Protocol

1. Preparation of Injection Solution

1. Prepare 0.2% Evans Blue dye in phosphate buffered saline (PBS) or other aqueous solution of interest. This vital dye is useful for assessing the success of intraductal injections and is recommended as a visualization aid when developing expertise in the technique.
The volume required will depend on the number and location of the glands to be injected. All 10 mammary glands of female mice can be injected but due to the smaller gland size, gland pairs 1 and 5 are typically injected with 10 μl of solution. All other mammary pairs are injected with 20 μl. These volumes are sufficient to fill the entire ductal tree.

2. Pre-operative Preparation

1. Record the body weight of each mouse. As with all preclinical studies, animal weights should be monitored regularly (twice a week or greater) to assess potential toxicity.
2. Anesthetize the mouse using an isoflurane chamber and apply eye lubricant. During the procedure mice will continue to be anesthetized using inhaled 2-4% concentration of isoflurane in oxygen via a nose cone. Carefully monitor the mouse for changes in respiratory rate, adjusting the level of isoflurane accordingly.
3. Inject meloxicam (5-10 mg/kg) subcutaneously prior to the procedure as analgesia.
4. Apply an over-the-counter hair removal cream to the nipple area. Wait 5 min and gently remove loose hair with a cotton-tip applicator using a circular motion. Remove the cream using damp paper towels wetted with warm water. Shaving is not recommended due to the risk of damaging the nipples.
5. Secure the mouse under the stereoscope by gently taping down the extremities.
6. Clean injection sites with alcohol swabs.

3. Intraductal injection

1. Locate appropriate nipples to be injected under the stereoscope. Use fine micro-dissecting tweezers to remove any dead skin that covers the nipple opening.
2. Load 10-20 μl of injection solution into a 50 μl syringe with a 33 G metal hub needle affixed. Sometimes, after injection, small amounts (1 μl or less) can leak out of the nipple upon pulling out the needle. Therefore, it is recommended to inject 11 or 21 μl respectively to account for the potential loss.
3. Hold the nipple gently with the fine tweezers and lift it slightly to position it for injection. It is not necessary to cut the nipple.
4. Inject the solution slowly to minimize potential damage caused by rapidly moving fluid within the ductal lumens. The injection rate should be maintained at approximately 40 μl/min.

4. Post-operative Care

1. Observe the injection site. There should be no signs of trauma to the nipple region or surrounding tissue. Swelling in the area surrounding the nipple likely indicates a mammary fat pad injection rather than a successful intraductal injection.
2. Remove the animal from the nose cone and move to a separate cage for recovery. Place the cage under a heat lamp to prevent hypothermia and assist in recovery. Mice are housed singly and will be monitored closely until they regain consciousness and mobility.

5. Analysis of Mammary Gland Tissue

1. Upon completion of the study, mice are euthanized by cervical dislocation following CO₂ compressed gas in an isolation chamber. Mammary glands are excised and may be used for whole mount preparation, histology, or RNA and protein isolation.

Representative Results

The nipples can be easily localized using a stereomicroscope once hair has been removed in the area surrounding it (Figure 1A). To master the injection technique, it is recommended to inject Evans blue dye and monitor the integrity of the gland (Figures 1B,C). This approach also allows for the determination of appropriate volumes to be injected into each gland as one can visually assess whether the dye reaches the entire ductal system (Figures 1C, 2A). An image of the injected Evans blue image can then be compared to the whole-mounted mammary gland, in which the entire ductal tree is stained with Carmine alum dye (Figure 2B).

Please note that robustness of this injection method is highly dependent on the operator. For example, perforating the duct will result in a mammary fat pad injection and injecting the solution too fast may damage the ductal epithelial cells and provoke an inflammatory response. Successful intraductal injection enables the localized drug delivery to the mammary gland and reduces non-specific side-effects often observed otherwise.

An example image of a gland injected with fluorescently tagged siRNA targeting cyclophilin (a non-essential gene) is shown in Figure 3.
Figure 1. An inguinal mammary gland injected through the nipple with Evans blue dye. A) Appearance of the nipple before injection and B) after injection with 20 μl Evans blue dye. No swelling or tissue damage is observed. C) Upon opening the skin, the dye permits visualization of the entire mammary ductal tree.

Figure 2. Whole-mount analysis of Evans blue-injected gland. A) Representative image of a dissected gland excised from the animal and spread onto a glass slide immediately after injection. B) Whole mount staining of the same gland with carmine dye after fat had been cleared. Comparison with A) confirms that the injected solution fills the mammary ductal tree and the gland is anatomically intact.
Figure 3. Intraductal delivery of fluorescent siRNA. Representative image of fresh tissue sample, excised 48 hr post-injection.

Discussion

Transgenic and knockout mice are invaluable tools for studying the in vivo role of individual genes in breast cancer. However, it is costly and time-consuming to generate these animals and the gene knockdown is usually ubiquitous which sometimes precludes us from recognizing the specific effects of that gene on the mammary gland. Therefore, a targeted knockdown would alleviate issues of non-specific side-effects and toxicities in other organs.

The intraductal injection of siRNA presented here enables the localized gene knockdown in the mouse mammary gland. It is a rapid method for a targeted and localized drug delivery to the mammary gland not limited to siRNA delivery. Such a mammary gland-specific drug delivery avoids unspecific side-effects and toxicity to other organs and allows for the study of gene alterations at specific time points during mammary gland development and breast tumor formation. There is no evidence of siRNA particles in non-injected glands, the liver or other organs providing further evidence that this method presents a preferred way for localized gene knockdown in the mammary gland. Furthermore, the intraductal injections can be repeated weekly or biweekly over several months to allow for long-term monitoring of therapeutic agents.

This technique opens up the possibility for assaying the intraductal delivery of various reagents to the mouse mammary ducts. Advances in localized delivery in well-characterized mouse models should accelerate the application of non-invasive, targeted therapeutic strategies in humans.

Disclosures

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References