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Video Article

Novel Method of Plasmid DNA Delivery to Mouse Bladder Urothelium by Electroporation

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Abstract

Genetically engineered mouse models (GEMMs) are extremely valuable in revealing novel biological insights into the initiation and progression mechanisms of human diseases such as cancer. Transgenic and conditional knockout mice have been frequently used for gene overexpression or ablation in specific tissues or cell types *in vivo*. However, generating germline mouse models can be time-consuming and costly. Recent advancements in gene editing technologies and the feasibility of delivering DNA plasmids by viral infection have enabled rapid generation of non-germline autochthonous mouse cancer models for several organs. The bladder is an organ that has been difficult for viral vectors to access, due to the presence of a glycosaminoglycan layer covering the urothelium. Here, we describe a novel method developed in lab for efficient delivery of DNA plasmids into the mouse bladder urothelium *in vivo*. Through intravesical instillation of pCAG-GFP DNA plasmid and electroporation of surgically exposed bladder, we show that the DNA plasmid can be delivered specifically into the bladder urothelial cells for transient expression. Our method provides a fast and convenient way for overexpression and knockdown of genes in the mouse bladder, and can be applied to building GEMMs of bladder cancer and other urological diseases.

Video Link

The video component of this article can be found at https://www.jove.com/video/57649/

Introduction

Genetically engineered mouse models (GEMMs) have been playing an essential role in expanding our knowledge about animal development, gene functions *in vivo*, and mechanisms of disease progression[1,2]. Germline GEMMs are traditionally developed in two ways. The first is the creation of transgenic mice by pronuclear injection of DNA plasmid followed by the transplantation of zygotes into pseudopregnant females. The other is the generation of knock-out/knock-in mice by homologous recombination in embryonic stem cells and the development of chimeras. Conditional knockout of genes in specific tissue or cell type of interest involves the breeding of genetically engineered alleles with Cre and flox sites for multiple generations. The whole process can be expensive and time-consuming, especially if the goal is to target multiple genes tissue-specifically. Recently, gene-editing technologies such as clustered regularly interspaced short palindromic repeats (CRISPR) have been applied to several organs of the mice to induce tissue-specific genetic alterations for autochthonous cancer modeling[3,4,5,6,7] or correct disease mutations *in situ*[8,9]. In these studies, the delivery of the gRNA vectors was usually achieved through adenoviral or lentiviral infection of the organ or hydrodynamic tail-vein injection. The relative ease of delivery of the CRISPR components to the lung and liver has greatly improved the convenience and efficiency of cancer modeling in these organs compared to the traditional Cre-Lox based mouse models.

The bladder urothelium is where most bladder tumors originate. The delivery of DNA vectors to the bladder urothelium for cancer modeling or gene therapy is hampered by a glycosaminoglycan layer on the apical urothelial surface, which acts as a barrier for adherence of infectious viruses[10,11]. Several pretreatment agents such as Syn3 and dodecyl-β-d-maltoside have been used to disrupt this barrier and enhance adenovirus infection efficiency[12,13,14]. Whether adeno-associated viruses (AAVs) can effectively infect the bladder has not been reported. Overall, a non-viral based delivery method would be desirable. Here, we describe a novel method developed in the lab for efficient DNA plasmid delivery to the mouse urothelium through urethra catheterization and electroporation. Because our approach does not involve chemical pretreatment of the glycosaminoglycan layer or virus production, it significantly simplifies the delivery of DNA plasmids into the mouse bladder urothelium, and should facilitate various *in vivo* studies of bladder diseases.

Protocol

All procedures described here were performed in accordance with the guidelines and regulations from the Institutional Animal Care and Use Committee (IACUC) of University of California Santa Cruz.

1. Plasmid and Tools Preparation

1. For each bladder to transfect, prepare at least 20 µL of DNA plasmid (1 µg/µL).
2. Add 1 µL of Trypan Blue to the 20 µL of plasmid solution in a 1.5 mL microcentrifuge tube. Pipette to mix well.
3. Autoclave all surgical instruments and sterilize the workspace with 70% ethanol. Spray surgical instruments and gloves with 70% ethanol frequently or use a glass bead sterilizer when performing the following steps to maintain sterile conditions.

2. Anaesthetizing Animal

1. Use a table top anesthesia system to anesthetize the animal with isoflurane. Turn on the O_2 tank and adjust the oxygen flowmeter to 1 L/min.
2. Place an adult female C57BL/6J mouse in the induction chamber. Turn on and adjust the isoflurane vaporizer to 3% for induction. The mouse should be in anesthesia within 2 min. Administer buprenorphine analgesic (0.1 mg/kg) subcutaneously upon removal of the mouse from the induction chamber for preemptive analgesia.
   NOTE: In this protocol, female mice are used, as they are easier to work with during urethra catheterization. The protocol also works for male mice, but extra caution is needed when performing step 3.
3. Apply ophthalmic ointment to the eyes of the animal to prevent dryness. Place the anaesthetized mouse facing up on a heating pad with its nose in the nose cone. Turn the air circuit switch to let isoflurane go through the nose cone.
4. Restrain the head and the nose cone with adhesive tape. Adjust the isoflurane vaporizer to 2% for maintenance.
5. Restrain the limbs with adhesive tape. Perform toe-pinch tests to make sure the animal is in deep anesthesia and then shave the abdominal area.

3. Urine Depletion and Bladder Rinsing

1. Apply lubricant to the catheter (24G, length of 2.11 cm, outer diameter of 0.045 cm) and ensure that its outer surface is fully covered.
2. Insert the catheter into the urethra opening and slowly push it until it reaches the bladder, at which time the urine should flow out through the catheter. Gently press the abdomen to help urine depletion.
   1. Check if the catheter is inserted correctly into the bladder by observing automatic urine out-flow. Avoid piercing the urethra and bladder wall, and apply lubricant multiple times if needed.
3. Discard the urine with a pipette into a waste beaker.
4. Pipette 80 µL of phosphate buffered saline (PBS) into the outer end of the catheter, with the other end still in the bladder. Carefully attach a 1 mL syringe to the catheter. Gently push the syringe to inject the PBS into the bladder. Leave some PBS in the catheter to avoid creating air bubbles in the bladder.
5. Remove the syringe. Wait for PBS to drain out of the bladder. Gently press the abdomen to evacuate PBS.
6. Discard the PBS in the catheter with a pipette into the waste beaker.
7. Repeat PBS washing (steps 3.4 - 3.6) for two more times.
   NOTE: It is essential to work gently during these washing steps. Blood in the catheter or failure of automatic out-flow of liquid usually indicates the urethra is pierced through. It is also important to avoid introducing air bubbles, as they will decrease the electroporation efficiency.

4. Plasmid Delivery

1. Apply 70% ethanol to the mouse abdomen and use a scalpel to make a vertical incision of 1 cm to open the abdominal skin above the bladder.
2. Pipette at least 20 µL of plasmid plus Trypan Blue solution into the outer end of the catheter, and attach the syringe.
3. Inject the plasmid solution into the bladder. If the bladder turns blue, the injection is successful. Leave some liquid in the catheter to avoid creating air bubbles in the bladder.
4. Grab the external urethral orifice using tweezers, and then remove the catheter and syringe. Use a string to tighten the external urethral orifice. Make two loops with the string and then tie with two knots.
   NOTE: Tightening of the urethral is important for preventing backflow of plasmid liquid.
5. Turn on the electroporation generator with the following parameters: 33V, 50 ms working time, and 950 ms interval time.
6. Hold the bladder with tweezers and pinch the bladder with two electrodes. Push the foot pedal to perform the electroporation.
   NOTE: The electric pulses are set to occur 5 times with short intervals after each pedal push. The mouse may twitch in this process. Pushing the foot pedal more than once can increase the delivery efficiency, but too many electric pulses can damage the tissue integrity of the urothelium.
   1. Avoid any contact between the tweezers and the electrodes, as this will generate a spark.

5. Recovery

1. Suture the abdominal and skin opening with sterile surgical suture and clips. Apply iodine onto the wound.
2. Remove the animal from the isoflurane system. Administer buprenorphine analgesic (0.1 mg/kg) subcutaneously to alleviate post-surgical pain.
3. Keep the animal on the heating pad and return it to the home cage after full recovery. Check the animal at least once daily for normal mobility, drinking and feeding behaviors and no signs of infection until the incision is healed and then remove the clips. Administer subsequent

injections of buprenorphine analgesic if the animal displays pain symptom such as reduced grooming, increased aggressiveness, and vocalization.

Representative Results

To demonstrate the success of gene delivery into the bladder urothelium, we used the pCAG-GFP[15] plasmid for electroporation. 20 µL of plasmid and Trypan Blue solution was injected through the urethra and 5 electric pulses were administered. After 48 h, we dissected the mouse bladder and observed patches of GFP fluorescence under a dissection fluorescence microscope, whereas a negative control bladder that did not undergo electroporation showed no GFP signal (**Figure 1A**). When we performed immunofluorescence staining of sectioned bladder tissues with cytokeratin (CK)5, CK18, and GFP antibodies, we found that GFP signal was present in umbrella, intermediate, and basal cells, but not in the muscle layer (**Figure 1B**), indicating good specificity of plasmid delivery into the urothelium. Approximately 15% of the urothelial cells are GFP^+, and such clonal nature should be ideal for cancer modeling since tumors usually initiate from individual cells.

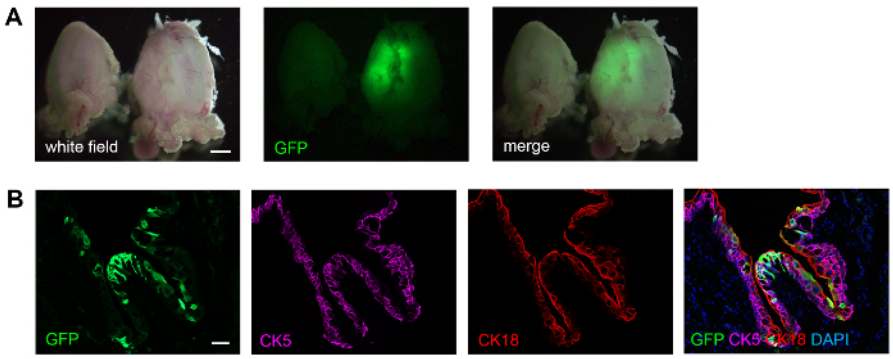

Figure 1: Successful delivery of pCAG-GFP plasmid to the mouse bladder urothelium. (**A**) Comparison of a bladder that underwent electroporation (right) to a negative control bladder (left) showing that electroporation successfully turned the bladder green. (**B**) Immunofluorescence staining of sectioned bladder tissues showing mosaic expression pattern of GFP^+ cells in umbrella ($CK18^+$), intermediate ($CK5^+CK18^+$), and basal ($CK5^+$) urothelial layers. Scale bar in **A** corresponds to 1 mm, and in **B** to 50 µm. Please click here to view a larger version of this figure.

Discussion

Electroporation enhances the cell membrane permeability and is a powerful technology for gene electrotransfer[16]. Gene delivery into living rodents by electroporation has been frequently used in neurobiology fields[17,18,19,20,21]. Its potential applications in many other organs have not been extensively explored. To our knowledge, our method described here is the first report of successful gene delivery by electroporation to the bladder urothelium. It offers several advantages to the adenovirus/lentivirus-based infection methods. First, the electroporation method is easier, safer, and cheaper to adopt because non-viral genetic vectors are used and the delivery of DNA plasmids to specific organ sites *in vivo* is relatively straightforward. Second, it avoids the undesirable host immune responses that are often associated with the delivery by virus vectors[22]. Third, in the case of bladder, it negates the need for chemical pretreatment of the glycosaminoglycan layer, which normally blocks virus binding to urothelial cells.

The most critical steps in this protocol are the catheterization, rinsing, and plasmid injection. Extra care and gentle handling are needed to ensure that the catheter does not pierce through the urethra or the bladder wall when performing insertion and attaching/removing the syringe. Apply adequate lubricant to fully cover the outer surface of the catheter is also essential. Another likely cause of failure is the introduction of air bubbles into the bladder during rinsing and plasmid injection, since it will decrease electrical conductivity. To prevent this, we recommend always leaving some liquid in the catheter when performing those steps.

Our protocol should work for adult mice of any age, because we did not observe a difference in delivery efficiency between younger (2 months) and older (1 year) mice. Several factors can influence plasmid delivery efficiency. We recommend 20 µL of plasmid as the minimal volume for injection as lower amount may not sufficiently cover the inner surface of the bladder. Increasing plasmid volume and concentration generally yields higher penetration, but no additional benefit was observed for volumes beyond 60 µL. Giving more electrical pulses and touching different surface areas of the bladder with electrodes will have the largest impact on enhancing delivery efficiency. The electroporation parameters described in this protocol is set to allow permeabilization of urothelial cells while preserving tissue integrity, since higher voltage tends to induce cell death. It is possible to tweak these parameters for further optimization. Mouse-to-mouse variations and the handling of the researcher can affect the efficiency greatly. Depending on the goal of the project, we recommend performing trials to determine the best parameters for each specific experiment. For example, in modeling cancer initiation and clonal growth, a lower penetration may be desired.

As a proof-of-principle for our delivery method, GFP expression can be visualized in the bladder as early as 36 h after electroporation, and can persist for at least two weeks. With this new method, it is now possible to deliver DNA plasmids to the mouse bladder fast and inexpensively for gene overexpression or down-regulation and genome editing. It opens new avenues for studying bladder development and diseases, as well as building novel autochthonous bladder cancer models.

Disclosures

The authors declare no competing interest.

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