

Video Article

Cutaneous Surgical Denervation: A Method for Testing the Requirement for Nerves in Mouse Models of Skin Disease

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URL: <https://www.jove.com/video/54050>

DOI: [doi:10.3791/54050](https://doi.org/10.3791/54050)

Keywords: Medicine, Issue 112, Mouse models, skin, epidermis, hair follicle, touch dome, nerves, denervation, Cre recombinase, biopsy

Date Published: 6/26/2016

Citation: Peterson, S.C., Brownell, I., Wong, S.Y. Cutaneous Surgical Denervation: A Method for Testing the Requirement for Nerves in Mouse Models of Skin Disease. *J. Vis. Exp.* (112), e54050, doi:10.3791/54050 (2016).

Abstract

Cutaneous somatosensory nerves function to detect diverse stimuli that act upon the skin. In addition to their established sensory roles, recent studies have suggested that nerves may also modulate skin disorders including atopic dermatitis, psoriasis and cancer. Here, we describe protocols for testing the requirement for nerves in maintaining a cutaneous mechanosensory organ, the touch dome (TD). Specifically, we discuss methods for genetically labeling, harvesting and visualizing TDs by whole-mount staining, and for performing unilateral surgical denervation on mouse dorsal back skin. Together, these approaches can be used to directly compare TD morphology and gene expression in denervated as well as sham-operated skin from the same animal. These methods can also be readily adapted to examine the requirement for nerves in mouse models of skin pathology. Finally, the ability to repeatedly sample the skin provides an opportunity to monitor disease progression at different stages and times after initiation.

Video Link

The video component of this article can be found at <https://www.jove.com/video/54050/>

Introduction

Over the past few years, there has been a widening appreciation for the influence of nerves on diseases not typically regarded as classical neuropathies¹⁻⁴. In the skin, recent experimental evidence has suggested that sensory nerves can modulate diverse pathologies ranging from psoriasis to cancer⁵⁻⁹. This has been demonstrated using techniques such as surgical denervation and pharmacological inhibition of neural function in rodents. In the case of psoriasis, these studies have provided a mechanistic framework for understanding why human psoriatic plaques regress following loss of neural function^{7,10-12}.

Cutaneous nerves can also affect gene expression^{13,14} and are critical for mechanosensing in normal skin¹⁵. In particular, touch dome (TD) epithelia are comprised of a patch of columnar epidermal cells in juxtaposition with neuroendocrine Merkel cells innervated by slowly adapting type 1 (SA1) nerve fibers¹⁶⁻¹⁸. TDs mediate light touch sensation and have been shown to display Hedgehog pathway activity^{5,19}. TD maintenance depends on innervation^{20,21}, as nerves secrete Hedgehog ligands to sustain normal TDs and their associated Merkel cells¹⁹. In addition, innervation promotes Hedgehog-dependent tumor formation from TD epithelia⁵. Together, these studies reinforce the notion that intricate molecular interactions occurring between nerves and the surrounding cells in their niche are crucial for normal TD physiology as well as disease.

To interrogate the nature of these interactions, we describe here a series of *in vivo* techniques for manipulating gene expression in the TD, as well as for harvesting skin biopsies for TD visualization after lineage tracing. Finally, we describe procedures for performing unilateral surgical denervation, wherein nerves are removed from one side of the mouse dorsal skin, while leaving the contralateral side intact as a sham internal control. Several weeks after surgery, denervated and sham control skin are compared to assess changes that occur when nerves are ablated. Although these techniques are described in the context of normal TDs, the denervation procedure has been used to examine the requirement for nerves in mouse models of psoriasis⁶, wound healing¹³ and tumorigenesis⁹. Finally, since the skin is amenable to repeated biopsies, this provides an opportunity to monitor the long-term fates of labeled cells or to assess disease progression over multiple time points.

Protocol

All procedures described in this protocol were performed in accordance with regulations established by the University of Michigan Unit for Laboratory Animal Medicine.

1. Induce Genetic Recombination in Mice

Note: The *Gli1^{tm3(cre/ERT2)Alj/J}* mouse strain (*Gli1-CreERT2*)¹³ enables targeting of tamoxifen-induced genetic recombination to TD epithelia. Cross this strain with *B6.129S4-Gt(ROSA)26Sor^{tm1Sor/J}* reporter mice (*LacZ*)²² to generate *Gli1;LacZ* animals to visualize TD cells by whole-mount staining below.

1. Prepare tamoxifen solution to a concentration of 12.5 mg/ml in corn oil.
 1. In a 1.5 ml tube, add up to 20 mg of crystalline tamoxifen, and then 1 ml of corn oil. Firmly tape the tube to a vortex mixer, and vortex continuously at the highest setting at RT until the tamoxifen has fully dissolved (2-4 hr), as confirmed by examining the tube under a dissecting microscope for the absence of tamoxifen particulates.
 2. Transfer the solution to a 15 ml tube and dilute the tamoxifen to a final concentration of 12.5 mg/ml with additional corn oil. Mix by vortexing the viscous solution for an additional 30 sec. Store this solution for up to 1 week at 4 °C in the dark.
2. Inject the tamoxifen solution intraperitoneally into *Gli1;LacZ* mice, at a volume of 200 µl per 20 g of mouse body weight, for an effective tamoxifen dose of 2.5 mg per 20 g mouse weight.

2. Harvest Skin Biopsies

Note: Depending on the experiment, harvest skin biopsies several days to weeks after tamoxifen induction. For all surgeries, follow standard protocols for rodent surgery, including using sterile gloves, wearing a surgical mask or hair net, and covering the animal with a sterile surgical drape during the procedure.

1. Prepare 10x stock anesthetic solution by mixing 90 mg/ml ketamine and 6.5 mg/ml xylazine in water. Dilute this stock solution 1:10 into sterile PBS just prior to use, and store at RT in the dark for up to 8 months.
 1. Alternatively, anesthetize mice by isoflurane inhalation, beginning with a gas concentration of 4% with oxygen to fully anesthetize the animal, and then subsequently lowering this to 1-2% for the duration of the procedure.
2. Inject the anesthetic solution intraperitoneally at a dose of 200 µl per 20 g mouse body weight. Check that the animal has reached the proper plane of sedation by toe pinch assay, and confirm that heart and respiratory rates are normal (approximately 600 beats and 160 breaths per min, respectively).
3. Use an electric clipper to remove the hair from the site of biopsy on the dorsal back skin, being careful not to nick or damage the underlying skin.
4. Prepare the surgical site by wiping the shaved area in an anterior-to-posterior direction using Betadine and alcohol wipes. Ensure all hair clippings are removed from the site.
5. Outline the biopsy site using a black marker, place the animal on a warming pad in an aseptic surgical area, and cover with a sterile surgical drape (for demonstration purposes, sterile drape was omitted to increase visibility).

Note: To obtain longitudinal sections of hair follicles, the longer edge of the biopsy (the edge to be sectioned for histology, ~1 cm) should run in an anterior-posterior direction (parallel to the direction of the hair follicles), parasagittal to the dorsal midline (**Figure 1A**).
6. Use a sterile #11 scalpel to make a full thickness excision along the marked area without damaging the underlying muscular fascia.

Note: The excised skin tissue includes the epidermis, dermis, subcutaneous fat and panniculus carnosus. Bleeding is typically minimal.
7. Flatten the excised skin sample on a dry paper towel, dermis side down, trim away the excess paper towel, and store the sample in cold PBS for up to 1 hr if other samples need to be collected. When ready, proceed to Steps 3.1 or 3.2 to process samples for histology, or Step 4 for whole-mount β-galactosidase (LacZ) staining.
8. Suture close the biopsy site using 6-0 nylon sutures, in a simple interrupted pattern spaced roughly 3 mm apart.
9. Do not return mice that have undergone surgery into the same cage as other animals until after full recovery.
10. Monitor animals immediately after surgery until they regain consciousness, and also daily until the surgical area has healed, typically within 1 week. Use analgesics in accordance with designated institutional animal care and use guidelines if mice exhibit signs of pain or distress. Remove sutures within 7-10 days after surgery.

Note: If needed, prepare analgesic solution by diluting carprofen (50 mg/ml stock solution) 1:100 in sterile water. Inject the solution subcutaneously between the shoulder blades near the scruff of the neck, at a dose of 200 µl per 20 g body weight (5 mg/kg mouse body weight).

3. Process Samples for Histology

Note: To fix and process the excised tissue, use either method below depending on application.

1. To generate paraffin-embedded histological samples, fix the skin in 3.7% formalin in PBS O/N at RT and store in 70% ethanol for up to 2 weeks. Remove the paper towel before embedding into paraffin.
2. For generating frozen histological samples, submerge the tissue in cold 4% paraformaldehyde in PBS and gently shake for 1 hr. Remove the solution and wash the sample with 3 changes of PBS, roughly 5 min each. Next, submerge the sample in 30% sucrose in PBS to cryoprotect the tissue ("sucrose sinking").
3. Incubate with gentle shaking O/N at 4 °C. The next day, remove the paper towel and trim away excess adipose tissue from the dermal side of the skin. Embed the tissue directly into OCT and store the frozen block at -80 °C.

Note: After sectioning, either paraffin or frozen samples can be stained by immunohistochemistry to identify TDs, Merkel cells and nerves using antibodies against Keratin 17, Keratin 8 and Neurofilament, respectively, as previously described^{5,19}.

4. Visualize Samples by Whole-mount LacZ Staining

1. Prepare X-gal staining solution.
 1. Combine 0.94 g sodium phosphate monobasic, and 2.6 g sodium phosphate dibasic in 250 ml of sterile water. Adjust pH to 7.3. To this, add 0.5 ml of 1 M magnesium chloride, 0.528 g of potassium ferrocyanide, and 0.412 g of potassium ferricyanide. Add 250 μ l of octylphenyl-polyethylene glycol and 125 mg of deoxycholate. The base solution can be stored at 4 °C for up to 6 months in the dark.
 2. Prepare 50x stock X-gal solution by adding dimethylformamide to the X-gal stock bottle to generate a 50 mg/ml solution. Store this solution at -20 °C in the dark.
 3. Just prior to use, dilute stock X-gal solution 1:50 into X-gal base solution to generate staining solution. For smaller biopsies (<1 cm²), aliquot 1-2 ml of staining solution per sample.
2. Fix the skin sample collected in Step 2.7 in a solution containing 2% paraformaldehyde/0.2% glutaraldehyde in cold PBS for 30 min, gently shaking on ice. For smaller biopsies (<1 cm²), use 1-2 ml of fixative solution per sample.
 Note: Alternatively, fix samples in 2-4% paraformaldehyde only, or in 0.5% glutaraldehyde only. Optimal fixation conditions depend on the tissue, degree of LacZ expression and application.
3. Remove the fixative solution, and rinse samples with 3 changes of PBS, 5 min each, on a shaker at RT.
4. Remove the paper towel underneath the sample and cut away excess adipose tissue from the dermal side of the skin by gripping the fat with blunt forceps and trimming with dissecting scissors.
5. Submerge the sample in X-gal staining solution, and incubate at 37 °C O/N. LacZ expression will be visible as a blue stain under a dissecting microscope (**Figure 1B**).
 Note: If the signal intensity is weak, replace the staining solution the next day and repeat the O/N incubation. If the background staining is too intense, reduce the time of staining, or incubate the sample at RT instead of 37 °C.
6. Remove the staining solution and wash the samples in 3 changes of PBS containing 3% DMSO for approximately 5 min, gently shaking at RT.
7. Wash samples in 2-3 changes of 70% ethanol for 5 min each. Store samples in 70% ethanol.

5. Surgical Denervation

1. Anesthetize the animal as in Step 2.2 and shave the entire dorsal skin.
2. Prepare the surgical area of the back skin using Betadine and alcohol wipes, and cover the animal with a sterile surgical drape (for demonstration purposes, sterile drape was omitted to increase visibility). Keep the animal warm using a heating pad while operating in an aseptic surgical area.
3. Make an incision using a sterile #11 scalpel along the dorsal midline from the base of the neck to roughly 0.5 cm above the tail.
4. Using blunt forceps, gently reflect the skin on the left side away from the flank to visualize the underlying tissue from the scapular fat pads near the neck to just above the hind limb.
 Note: Dorsal cutaneous nerves appear as white strands that travel caudally through the translucent fascia of the trunk wall before making sharp bends and entering the loose connective tissue underneath the skin (**Figure 2**).
5. Using ultra-fine forceps under a dissecting light microscope, remove the nerves exclusively from the left side of the animal located at anatomical sites T3-12 by plucking from where the segments bend at the trunk wall to their entry sites into the skin (**Figure 2**).
 1. Orient forceps vertically and remove the nerves by grasping approximately 0.5 cm below their bend sites and pulling upwards, causing the nerve to stretch and separate from the surrounding tissue (**Figure 2C-E**). Be careful to avoid rupturing adjacent blood vessels.
 2. Continue until all nerves extending from the trunk wall to the skin are removed. Do not disrupt the nerves within the dense fascia of the trunk wall. Keep the tissue moist throughout the procedure by periodically applying drops of sterile 0.9% saline solution.
 3. Alternatively, remove nerves by grasping their proximal ends near the trunk wall with forceps and snipping with fine scissors. Afterwards, sever the distal ends near the skin (**Figures 2F-H**). Finally, remove the intervening nerve segments (**Figure 2I**).
6. Remove any nerves from the skin flap exposed in Step 5.4. These fibers comprise the distal branches of the dorsal cutaneous nerves and appear as white branching strands located sporadically within the connective tissue on the dermal side of the skin flap (**Figures 2J-K**).
 1. To remove these fine branches, position the fine forceps roughly parallel to the dermal surface, grasp the nerves and pluck upwards to avoid disrupting blood vessels and puncturing the skin. Continue until all visible nerves have been removed.
7. Using blunt dissection, reflect the skin on the right side of the dorsal midline incision, but do not remove the nerves. This will serve as the contralateral sham-operated control.
8. Suture along the dorsal midline in a simple interrupted pattern to close the incision. Monitor the animal during recovery and post-operatively as previously demonstrated (Steps 2.8-10). Remove sutures within 7-10 days after surgery.
9. To functionally assess stable denervation up to several weeks after surgery, remove the hair from the dorsal skin using an electric clipper.
10. Gently prick the denervated skin area using a hypodermic needle, and note whether the animal responds, typically by shuddering or turning its head. If the skin area has been stably denervated, the animal will exhibit little or no response.
11. Using a black marker, outline the area of no response, as well as an area of similar size and location on the contralateral sham side.
12. Collect biopsies from these sites as in Steps 2.1-2.9 for analysis.
 Note: Alternatively, the entire dorsal back skin, including denervated and sham-operated regions, can be removed as a single sheet for whole-mount staining, similar to as described in Step 4.

Representative Results

By generating mice expressing tamoxifen-inducible *Gli1-CreERT2* and a *LacZ* reporter allele, it is possible to visualize TD epithelia and track the fates of these cells over time. The entire denervation procedure typically can be completed within 1 hr per mouse and should cause minimal distress to the animal.

Our previous studies have indicated that nerves are crucial for maintaining both normal TDs as well as their associated Keratin 8⁺ Merkel cells (**Figures 3A-C**)^{5,19}. Nerves are also critical for promoting *Gli1* expression in the TD (**Figure 3D**). Given the relatively infrequent appearance of TD clusters throughout the skin (**Figure 1B**), it is imperative to sample multiple frozen sections to accurately quantitate TD frequency. Typically, we assess 15 non-consecutive sections (each 10 μ m thick and ~1 cm long) from both sham and denervated skin from each animal. Following denervation, stable loss of nerves, both at the TD and throughout the skin, can be confirmed by the absence of immunohistochemical staining for standard pan-neural markers such as Neurofilament in either frozen or paraffin sections (**Figures 3A** and **3C**, and as previously reported⁵). Alternatively, nerves can also be identified by expression of β 3-tubulin or PGP9.5^{6,9}.

By using *Gli1;LacZ* mice, it is also possible to confirm both the requirement for nerves in activating Hedgehog signaling in the TD and in maintaining TD cell fate by varying the sequence of tamoxifen-induced recombination and denervation. If denervation is performed prior to recombination, for instance, this would test the requirement for nerves in activating the Hedgehog pathway, as monitored by Cre recombinase activity and levels, which are correlated with *Gli1* expression in these animals. On the other hand, if denervation is performed after recombination, this would assess the requirement for nerves in maintaining already-labeled cells in the TD.

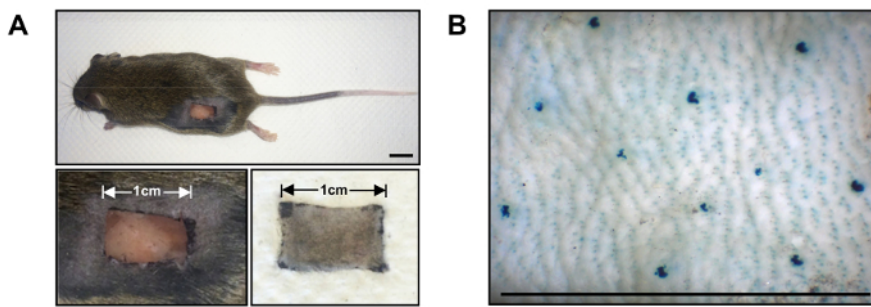


Figure 1. Skin biopsy and whole-mount LacZ staining of TD epithelia. (A) (Top) Photograph of mouse after biopsy and prior to suturing. (Lower left) Enlarged image of biopsy site. (Lower right) Skin sample obtained from biopsy with its dermis side spread flat on a dry paper towel. (B) Whole-mount LacZ staining of skin from a *Gli1;LacZ* mouse, 7 days after tamoxifen induction, depilated just prior to biopsy to improve skin visualization. *Gli1*⁺/*LacZ*⁺ TDs are labeled as intense blue clusters. Scale bar = 1 cm. [Please click here to view a larger version of this figure.](#)

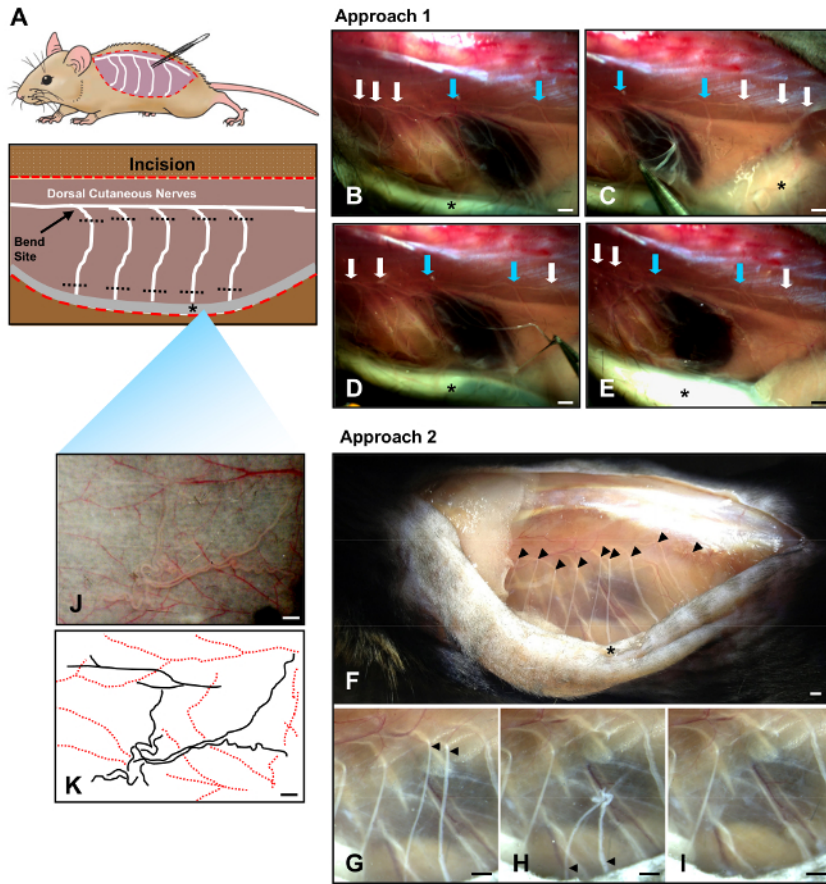


Figure 2. Two approaches for denervating dorsal skin. (A) Cartoon diagram of innervated mouse skin. Red dotted lines indicate a single excision made along the dorsal midline to expose the underlying musculature on the trunk wall (purple) as well as the dermis (grey, asterisk) beneath the reflected skin. Dorsal cutaneous nerves traveling caudally appear to "bend" as they leave the trunk wall (the black arrow indicates one such bend). Nerve segments to be excised are demarcated by black dotted lines. (B) Photograph of intact nerves with sites of bending indicated (arrows). Blue arrows point to 2 nerve segments that will be removed. (C-D) Photographs showing denervation technique. Using ultra-fine forceps, grip the nerves 0.5 cm below their sites of bending and pull outwards. (E) Photograph showing the body cavity after removal of 2 nerve segments (blue arrows). The remaining nerves also need to be removed. (F-I) An alternative approach for nerve removal is depicted, where nerves are snipped at their proximal ends just below where they bend (arrowheads) (G), and also distally, close to their site of entry into the skin (arrowheads) (H). Note that the midline incision in these images is longer than typical for the purpose of better visualization. (J) Photograph of dermal side of the reflected skin flap to one side of the midline. (K) Nerves are outlined (black lines), with larger blood vessels indicated in red. The nerves located on the dermis side of the skin flap also need to be excised. Asterisk, underlying dermis from the reflected skin flap. Scale bar = 1 mm. [Please click here to view a larger version of this figure.](#)

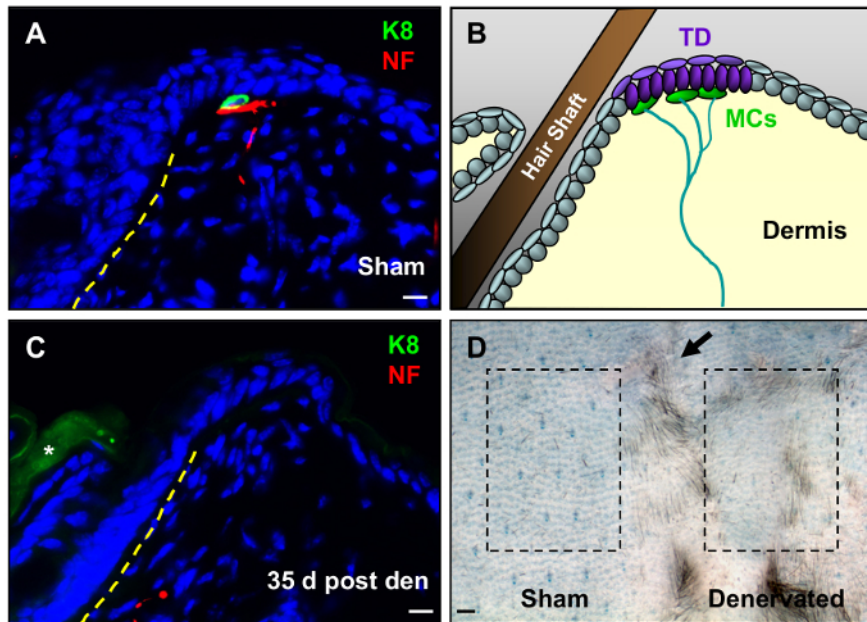


Figure 3. Stable loss of nerves and deterioration of TDs after denervation. (A) Immunohistochemistry showing TD epithelia with Keratin 8⁺ Merkel cells (K8, green) and Neurofilament⁺ nerves (NF, red) in sham-operated skin. (B) Cartoon depiction, with TD epithelia highlighted in purple, Merkel cells in green, and sensory nerves in blue. (C) Immunohistochemistry showing denervated skin (den) lacking Merkel cell-neurite complexes within the TD area. Dashed yellow lines, hair follicle epithelium. Asterisk, background staining. (D) Whole-mount LacZ staining of dorsal back skin from *Gli1^{LacZ/+}* mouse 2 weeks after unilateral skin denervation. In the box to the left of the healed midline incision (arrow), abundant labeled TD epithelia are observed in sham-operated skin. To the right of the midline, TDs are not visible in denervated skin. Scale bar = 10 μ m for (A), (C); and 1 mm for (D). [Please click here to view a larger version of this figure.](#)

Discussion

Nerves serve crucial functions not only in sensation, but also in mammalian organ development, maintenance and regeneration^{13,24-27}. As nerves have recently been implicated in diverse skin disorders, the techniques described here can be used to study the requirement for innervation in a variety of animal disease models. Indeed, the unilateral denervation technique allows for the direct comparison of skin with either intact or disrupted nerves from the same mouse. This provides an ideal internal control to compensate for animal-to-animal differences, with subsequent data analyses making use of a paired t-test.

While the procedures described here largely utilize the *LacZ* reporter gene, these experiments can be adapted such that the *Gli1-CreERT2* allele is combined with other fluorescent reporter or conditional alleles to modify gene expression in the TD. For instance, *Gli1-CreERT2* mice can be crossed with animals harboring conditional alleles of *Patched1* (*B6N.129-Ptch^{1tm1Hahn/J}*)²⁸ to generate mice that form TD-derived tumors after tamoxifen induction⁵. It is important to note that the *Gli1-CreERT2* strain also induces recombination in a subset of *Gli1*⁺ hair follicle stem cells that are physically separated from those in the TD¹³.

Following denervation, nerves in the skin remain stably ablated for several months (Figure 3C)^{5,19}. In other studies, however, some re-innervation has been reported to occur over time⁶. The perdurance of the denervated phenotype may depend on the thoroughness of nerve removal, as it is absolutely critical to excise nerve segments between their exit from the chest wall to a point close to the sites of insertion into the dermis of the skin.

Completely removing the hair from the skin prior to biopsy can enhance the ability to subsequently visualize TDs by whole-mount staining (Figure 1B). This is accomplished by applying depilatory cream to clipped skin for 2 min, and then wiping the hair away in an anterior-to-posterior direction using cotton balls. Please note that depilation can affect hair cycle kinetics by promoting entry into the anagen growth phase. Alternatively, hair can be completely removed using a razor blade. In addition, whole mount immunohistochemistry can be performed to visualize TDs and Merkel cells on epithelial sheets separated from the dermis, as has been previously described²³.

The possibility remains that surgical denervation may cause inflammation at the surgical site, potentially confounding any observed phenotypes. In our experience, we have not observed significant inflammation after denervation, likely because the collateral tissue damage incurred in the skin is slight if the procedure is done properly. To minimize the possibility that inflammation may affect results, additional controls can be incorporated into the experiment. For instance, we observed that denervation specifically inhibited TD-derived tumors, but not adjacent hair follicle-associated lesions in the same skin samples, arguing that denervation—and not a general wound-induced inflammatory response—likely inhibited tumorigenesis at the TD⁵.

It is important to note that surgical denervation ablates all cutaneous nerves, including sensory and sympathetic fibers⁵, and thus provides a general overall assessment of the influence of these nerves on either normal or diseased skin. Other experimental approaches, for instance using pharmacologics such as Botulinum neurotoxin to block neurotransmission, may yield more detailed mechanistic insights⁷, although it is unclear whether these agents inhibit retrograde secretion of cytokines such as Hedgehog ligands. Alternatively, compounds such as 6-

hydroxydopamine have been used to ablate sympathetic nerves in the skin⁹. In addition, targeting the receptors for nerve-derived factors such as Calcitonin gene-related peptide and Substance P may be useful for interrogating specific interactions between nerves and the surrounding cells within their niche⁶. Ultimately, multiple strategies may be utilized in combination to identify, or at least rule out, potential signaling mechanisms.

Finally, targeted genetic deletion of nerve-derived factors in the neural lineage using either Wnt1-Cre or Advillin-Cre may represent the gold standard for elucidating the signals that are exchanged between nerves and their niche¹⁹. As neither of these strains are tamoxifen-inducible, however, some caution needs to be taken to ensure that disruption of these signals does not impair nerve development or proper targeting of neural afferents. Use of a tamoxifen-inducible Cre such as Advillin-CreERT2 may help circumvent these issues²⁹.

Overall, the techniques described here—a combination of lineage tracing, cell visualization and surgical denervation—offer powerful approaches for studying the influence of nerves on normal and diseased skin. With experience, these procedures can be performed routinely and reliably, while causing minimal distress to the animal—or the investigator.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors would like to thank Autumn Peterson for assistance with mouse photography, Daniel Thoresen for assistance with mice, and Drs. Nicole Ward and Abdelmajid Belkadi for assistance with surgical denervation. These studies were supported by funding from the National Institute of Arthritis and Musculoskeletal and Skin Diseases (grants R00AR059796 and R01AR065409); the University of Michigan Department of Dermatology; the Biological Sciences Scholars Program; the Center for Organogenesis; the University of Michigan Comprehensive Cancer Center; and the John S. and Suzanne C. Munn Cancer Fund. S.C.P. was supported by funding from the National Institute of General Medical Sciences (grant T32 GM007315). This work was also supported by the NIH Intramural Research Program, Center for Cancer Research, National Cancer Institute.

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