx for better visualization of electroporation specificity (**A**). Electroporation area, identified by TdTomato expression, was restricted to the distal oviduct, labelled AMP (**B,C**). (**D-F**) Electroporation using 1 mm sized tweezer-type electrodes. The oviduct was uncoiled by removing the mesosalpinx for better visualization of electroporation specificity (**D**). The electroporation area was restricted to the infundibulum (labelled INF), the distal-most tip of the oviduct (**E,F**). (**G,H**) Transverse section of the distal oviduct 4 days following electroporation with PCS2 CreNLS plasmids. Electroporated cells labelled with TdTomato (**G**) were restricted to the epithelial monolayer (**H**). Please click here to view a larger version of this figure.

Discussion

Crucial steps in this detailed protocol are the microinjection of DNA/RNA/RNP solution into the oviduct lumen and control of the electroporation strength and area. DNA/RNA/RNP solution leakage during microinjection may cause transfection of undesired areas/cells. For consistent and efficient electroporation, it is preferable to fill the oviduct lumen with the solution (**Figure 3C,D**). This is because the area of electroporation is mainly controlled by electrode size and placement. Weak electroporation reduces the number of electroporated cells, and harsh electroporation may cause the electroporation of undesired cells or disrupt tissue structure. The number of electroporated cells can be varied by adjusting the DNA/RNA/RNP solution concentration or electroporation parameters. However, since high voltages/heat production during electroporation could damage the tissue, these parameters should be tested prior to use on live/anesthetized mice. Finally, due to the demanding nature of this procedure, it is recommended to practice tract exposure

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and microinjection on dead mice prior to performing this surgery on live/anesthetized mice.

It is recognized that multiple genes are involved in cancer initiation, thus, recapitulating this event requires multi-allelic modification of several genes. Additionally, it is also known that not all cells are equally susceptible to oncogenic mutations[9]. Cancer modeling, therefore, requires specific Cre mouse lines to achieve tight control of oncogenic insults. However, their availability and specificity causes various challenges, including effects in non-targeted tissues and lethality[19]. Further, traditional G-GEMMs incur high costs for maintenance and require time for mouse line generation. The development of CRISPR/Cas9 genome editing technology and improvement of gene delivery into specific somatic cells has allowed us to overcome these issues. In this protocol, we present a DNA/RNA/RNP delivery method for somatic genome manipulation that can be used for cancer modeling, without the absolute need for specific mouse lines[12]. By injecting DNA/RNA/RNP solutions into the lumen and using different sizes of tweezer-type electrodes for electroporation-based delivery, restricted areas of the mouse oviduct mucosal epithelium are targeted (**Figure 4A-F**). This is especially useful in modeling the initiation of HGSCs that originate from the distal end of the fallopian tube.

The presented microinjection and *in vivo* electroporation method is highly versatile for targeting tissues/organs with a lumen. Organ parenchyma is also targetable using this method[14]. Viral delivery approaches, like lentiviral and adenoviral systems, can also be used for similar purposes. However, the advantages of electroporation over viral delivery approaches are: 1) multiple plasmid delivery into single cells, 2) no restriction on the insert size[15], and 3) easy control of the area and timing of delivery. In addition, targeting specificity can be improved by using lineage specific promoters. This is sometimes difficult in viral systems due to the limit on viral packaging.

As is the nature of electroporation, electroporated epithelial cells are randomly interspersed with healthy, unedited cells (**Figure 4G**). However, this mosaicism in genetically modified and unmodified cells could be advantageous to study cancer initiation, as it recapitulates the sporadic nature of early cancer initiation within an immunocompetent microenvironment. The frequency of electroporated cells can be adjusted by varying the DNA/RNA/RNP solution concentrations and/or electroporation parameters. Using CRISPR-Cas-mediated gene editing in combination with the presented protocol, it is easy to screen multiple genes and generate heterogeneous patterns of mutations/allelic combinations in targeted genes; this is particularly useful in modeling cancers that present with a considerable number of genomic alterations like HGSCs[20,21]. Further, by sequencing targeted genes during cancer progression, it is possible to follow the *in vivo* clonal evolution of tumors and metastases in immunocompetent mice[12].

Disclosures

The authors have nothing to disclose.

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