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RNAi-Mediated Gene Function Analysis in Skin

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Abstract

We have recently developed a method for RNAi-mediated gene function analysis in skin (Beronja et al., *Nat Med* 16:821–827, 2010). It employs ultrasound-guided *in utero* microinjections of lentivirus into the amniotic cavity of embryonic day 9 mice, which result in rapid, efficient, and stable transduction into mouse skin. Our technique greatly extends the available molecular and genetic toolbox for comprehensive functional examination of outstanding problems in epidermal biology. In its simplest form, as a single-gene function analysis via shRNA-mediated gene knockdown, our technique requires no animal mating and may need as little as only a few days between manipulation and phenotypic analysis.

Keywords

Lentivirus; Intraamniotic; In utero; In vivo RNAi; shRNA; RNA interference; Ultrasound-guided microinjection

1. Introduction

Investigations of embryonic development and adult tissue homeostasis often employ gene loss-of-function analyses. While such studies can be done rapidly in lower metazoans, in mammalian model organisms, where links to human conditions are more direct, they require an often-prohibitive commitment in time, expertise, and money. To circumvent these hurdles, we have recently developed a technique that couples the strengths of mouse epidermis as a well-defined and accessible epithelial tissue, with the utility and expediency of RNAi-mediated gene knockdown and commercially available shRNA libraries (1, 2).

Mammalian skin epithelium serves as the protective interface between the vital stability of the milieu intérieur and the harsh, dry, and fluctuating conditions of the world we inhabit. At the body surface, skin is further exposed to constant assaults not only from physical damage but also microbial infections. The ability of skin to protect and counter additional assaults depends on precisely regulated programs of growth and differentiation during its embryonic and postnatal development (3).

Skin is composed of an outer epithelium, the epidermis, which is separated from an underlying dermis by a basement membrane rich in extracellular matrix and growth factors. Epidermis is a stratified epithelium composed of an inner (basal) layer, which maintains contact with the basement membrane, and overlying spinous layers, granular layers, and

stratum corneum. Proliferative cells within the basal layer give rise to the differentiated layers above it (4, 5).

Any technique designed to permanently modify epidermis requires the ability to reach and modify progenitor cells that are in the innermost (basal) layer. Postnatally, this is prevented by the skin's barrier function. While approaches to circumvent the barrier may include its chemical and physical disruption (6), the requirement to maintain the tissue integrity suggests an alternative approach of targeting progenitor cells during embryogenesis, before any barrier to exogenous agents forms.

To manipulate early embryos without compromising their development and viability, several groups have pioneered the use of high-frequency ultrasound-guided microinjections (7, 8). We have adapted this approach for microinjections of lentivirus, proven to be an effective method for gene delivery in vivo for many tissues including the CNS and skin (9, 10). By targeting our injections to the amniotic cavity and the amniotic fluid that surrounds the embryo, we achieve highly efficient, immediate, and stable epidermal transduction of the mouse skin.

2. Materials

1. Glass capillaries (Drummond Scientific, Broomall, PA, USA).
2. Micropipette puller (e.g., P-97, Sutter Instrument, Novato, CA, USA).
3. Needle sharpener (e.g., BV-10, Sutter Instrument, Novato, CA, USA).
4. High-frequency ultrasound system with RMV707B scanhead (Vevo 770 High-resolution Imaging System, VisualSonics, Toronto, ON, Canada).
5. Micro-injection system, with heated animal platform and scanhead mount (e.g., Vevo Integrated Rail System 2, VisualSonics, Toronto, ON, Canada).
6. Nanoject II nanoliter injector with micromanipulator (Drummond Scientific, Broomall, PA, USA).
7. Small animal anesthesia system with compressed oxygen tank, oxygen regulator, vaporizer, and induction chamber (e.g., Vevo Compact Anesthesia System, VisualSonics, Toronto, ON, Canada).
8. Isoflurane inhalation anesthetic (IsoFlo, Abbott Laboratories, Abbott Park, IL, USA).
9. Hair removal cream (e.g., Veet or Nair).
10. Ultrasound gel (Aquasonic, Parker Laboratories, Fair field, NJ, USA).
11. Cotton-tipped applicator (e.g., Kendall Healthcare Q-Tips, Covidien, Mansfield, MA, USA).
12. Ethanol (70% in water).
13. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄. Adjust the pH to 7.4, sterilize.

14. Mineral oil (M5310, Sigma, St. Louis, MO, USA).
15. 0.2 μ m red fluorescent microspheres (F-8810, Invitrogen, Carlsbad, CA, USA).
16. Plasmid vectors for packaging and production of shRNA-expressing lentivirus (see Note 1).
17. Gauze or tissue paper (e.g., Kimtech Science Kimwipes, Kimberly-Clark, Roswell, GA, USA).
18. Surgical tape (e.g., Micropore paper tape, 3M, St. Paul, MN, USA).
19. Parafilm M laboratory film (Pechiney Plastic Packaging, Chicago, IL, USA).
20. Buprenorphine hydrochloride (Buprenex, Reckitt Benckiser Healthcare, Slough, UK).
21. Permoplast modeling clay (e.g., Nasco).
22. Petri dish with central opening (SA-11213, VisualSonics, Toronto, ON, Canada).
23. Silicone membrane (SA-11054, VisualSonics, Toronto, ON, Canada).
24. Membrane tape (SA-11053, VisualSonics, Toronto, ON, Canada).
25. Semicircular silicone plug (Silastic E RTV silicone rubber, Dow Corning, Midland, MI, USA).
26. Chromic gut absorbable suture, with a 3/8 16 mm needle (SG-535, Covidien, Mansfield, MA, USA).
27. Surgical instruments including micro-dissecting scissors (11-2070), sharp tip forceps (10-1425), two pairs of blunt tip micro-dissecting forceps (10-2950), needle holder (25-2265), wound clip applier with 9 mm staples (43-1000, 43-1010). All from Biomedical Research Instruments, Silver Spring, MD, USA.
28. ½cc Insulin syringe (329461, BD, Franklin Lakes, NJ, USA).
29. 10 ml luer-lok syringe with a 26 G1/2 needle (BD, Franklin Lakes, NJ, USA).
30. 60 ml Eccentric tip syringe (BD, Franklin Lakes, NJ, USA).

3. Methods

3.1. Microinjection Needle Pulling, Sharpening, and Loading

1. Microinjection needle should be made from a thick-walled glass capillary and pulled to a long taper. Using a Sutter micropipette puller P-97 use the following program variables: Pressure = 200; Heat = 769; Pull = 0; Velocity = 140; Time = 100.

¹Reagents required for production of shRNA-compatible lentivirus are available from Addgene (Cambridge, MA, USA). For generating high-titer lentivirus we use the 2nd generation packaging system (Addgene plasmids 12259 and 12260), and a lentivirus production and concentration protocol from the Deisseroth lab (11). The shRNA expressing lentivirus can be obtained from commercial sources, or generated using the pLKO.1 cloning vector (Addgene plasmid 10878) and protocols from the RNAi consortium (2). For use in skin transduction we have modified the basic pLKO.1 vector to express various several fluorescent proteins (1). These are also available (Addgene plasmids 25997-26001) along with others.

2. Using fine tip forceps, snap off the needle tip at the level where its diameter is ~ 30 μm . Bevel the needle to 25° on a fine-grade abrasive plate with regular wetting for 20 min (Sutter instrument BV-10). The outer diameter of the finished needle should be between 40 and 60 μm .
3. Using a 10 ml syringe with a 26 G1/2 needle, push ddH₂O through the needle to clear it of any debris that may have accumulated during the sharpening process (see Note 2). Sterilize the needle by filling it with 70% ethanol. Expel all liquid to dry. The finished needle can be used immediately or stored indefinitely at room temperature.
4. To prepare the needle for microinjection, back fill it with mineral oil using a 10 ml syringe with a 26 G1/2 needle, ensuring that there are no bubbles along its length. While the specific instructions for mounting the needle onto the microinjection apparatus will depend on the system used, the following apply to a common Nanoject II injector and should be broadly applicable. Disassemble the injector tip by removing the cap and three rubber gaskets, and wipe components clean with a Kimwipe. Place the first black gasket with a small hole onto the piston, followed by the white gasket. Note that the white gasket has a small opening at one end and a large one at the other. Mount the white gasket onto the piston so that the side with a small hole faces the piston base. Next, put the needle through the injector cap, and place the second black gasket onto the base of the needle. Mount the needle onto the extended piston and screw the cap onto the injector. Place the injector with a needle into the holder.
5. For in vivo RNAi experiments, the needle is filled with lentivirus (see Note 3) by aspiration. First, expel the mineral oil from the needle by extending the piston to its furthest position. Note that a small volume of oil will remain in the needle, preventing the lentivirus from contacting the piston. Dispense a 10 μl drop of lentiviral suspension onto a piece of parafilm. The hydrophobic surface will ensure that the lentivirus stays in a drop rather than spread out too thin. Lower the tip of the needle into the drop and load by slowly retracting the piston to its maximum (see Note 4). This will result in ~ 4.5 μl of lentivirus loaded, sufficient for 3–4 embryo injections.
6. Once the needle is loaded with lentivirus, place its tip into a dish with PBS to prevent the liquid in the tip from drying out and clogging the needle. Next, prepare the pregnant female for the surgical procedure (see Note 5).

²The needle tip can often get filled with small glass particles, especially if the grinding surface is not kept wet for the duration of beveling. This debris can be viewed with a light microscope and a 10 $\times$ objective, and it is essential that it be removed, as it will interfere with all subsequent steps. Note that some blockages will resist any attempt to be cleared. In such cases, it is best to start over with a new needle.

³While lentivirus is preferable vehicle for gene transfer into mouse skin, due to high integration efficiency and ability to concentrate to high titers, our technique is not limited to it. Using the protocol outlined here, we have also successfully used retrovirus, adenovirus, and small molecules in our ultrasound-guided microinjections.

⁴It is essential to avoid getting air bubbles into the needle during loading. This will interfere with regulating the viral-injection volume, result in lentivirus degradation, and, if injected into the amniotic cavity, can affect survival of injected embryos. Keep a close eye on the needle loading process, and if any air gets aspirated, expel it immediately. Reload the needle making sure that its tip stays submerged and that the lentivirus drop does not contain any air bubbles.

3.2. Ultrasound- Guided Embryonic Microinjection

1. Turn on the high-frequency ultrasound system and set the heated animal platform to 40°C. Assemble the modified Petri dish as follows: (a) peel off the backing of one side of double sided membrane tape, and adhere it to the bottom of the Petri dish such that it surrounds the opening; (b) remove the backing off the other side of the double-sided membrane tape; (c) place a square of silicone membrane over the exposed membrane tape and press down firmly to ensure tight adhesion; (d) using micro-dissecting scissors, cut a rectangular opening in the silicone membrane measuring 2 × 10 mm.
2. Anesthetize the pregnant mouse in the induction chamber by setting the oxygen regulator to 1 L/min and isoflurane vaporizer to 2%. After ~3 min, check to determine if animal is anesthetized by performing the paw pinch test (see Note 6).
3. Switch the oxygen/isoflurane flow from the induction chamber to the nose cone attached to the heated animal platform. Remove the anesthetized mouse from the induction chamber and place it ventral side up onto the heated animal platform, ensuring that its head is firmly held within the nose cone. Immobilize the mouse by taping its hind legs to the animal platform using surgical tape (Fig. 1a).
4. Using the insulin syringe administer subcutaneous injection of 0.03 cc of Buprenex, or similar analgesic, to the pregnant female (see Note 7).
5. Apply a small quantity of a depilatory cream to the abdomen and spread it over a 4 × 4 cm area using a cotton-tipped applicator. The solution will dissolve abdominal hair within 1–3 min, which can be aided by continuous rubbing with the applicator. Gently remove the depilatory cream with gauze or tissue and wipe the area clean using 70% ethanol. At this point ultrasound can be used to confirm that the female is pregnant with E9.5 embryos (see Note 8).
6. With blunt tip micro-dissecting forceps, grab a bit of abdominal skin and make a ~2 cm incision along the midline of the mouse. Using sharp tip micro-dissecting forceps, grasp the peritoneum, and make a similar sized incision in the abdominal wall (see Note 9). There is a faint, poorly vascularized region along the midline (linea alba), and an incision there should result in little or no bleeding.

⁵Ultrasound-guided lentiviral microinjection results in the most efficient epidermal transduction and highest survival rate when performed on embryonic day (E) 9.5 embryos. The morning when a copulatory plug is first detected is considered as 0.5 days of gestation.

⁶Animal's age and genetic background can have a strong effect on its sensitivity to anesthesia. From experience, C57Bl6 mice require less isoflurane than CD1 mice (1.5% vs. 2.5%), and older animals may take longer to anesthetize compared to younger ones. It is advisable to test your mice with a paw pinch test while varying isoflurane concentration (1–3%) and the time in induction chamber (2–5 min). Importantly, stay vigilant throughout the procedure for any signs of movement (indicative of an incomplete anesthesia) or abnormally slow breathing (sign of too deep an anesthesia).

⁷We have observed that the postoperative recovery time is reduced and that animals display signs of normal behavior, such as burrowing and nest building, more rapidly if injected with an analgesic prior to the procedure. No effect was observed on survival rate of injected embryos.

⁸To visualize embryos within the abdominal cavity, apply ultrasound gel to the skin after depilation. Using a slow sweeping motion of the ultrasound scanhead inspect the abdominal region until embryos are detected. For detailed instructions on embryo staging consult The Atlas of Mouse Development (12).

⁹When making an incision in the abdominal wall, be careful not to damage the underlying internal structures, such as the bladder, uterus, or the intestines. It is helpful to only grab a hold of a very small portion of the abdominal wall and also lift it up and away from the internal organs for the initial cut.

7. At this stage, uterine horns can be identified based on their dark red color, and due to presence of embryos, a string-of-pearls appearance. Using blunt tip micro-dissecting forceps, take hold of one uterine horn between implantation sites and gently pull out, and expose a segment containing 3 embryos (Fig. 1a).
8. Place four squares of modeling clay around the mouse. With the modified Petri dish held over the abdominal incision, grasp the exposed uterine segment through the inside of the dish, and pull the exposed embryos into the dish (see Note 10). Stabilize the dish by pressing it into the modeling clay (Fig. 1b).
9. Next, place the semicircular silicone plug behind the uterine segment (see Note 11), on the side away from the injection apparatus (Fig. 1c). This will prevent embryos from getting pushed away by the needle during injection.
10. Fill up the modified Petri dish with room temperature PBS. The silicone membrane of the modified Petri dish will adhere well to the mother's abdominal skin and will prevent PBS from leaking out during the procedure. Lower the ultrasound scan-head into the PBS and adjust the animal platform and vertical position of the scanhead to visualize an embryo.
11. Move the injection apparatus towards the animal platform, and bring the injector with needle into the modified Petri dish. Using the micromanipulator, position the tip of the needle within ~5 mm of the middle embryo, and bring the needle into the plane where its tip appears the brightest (Fig. 1d).
12. Using gentle adjustments of the animal platform and injector micromanipulator, confirm that the needle is at its brightest and that amniotic cavity is clearly visible in the ultrasound view. Also, confirm that the silicone plug is directly behind the embryo (Fig. 2a).
13. Using the injection knob, move the needle forward until it is in contact with the uterine wall. Gently push forward until the tip of the needle passes through it and is clearly positioned within the amniotic cavity (see Note 12; Fig. 2b). Inject lentivirus by pressing the Inject button for prespecified volumes, or Empty button for custom volumes (see Note 13).
14. When three embryos are exposed, they sit in a triangular formation. Inject the middle one (at the top) first, then move the silicone plug behind the two side

¹⁰Note that each implantation site appears dark red on the placental side and lighter and slightly opaque on the side where the embryo is. Always try to inject from the lighter side as that ensures that blood vessel-rich placenta stays intact (if the uterus is exposed with light side away from the injection apparatus, turn the heated animal platform 180°).

¹¹To make the semicircular silicone plug, follow the manufacturer's instructions for combining the two-component Silastic mixture. Pour it into a mold (10 cm Petri dish) and after the mass solidifies, cut in half. In the region of the plug where it will contact the uterus during injections further carve a slight depression using a scalpel.

¹²At times, to properly target the amniotic cavity, you may need to inject through the embryo. Based on empirical evidence, this is quite safe to do. We have not noticed any morbidity or mortality to result from it, presumably due to documented plasticity of the embryo at this stage and very small diameter of the needle.

¹³During injection, you may observe particulate matter expelled into the amniotic cavity, depending on granularity of your virus preparation, or a deflection of the amnion consistent with the expansion of the amniotic space. Both are signs of properly targeted injections. Also, it may be useful when learning the technique to inject fluorescent microspheres only or add them to the lentiviral prep. They can be used to identify injected embryos, and easily assay injections correctly targeted to the amniotic cavity (see supplementary material in 1). We normally inject up to 1.25 l of lentivirus into the amniotic cavity of E9.5 embryos. Higher volumes result in higher incidence of embryonic mortality.

(bottom) ones and lower the scan head to get them in plane. Inject in similar fashion.

15. Once three embryos in a uterine horn are injected, lift out the scanhead and remove the silicone plug from the modified Petri dish. Aspirate the PBS with a 60 ml syringe. Return the exposed embryos back into the abdominal cavity using a cotton-tipped applicator and remove the Petri dish (see Note 14).
16. Wipe the abdominal area with tissue paper and remove PBS that may have leaked into the abdominal cavity (see Note 15). Close the peritoneal incision using absorbable sutures. Staple the incision in the abdominal skin. Two staples should be sufficient.
17. Remove the mouse from the animal platform, and place her in a heated recovery cage. Monitor her until she is fully recovered (15–30 min). Clean up (see Note 16).

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¹⁴At this point you can repeat the procedure with the remainder of the embryos in the injected horn, or embryos in the other, non-injected uterine horn. In our experience, up to 9 embryos can be injected even though we normally limit the number to 6. The limitation is due to a requirement to keep the procedure to under 45 min. Longer anesthesia results in a marked increase in embryonic mortality.

¹⁵You will notice that some PBS has seeped into the female's abdominal cavity. It is essential to expel that extra liquid as it can place extensive stress onto her system, possibly resulting in death or abortion. That is easily done by placing a tissue paper over the opening in the abdomen, and gently pressing the abdominal sides until most of the liquid is removed.

¹⁶Note that everything that comes into contact with the lentivirus should be considered infectious and should be handled during and after the procedure according to your institutional biosafety guidelines. All protocols for this work should also be reviewed and approved by your institutional and governmental guidelines for the proper care and handling of animals.

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Fig. 1.

Ultrasound-guided microinjection set-up. (a) Anesthetized mouse is taped to a heated platform with a uterine segment exposed. (b) Three embryos are pulled into the modified Petri dish stabilized with modeling clay. (c) Semicircular silicone plug is placed behind the exposed embryos. (d) Complete set-up with PBS filled modified Petri dish, microinjection apparatus with the injector (on the *left*), ultrasound scanhead and mount (above), and a monitor showing live ultrasound view of the embryo and needle (in the *background*)

Fig. 2.

Ultrasound view of an *in utero* intraamniotic microinjection. **(a)** Ultrasound scan shows the microinjection needle (n), embryo (e), amnion (a), and silicone plug (p). **(b)** Microinjection is targeted to the amniotic cavity (ac). Note the larger exocoelomic cavity (ec), which is not in direct contact with the embryo.