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Investigating the Origins of Somatic Cell Populations in the Perinatal Mouse Ovaries Using Genetic Lineage Tracing and Immunohistochemistry

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Abstract

Genetic lineage tracing (or fate mapping) techniques are designed to permanently label progenitor cells of target tissues, thereby allowing delineation of the progenies of labeled cells during organogenesis. This technology has been widely used in the study of cell migration and lineage specification in various organs and organisms. Here, we describe how to apply the genetic lineage tracing model in combination with immunohistochemistry to identify the potential origins of somatic cell precursors in perinatal mouse ovaries.

Keywords

Lineage tracing; Fate mapping; Immunohistochemistry; Progenitor; Somatic cells; Ovary; Organogenesis

1. Introduction

Traditional lineage tracing experiments utilize mostly fluorescent dyes to stain living cells, which pass the dye onto their progenies. One concern of this approach is that the dyes could diffuse to adjacent but unrelated cells (1). The Cre recombinase-mediated genetic lineage tracing technique is a reliable method that marks particular cell populations and their descendants during development (1, 2). The power of mouse genetics has allowed us to trace the fate of certain cells in not only a cell type-specific, but also a time-course dependent, manner. One of such systems requires a mouse line that carries the tamoxifen-inducible Cre recombinase gene under the control of a cell type-specific promoter. Treatment of tamoxifen activates the Cre recombinase exclusively in the cells that express the cell type-specific promoter. When a Cre reporter gene, such as *Rosa26YFP* or *Rosa26LacZ*, is also present, the induced Cre recombinase turns on the reporter gene permanently in the target cells, therefore marking the cells and their progenies. Cre recombinase is active only in the presence of tamoxifen, allowing cell labeling to occur only in a defined period of time (3–6). This inducible lineage tracing model is different from traditional reporter lines in which the expression of the reporter is under the control of the promoter elements of the cell type-specific gene (7). The reporter in the traditional model reflects the endogenous expression pattern of the cell type-specific gene. If the gene is turned off during development, the reporter expression is off, consequently resulting in the inability to track the progenies of this particular cell type.

The inducible Cre-mediated lineage tracing technique has been successfully used in various organ systems, such as limb, brain, etc. (5, 8–10). However, such a genetic system has not

been applied to the ovary, mainly because we have limited knowledge on genes implicated in establishing cell lineages in the ovary. We have been searching for the potential progenitor cell source of the theca cells, the mesenchymal cell types that produce androgens during folliculogenesis. It was found that the mesenchyme surrounding the follicles in the adult mouse ovary is positive for intracellular components of the Hedgehog signaling pathway, such as *Gli1* transcription factor (11–13). During fetal life, *Gli1* expression is absent in the ovary but is positive in the neighboring mesonephros, which contains the Wolffian and Müllerian ducts. After birth, *Gli1*-expressing cells first appear in the junction between the ovary and mesonephros and extend their presence to the mesenchyme of the neonatal ovary (unpublished data). We, therefore, hypothesize that *Gli1* can be used as a lineage tracing marker to investigate whether cells in the fetal mesonephros contribute to mesenchymal cell population in the neonatal ovary.

The lineage tracing experiments were accomplished by crossing the *Gli1^{tm3}(cre/ERT2)Alj/J* (or *Gli1-CreER^{T2}* hereafter) to the Cre reporter line (*Rosa26YFP* or *Rosa26LacZ*) (8, 14, 15). *Gli1-CreER^{T2}* mice express the tamoxifen-inducible Cre recombinase (CreER^{T2}) from the endogenous *Gli1* locus (8, 16). The *Rosa26YFP* mice contain an enhanced yellow fluorescent protein (*EYFP*) gene inserted into the ubiquitous *Rosa26* locus (Fig. 1). The expression of *EYFP* is blocked by an upstream *loxP*-flanked STOP sequence (15, 17). Tamoxifen treatment activates the *Gli1-CreER^{T2}*, which in turn excises the STOP sequence and allows the permanent expression of *EYFP* in the *Gli1*-positive cells and their progenies (Fig. 1). By giving tamoxifen during fetal stages when *Gli1* expression is present in the mesonephros but absent in the ovary, we can investigate whether the *Gli1*-positive cells in the mesonephros eventually become a part of the neonatal ovaries.

2. Materials

2.1. Strains of Mice

Mice were maintained on a C57BL/6 J background and were used for the experiment at 3–5 months old for female and 3–8 months old for male.

1. *Gli1^{tm3}(cre/ERT2)Alj/J* mice (8) (Heterozygote; Stock # 007913; The Jackson Laboratories).
2. B6.129X1-*Gt(ROSA)26Sortm1(EYFP)Cos/J* mice (15) (Homozygote; Stock # 006148; The Jackson Laboratories).
3. B6.129 S4-*Gt(ROSA)26Sortm1Sor/J* mice (14,18) (Homozygote; Stock # 003474; The Jackson Laboratories).

2.2. Tamoxifen Stock Solution

1. 70% ethanol: Add 30 mL of sterile water to 70 mL of 100% ethanol.
2. 50 mg/mL tamoxifen solution: Dissolve 0.05 g of tamoxifen in a solution of 950 µL corn oil and 50 µL 100% ethanol at 55°C in water bath. Vortex every few minutes until the powder is completely dissolved (see Note 1).

2.3. Genotyping

1. 50 mM NaOH solution.

¹Tamoxifen solution made in corn oil is stored at 4°C for a week or at –20°C for several months. Warm the solution in 55°C water bath every time before use to ensure complete dissolution. Cool the dissolved solution to room temperature before dosing (16).

2. 1 M Tris-HCl: Dissolve 121 g Tris base in sterile water. Adjust pH to 8.0 by adding 42 mL of HCl. Bring volume up to 1 L. Autoclave for 30 min.
3. PCR primers:
 Cre.465.633: 5'-GAG TGA ACG AAC CTG GTC GAA ATC AGT GCG-3'.
 Cre.58.87: 5'-GCA TTA CCG GTC GAT GCA ACG AGT GAT GAG-3'.
4. Mango PCR Mix (BioLine).
5. 50× TAE buffer: Dissolve 242 g Tris base in 57.1 mL glacial acetic acid, and 100 mL 0.5 M EDTA; add 750 mL distilled H₂O (dH₂O) and adjust pH to 8.0. Bring the final volume of solution to 1 L with dH₂O and autoclave for 30 min.
6. 0.5 M EDTA (pH 8.0): Add 186.1 g EDTA to 800 mL of double-distilled H₂O. Adjust pH to 8.0 and bring final volume to 1 L. Autoclave for 30 min.
7. 4% paraformaldehyde in 1× PBS.
8. 1.5% agarose gel (100 mL gel): In a small beaker, add 1.5 g agarose to 100 mL 1× TAE (2 mL 50× TAE with 98 mL ddH₂O). Heat the solution to boiling in the microwave to dissolve the agarose until no residues are seen in the solution. Let the solution cool for about 2 min and add 5 µL of ethidium bromide (0.005%) to the solution and mix. Pour the agarose solution into a gel box mold and let the gel cool to room temperature.

2.4. Organ Culture

1. 4-well culture plate.
2. 10× PBS: Weigh 80 g NaCl, 2 g KCl, 14.4 g NaH₂PO₄, and 2.4 g KH₂PO₄; add 800 mL of ddH₂O and pH to 7.4; adjust the final volume to 1 L and autoclave for 30 min.
3. Dissecting PBS: 1× PBS with 5–10% fetal bovine serum (FBS).
4. Bottle-top filters (150 mL cellulose acetate, 0.4 µm, 35-mm neck).
5. Culture medium: 1 mg/mL BSA, 1 mg/mL Albumax, 0.05 mg/mL ascorbic acid, 0.0275 mg/mL transferrin, 0.005 mg/mL streptomycin in Ham's F-12/DMEM.
 - a. In 50 mL Ham's F-12/DMEM, add 250 µL of 200 mg/mL BSA, 500 µL of 100 mg/mL Albumax (Invitrogen), 50 µL of 50 mg/mL ascorbic acid, 50 µL of 27.5 mg/mL transferrin (Sigma-Aldrich), 50 µL of 5,000 µg/mL streptomycin (1,000×) (Invitrogen).
 - b. Filter the media through a bottle-top filter. Aliquot the media in sterile 50-mL tubes and store at 4°C. The medium is good for at least 2 weeks.
6. Millicell culture plate inserts (0.4 µm, 35-mm diameter).

2.5. Immunohistochemistry

1. 1× PBS.
2. 10, 15, and 20% sucrose in 1× PBS.
3. Optimum cutting temperature (OCT) compound.
4. 20% sucrose and OCT mix (1:1 and 1:3; v/v).

5. Blocking buffer: Combine 2.5 mL donkey serum (final concentration 5%; Jackson's Immunoresearch Laboratories), 50 μ L of Triton X-100 (final concentration 0.1%), and 47.5 mL PBS.
6. Washing solution: Combine 0.5 mL donkey serum (final concentration 1%), 50 μ L of Triton X-100 (final concentration 0.1%), and 49.5 mL PBS.
7. VECTASHIELD® Mounting Medium with DAPI.
8. Superfrost Plus Microscope Slides.
9. PAP/ImmEdge pen.
10. Microtome Cryostat.
11. Primary and secondary antibodies.
 - a. Primary antibody: Goat anti-FOXL2 antibody (Imgenex Corp).
 - b. Secondary antibody: Cy3-conjugated Donkey Anti-Goat IgG (Jackson ImmunoResearch Laboratories).

3. Methods

3.1. Breeding of Animals and Tamoxifen Treatment

1. Set up breeding cages for male *Gli1-CreER^{T2}* and female *Rosa26YFP* or female *Rosa26LacZ* mice in the late afternoon (see Note 2).
2. The following morning, check each female for the presence of a vaginal copulation plug. If a copulation plug is observed, noon of that day is considered 0.5 days post coitum (dpc). Noon of the following day is considered 1.5 dpc and so forth.
3. On 15.5 dpc, the tamoxifen solution is fed orally to the pregnant female at 9 a.m. in the morning. Tamoxifen is given once per day for 2 consecutive days based on the body weight of the pregnant female (20 μ L of the 50 mg/mL of tamoxifen stock solution per 10 g of body weight) (see Notes 3 and 4).
4. Euthanize the pregnant female at 18.5 dpc (following the IACUC guidelines) and dissect out the ovaries from the embryos. For each embryo, one ovary is used for organ culture (see Subheading 3.2) and the other is fixed in 4% paraformaldehyde at 4°C overnight (see Subheading 3.4 and Note 5). Collect a small piece of tissue (tail or limb, ~0.5 cm) from each embryo for genotyping and transfer the tissue to a 1.5-mL microcentrifuge tube. Store the tissue pieces at -20°C if they cannot be genotyped immediately (see Subheading 3.3 and Note 6).

²To get a higher rate of plugging, one male can be set up with two female mice at the same time. The copulation plug remains for only 12–14 h and then dissolves. Checking for the copulation plug early in the morning prevents the likelihood of missing any pregnancies.

³It was shown that only a small percentage of cells were labeled by a single tamoxifen treatment. Multiple tamoxifen treatments provide a cumulative effect that mark more cells of interest (8).

⁴Tamoxifen remains active for about 36 h after dosing. In this experiment, we dose the mouse at 15.5 and 16.5 dpc and collect ovaries at 18.5 dpc when tamoxifen is metabolized and excreted already (19–21).

⁵Tamoxifen-treated dams often experience dystocia due to the inhibitory effects of tamoxifen on estrogen signaling. As a result of this complication, pups often die and cannot survive to adulthood. We, therefore, collect the ovaries before birth and put them in culture to study their development after birth. In addition to organ culture, one alternative is to perform cross fostering. Caesarian section is performed at 18.5 dpc and the pups are nursed by a foster dam. This allows the animals to survive to adulthood.

⁶Because the genotypes of the embryos are not known at the time of dissection, the organ culture is set up blindly. Proper labeling of the cultured tissue (or culture dishes) is necessary to track the genotypes once the culture is completed.

3.2. Organ Culture (see Note 5)

3.2.1. Setting Up the Floating Filter Culture System

1. Using sterile techniques, cut the membrane of a Millicell culture plate insert into six pieces using a razor or scalpel.
2. Pipette 500 μ L room temperature culture medium into each well of 4-well culture plates.
3. Lay one piece of Millicell membrane onto the medium in each well and allow the membrane to float on the medium. Put the culture plates in the incubator (37°C with 5% CO₂/95% air) for at least 30 min before culture to allow for equilibration of the media.

3.2.2. Tissue Collection and Culture Procedure

1. Dissect out the ovary with the mesonephros attached (referred to as the ovary complex hereafter) in dissecting PBS and wash them in room temperature culture medium for 5 min in the tissue culture hood. Use a single-channel pipettor with 200–1,000- μ L tip to transfer each ovary complex onto the membrane. Place a drop of medium from that well onto the membrane so that the drop (approximately 2 $\times$ the size of the ovary complex) of the medium covers the entire ovary (see Note 7).
2. The ovary complexes are cultured in a humidified incubator at 37°C with 5% CO₂/95% air for 3 days. Change culture medium every 24 h by removing most of the medium and replacing with the same volume of prewarmed medium (see Note 8). After 3 days of culture, collect samples for immunohistochemistry (see Subheading 3.4).

3.3. Genotyping (see Note 9)

1. Add 400 μ L of 50 mM NaOH to each tube that contains the tissue pieces from Subheading 3.1 and place the tubes on a heating block at 95°C for 15 min or until the tissue has been digested (see Note 10). Tubes can be left on the heating block for 1–2 h, but should not be left overnight.
2. Add 200 μ L of Tris-HCL (pH 8.0) to each tube, vortex the tubes, and centrifuge for 5 min at 16,100 $\times g$ at room temperature. This sample is referred as “Extracted DNA” thereafter (see Note 10). For PCR, dilute gene-specific primers to 20 μ M in PCR clean water (see Note 11).
3. For each PCR reaction, combine the following ingredients in a PCR tube:

⁷You can use forceps to pick up a small amount of medium and then release the medium onto the membrane around the ovary.

Alternatively, medium can be placed onto the ovary using a micropipette. One or two ovaries are cultured on each piece of membrane.

⁸When removing medium, tilt the dish slightly and place pipette tip against the bottom edge of the well at the deepest part of the medium. When replacing medium to tissue-containing wells, pipette the medium with the tip against the wall of the well so that the medium runs down the wall. Otherwise, the membrane may sink to the bottom.

⁹For *Gli1*^{CreERT2} (heterozygous) and *Rosa26*^{YFP} (homozygous) breeding, the possible genotypes of the pups are *Gli1*^{Cre/+}, *Rosa26*^{YFP/+} or *Gli1*^{+/+}; and *Rosa26*^{YFP/+}. The pups carrying the Cre allele are the experimental samples and Cre-negative samples serve as the negative control. Genotyping can be done anytime after the organs have been put into culture. Extracted DNA can be used immediately or stored at –20°C.

¹⁰Vortex the tubes frequently. Make sure that the cap is tightly closed and be extremely cautious with the hot liquid when opening the tubes. The tissue is considered fully digested when the tissue fragments are completely dissolved in the solution.

¹¹We do not need to genotype for *YFP* allele in the progenies from *Gli1*^{Cre/+} $\times$ *Rosa26*^{YFP/YFP}, since the genotype of all the progenies is *Rosa26*^{YFP/+} (see Note 9).

Mango PCR mix	12.5 μ l
Forward primer	0.625 μ l
Reverse primer	0.625 μ l
H ₂ O	10.25 μ l
Extracted DNA	1 μ l

4. Perform PCR using the following protocol:

Step 1	94°C for 2 min
Step 2	94°C for 45 s 60°C for 45 s 72°C for 1 min Repeat step 2 for 40 times
Step 3	72°C for 2 min and then hold at 10°C

5. Run PCR samples (25 μ L) in 1 $\times$ TAE buffer on a 1.5% agarose gel to determine the size of the PCR products (*Gli1*^{Cre/+}: 408 base pairs; *Gli1*^{+/+}: no band; see Note 9).

3.4. Immunohistochemistry (see Note 12)

Day 1

1. Collect tissues (18.5 dpc ovary complexes and the complexes after a 72-h culture; see Subheading 3.2) and fix them in 4% paraformaldehyde overnight at 4°C (no longer than 16 h) or 1–2 h at room temperature.

Day 2

1. Pipette off paraformaldehyde from the tube and add 1 mL PBS at room temperature. Wash the ovaries twice with 1 mL PBS at room temperature in 1.5-mL microcentrifuge tubes for 10 min each. The samples can be stored in PBS at 4°C for a few weeks. For long-term storage, store in 100% ethanol at –20°C.
2. Dehydrate the samples as follows:

1 mL 10% sucrose/PBS for 15 min
1 mL 15% sucrose/PBS for 15 min
1 mL 20% sucrose/PBS for 1 h
1 mL 20% sucrose/OCT (1:1) at room temperature for 4 h or overnight at 4°C

¹²Immunohistochemistry is performed on the ovary complexes collected at 18.5 dpc (time zero) and the ovary complexes after a 72-h culture. At 18.5 dpc, *YFP*-positive cells are mostly present in the mesonephros. After 72 h of culture, *YFP*-positive cells appear in the ovary, indicating that these *YFP*-positive cells came from the mesonephros. By double staining the section with granulosa cell marker FOXL2, we can identify whether these mesonephros-derived *Gli1*-expressing cells contribute to FOXL2-positive granulosa cells or FOXL2-negative mesenchymal cells in ovary. If *Rosa26LacZ* reporter is used instead of *Rosa26YFP*, then *LacZ* primary antibody should be included along with the FOXL2 antibody to mark the *LacZ*-positive cells.

3. Transfer samples to an embedding block and remove the sucrose/OCT as much as possible. Fill the embedding block with 1:3 20% sucrose/OCT and position the samples using a toothpick under a dissection scope if necessary. Freeze samples on dry ice (the frozen block can be stored in the microcentrifuge tubes at -20 or -70°C).
4. Use Microtome Cryostat to section the frozen block to $8\text{--}10\text{-}\mu\text{m}$ thickness. Put the sections on Superfrost plus microscope slides and dry the sections at room temperature for $15\text{--}30$ min. Slides can be stored at -70°C in a slide box if not used immediately.
5. Circle desired sections with a PAP/ImmEdge pen and let dry. Add $500\text{--}1,000\text{ }\mu\text{l}$ $1\times$ PBS at room temperature onto the circled area containing sections three times for a 5-min each wash.
6. Add $500\text{--}1,000\text{ }\mu\text{l}$ blocking buffer to the PAP/ImmEdge pen circled area till all the sections are immersed in the buffer. Put the slides in a humidified chamber for $0.5\text{--}1$ h at room temperature.
7. Remove the blocking buffer from the slides.
8. Apply $500\text{--}1,000\text{ }\mu\text{l}$ Goat anti-FOXL2 primary antibody solution (1:300 in the blocking buffer) onto the sections to make sure that all the sections are immersed with antibody solution. Incubate the slides in a humidified chamber overnight at 4°C .
9. Rinse the sections three times by adding $500\text{--}1,000\text{ }\mu\text{l}$ $1\times$ PBS at room temperature onto the sections for a 5-min each wash.
10. Apply $500\text{--}1,000\text{ }\mu\text{l}$ donkey anti-Goat IgG secondary antibody solution (1:500 in the blocking buffer) onto the sections and incubate the slides in a humidified chamber at room temperature for 1 h.
11. Rinse the sections three times by adding $500\text{--}1,000\text{ }\mu\text{l}$ $1\times$ PBS at room temperature onto the sections for a 5-min each wash.
12. Add one or two drops of the mounting medium (DAPI) onto the sections and cover the slides with coverslips. The volume of mounting medium should be able to immerse all the sections after covered with coverslips. The slides can be stored at -20°C in the slide box for 2 weeks. Observe staining under a fluorescent microscope (Leica DMI 4000B) (see Fig. 2 and Note 12).

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Fig. 1.

(a) The lineage tracing strategy that marks and tracks *Gli1*-expressing cells. *CreERT²* is under the control of the *Gli1* promoter elements and therefore expressed only in the *Gli1*-positive cells. In the presence of tamoxifen (TM), *CreERT²* is activated and able to remove the *loxP*-flanked STOP sequence from the *Rosa26* allele, allowing a permanent *YFP* expression in the *Gli1*-expressing cells and their progenies only during the period of TM treatment. (b) Timeline of the experiment. The pregnant female is treated with TM at 15.5 and 16.5 dpc. At 18.5 dpc, the embryos are isolated and their ovaries were collected. For each embryo, one ovary is fixed for immunohistochemistry (IHC) and the other is cultured for 72 h until postnatal day 3 (PD3). After 3 days of culture, ovaries are harvested for immunohistochemistry.

Fig. 2. Fluorescent immunohistochemistry for YFP-labeled *Gli1*-positive (*green*) cells and FOXL2-positive granulosa cells (*red*) in the ovary before culture (18.5 dpc) and after culture (PN3).