

Video Article

Renal Capsule Xenografting and Subcutaneous Pellet Implantation for the Evaluation of Prostate Carcinogenesis and Benign Prostatic Hyperplasia

Tristan M. Nicholson^{1,2}, Kristen S. Uchtmann¹, Conrad D. Valdez¹, Ashleigh B. Theberge^{1,3}, Tihomir Miralem¹, William A. Ricke¹

¹Department of Urology, University of Wisconsin-Madison

²Medical Scientist (MD/PhD) Training Program, University of Rochester School of Medicine & Dentistry

³Molecular and Environmental Toxicology Center, University of Wisconsin-Madison

Correspondence to: Tristan M. Nicholson at nicholson@urology.wisc.edu

URL: <https://www.jove.com/video/50574>

DOI: [doi:10.3791/50574](https://doi.org/10.3791/50574)

Keywords: Medicine, Issue 78, Cancer Biology, Prostatic Hyperplasia, Prostatic Neoplasms, Neoplastic Processes, Estradiol, Testosterone, Transplantation, Heterologous, Growth, Xenotransplantation, Heterologous Transplantation, Hormones, Prostate, Testosterone, 17beta-Estradiol, Benign prostatic hyperplasia, Prostate Cancer, animal model

Date Published: 8/28/2013

Citation: Nicholson, T.M., Uchtmann, K.S., Valdez, C.D., Theberge, A.B., Miralem, T., Ricke, W.A. Renal Capsule Xenografting and Subcutaneous Pellet Implantation for the Evaluation of Prostate Carcinogenesis and Benign Prostatic Hyperplasia. *J. Vis. Exp.* (78), e50574, doi:10.3791/50574 (2013).

Abstract

New therapies for two common prostate diseases, prostate cancer (PrCa) and benign prostatic hyperplasia (BPH), depend critically on experiments evaluating their hormonal regulation. Sex steroid hormones (notably androgens and estrogens) are important in PrCa and BPH; we probe their respective roles in inducing prostate growth and carcinogenesis in mice with experiments using compressed hormone pellets. Hormone and/or drug pellets are easily manufactured with a pellet press, and surgically implanted into the subcutaneous tissue of the male mouse host. We also describe a protocol for the evaluation of hormonal carcinogenesis by combining subcutaneous hormone pellet implantation with xenografting of prostate cell recombinants under the renal capsule of immunocompromised mice. Moreover, subcutaneous hormone pellet implantation, in combination with renal capsule xenografting of BPH tissue, is useful to better understand hormonal regulation of benign prostate growth, and to test new therapies targeting sex steroid hormone pathways.

Video Link

The video component of this article can be found at <https://www.jove.com/video/50574/>

Introduction

Prostate cancer (PrCa) and benign prostatic hyperplasia (BPH) are significant health burdens. PrCa is the second most prevalent solid organ cancer in men and a leading cause of cancer related death¹. BPH is also highly prevalent among older men, and it is estimated that the clinical manifestation of BPH, lower urinary tract symptoms (LUTS), will affect 50-90% of men². While androgens and estrogens are known to be important in PrCa and BPH, our understanding of the hormonal mechanisms that underlie carcinogenesis and growth remains incomplete^{3,4}. Simple and genetically tractable animal models underpin the studies that will address these priorities in prostate research. In hormone responsive diseases such as PrCa and BPH, the use of subcutaneous, slow release hormone pellets, in isolation or in combination with renal capsule xenografting (the transfer of cells, tissues or organs from one species into another) into the immunocompromised mouse host, provides a simple and reproducible method for studying hormonal regulation of growth and carcinogenesis.

Subcutaneous hormone pellet implantation is a simple and reproducible technique to study hormonal regulation of carcinogenesis and benign growth in the prostate⁵. Subcutaneous implantation of adult male mice with 25 mg testosterone (T) and 2.5 mg 17 β -estradiol (E₂), causes an increase in serum E₂ and gradual decrease in T, recreating the dynamic hormonal environment of aging men^{5,6}. In addition, this model recapitulates many of the clinical features of BPH-LUTS^{5,7}. Alternative methods of hormone administration include oral/gavage administration or intraperitoneal injection, which cause distress to the rodent, are less consistent in delivering the same amount of drug over time, and are more labor-intensive than a one-time surgical implantation. Other subcutaneous modes for hormone and/or drug delivery include Silastic capsules⁸. While we also have experience with use of Silastic capsules, compressed pellets are favored for their ease of use and reproducibility. Furthermore, release rates of compressed hormone pellets better mimic the dynamic sex steroid hormone ratios observed in aging men^{5,7}.

Since the development of genetically immunocompromised mice, numerous *in vivo* model systems incorporating xenografting techniques have been developed for the study of a wide variety of normal and diseased tissues. There are several basic categories of xenografting. Tissue xenografts consist of a small piece of intact tissue, which can be a normal structure, malignant tumor, or benign growth. Szot *et al.* (2007) have recently illuminated renal capsule xenografting of pancreatic islets⁹. Aamdal *et al.* (1985) described renal capsule xenografting of 27 human cancer cell lines in immunocompromised mouse hosts¹⁰. Grafts can also be composed of a single immortalized cell line (cancerous or non-tumorigenic), or can consist of an immortalized cell line combined with cells isolated from mesenchyme (cell recombinant graft).

We favor xenografting of cell recombinants to probe the respective roles of stroma and epithelium in the development of PrCa and BPH. Cunha *et al.* (1980) was the first to report that when adult murine bladder epithelium is combined with embryonic urogenital sinus mesenchyme (UGM) and xenografted under the renal capsule of male mice hosts, the tissue develops into structures resembling prostate acini¹¹. Norman *et al.* (1986) showed that adult mouse prostatic ductal epithelium and UGM, when combined in tissue recombinants, undergo ductal growth and branching morphogenesis¹². Hayward *et al.* (1988) showed that human prostate epithelium responds to inductive fetal mesenchyme in a similar fashion¹³. A hormonal model of carcinogenesis in the prostate, by combining the immortalized human prostate epithelial cell line BPH-1 with UGM, was first reported by Wang *et al.*¹⁴ (2001) and has been used extensively in our laboratory¹⁵. This model is well suited for understanding PrCa progression because benign prostatic epithelium transforms to metastatic disease, modeling advanced PrCa in humans⁵. Because specific components can be genetically manipulated, cell recombinant grafting is particularly useful as an approach to evaluate stromal-epithelial interactions.

Other sites suitable for xenografting are intradermal, subcutaneous and orthotopic locations¹⁶. Depending on the tissue and disease process of interest, these may be suitable alternative approaches. For the study of prostate hormonal carcinogenesis and benign growth regulation, we choose the renal capsule site for xenografting due to its higher graft take rate, abundant blood supply, and ability to implant a greater number of xenografts into one confined site¹⁶. Moreover, hormonal manipulation of the host mouse that results in prostatic atrophy limits the use of prostate orthotopic grafting¹⁶.

This protocol outlines techniques of compressed hormone/drug pellet manufacture and surgical implantation, as well as renal capsule xenografting of human prostate cell and tissue grafts. Together, these techniques provide a sensitive assay to determine if a genetically modified mouse is more susceptible to PrCa and/or BPH initiation than control strains. Xenografting is a powerful technique for *in vivo* evaluation of the potential of experimental cells to develop histologic and molecular features of malignancy, as well as an assay for novel treatment strategies for a wide variety of benign and malignant diseases.

Protocol

1. Preparation of Compressed Hormone/Drug Pellet

1. This procedure should be done in a chemical safety hood while wearing a laboratory coat, gloves, mask, safety glasses, and bonnet. To prevent cross-contamination, it is critical that the pellet making equipment is thoroughly cleaned with 70% ethanol prior to and after manufacture of each type of pellet.
2. Using an analytical scale and weigh paper, measure desired amount of hormone/drug powder. Approximately 5% extra material is recommended to accommodate loss during pressing.
3. Some hormones/drugs require a binding agent or filler, which is mixed prior to pellet manufacture. For example, to manufacture a 2.5 mg pellet of 17 β -estradiol, combine the hormone with 22.5 mg of cholesterol, which results in a 25 mg pellet.
4. Carefully transfer into die set, place under the press and push lever firmly down to compress pellet. Use consistent pressure to maintain constant surface area to volume ratio.
5. Next reverse the die holder and push the lever down again to release the pellet.
6. Inspect pellet for integrity and determine if final mass is within desired range. An acceptable range is a $\pm$ 5% difference in the final mass (for example, an acceptable range for a 25 mg pellet is 23.8-26.3 mg).
7. Pellets can be made prior to surgery and stored (conditions and length of storage depends on stability of the hormone or drug used in pellet manufacture). Hormone pellets can be purchased as an alternative to pellet manufacture. Purchasing pellets avoids the use of non-pharmaceutical grade drugs since they will be formulated and compounded.

2. Preparation of Cell Recombinant Xenografts

1. Harvest and culture primary mouse urogenital mesenchyme (UGM) stromal cells for less than 4 weeks before being collected for counting.
2. Culture immortalized prostate epithelial cell lines in the usual fashion and collect for counting. For this example, prepare cell recombinant grafts by mixing 100,000 BPH-1 epithelial cells per graft with 250,000 UGM stromal cells per graft in suspension.
3. Pellet cells together and re-suspend in enough neutralized type 1 rat tail collagen to make desired number of grafts, each in a volume of 25 μ l to 50 μ l.
4. Set recombinant grafts at 37 °C for 15 minutes and then cover with growth medium for storage at 37 °C for up to 48 hours before grafting.

3. Preparation of Tissue Xenografts

1. Maintain universal precautions for blood borne pathogens during tissue procurement and surgical procedure. Following institutional review board approval and informed consent, obtain fresh prostate tissue from surgical resection. For studies of BPH growth, tissues can be harvested from transurethral resection of the prostate or simple prostatectomy procedures. Primary xenografts of PrCa or benign prostate tissue can be obtained from radical prostatectomy specimens.
2. Store harvested tissue in isotonic buffered cell media or normal saline on ice. Depending on the tissue and storage conditions, specimens may be stored for up to 24 hours prior to preparation of tissue grafts. Common practice is to divide the specimen into three pieces, with one section fixed in formalin and routinely processed for histology, one piece snap frozen for molecular analysis and one piece further divided into tissue xenografts (1-3 mm in size).

4. Preparation of Fire Polished Glass Pipette

1. Wearing eye protection, laboratory coat and heat resistant gloves, place glass Pasteur pipette tip into Bunsen burner flame at approximately a 60° angle to start.

2. Keep a constant, slight movement to the pipette so as to avoid hot spots.
3. Draw the tip thinly and fire-polish with the goal being a slightly curved end with a rounded, closed tip.

5. Preparation of Instruments and Surgical Suite

1. Autoclave all instruments. Prior to surgery, inspect all instruments, taking care to maintain sterile field.
2. Set up sterile surgical area with absorbent pads, dissection scope, anesthesia nose cone, surgical instruments and heating lamp directed toward the working area. To sterilize tools in between mice, prewarm bead sterilizer.
3. Assemble and test anesthesia apparatus; weigh gas scavenger.
4. Using the microwave, heat wax pads for up to 5 min (in 1 min increments, kneading the wax pad in order to combine melted and unmelted wax) at 70-80% power, until all the wax is evenly melted. By touch, ensure the pad is not too hot and place under the empty rodent recovery cage.
5. Don personal protective equipment for rodent surgical procedures, including surgical mask, bonnet, gloves and gown.

6. Surgical Procedure: Renal Capsule Xenografting

1. All procedures involving research animals should be performed with approval of institutional animal care and use committees. Prior to induction of anesthesia, observe the mouse to ensure health and well-being.
2. Place the mouse into chamber for induction of isoflurane anesthesia at 3-5%. When the animal has stopped moving and respiratory rate has decreased, transfer to nose cone in the prone position, and maintain anesthesia at 1-3%.
3. If necessary, use clippers to shave the back of the mouse. This is not needed when utilizing athymic nude (nu/nu) mice.
4. Apply firm pressure to the webbing of the extended hind foot to evaluate adequacy of anesthesia. If the mouse responds to pressure, more time is needed for the anesthesia to take effect. If necessary, adjust anesthesia flow slightly to achieve adequate anesthesia.
5. When the mouse is adequately anesthetized, disinfect the surgical site with surgical iodine (Betadine) solution followed by 70% alcohol.
6. Don sterile gloves and gown; apply sterile drapes to the surgical site. Lift the back skin with a pair of toothed forceps, and using the coarse scissors make a 2-3 cm dorsal midline incision.
7. Using blunt scissors or a probe, separate the underlying dermis from the body wall (on both sides of the incision for bilateral grafting or on one side for unilateral grafting).
8. Reposition the mouse into lateral position, and identify the location of the kidney by viewing the renal profile through the muscle wall. Applying gentle manual pressure with the thumb and index finger on the abdomen may assist with visualizing internal organs.
9. Using fine iris scissors, and taking care to avoid major vessels and spinal nerves, make a 1 cm incision in the body wall parallel to the spine. Widen this incision to 1.5-2.0 cm (slightly longer than the long axis of the kidney) by gently opening the scissors wider after placing them in the initial incision.
10. Exteriorize the kidney by applying gentle pressure outside the muscle wall on either side of the kidney using the index finger and thumb. Tuck the skin edges below the exteriorized kidney, which will rest on the body wall. While the kidney is exteriorized, maintain hydration of the renal capsule by applying sterile saline.
11. Using fine #5 forceps gently lift the kidney capsule from the parenchyma of the kidney and with a fine scalpel, make a 2-4 mm incision in the capsule. The size of the incision is determined by the size of the graft, but should be minimized in order to maintain integrity of the capsule.
12. Manipulate a glass Pasteur pipette that has been drawn thin and fire-polished with a rounded closed end under the capsule tangential to the surface of the kidney. Gently open a small capsule pocket for the grafts, using great care not to damage the kidney parenchyma.
13. The cut edge of the kidney capsule is lifted with the fine forceps, and the graft is inserted into the pocket under the capsule using the fire-polished glass pipette. Several grafts can be placed under the kidney capsule and evenly spaced on the kidney surface.
14. If, during the course of grafting, the capsule becomes dehydrated, it should be moistened by applying sterile saline.
15. When grafting is complete, gently lift the sides of the muscle wall incision to replace the kidney back into the body cavity. Observe that the grafts do not slip out from under the capsule.
16. Close the muscle wall with a single suture (4-0, FS-2 vicryl suture). The surgical procedure can be repeated on the contralateral kidney by repositioning the mouse.
17. When grafting is complete, the mouse may undergo subcutaneous pellet implantation during the same surgical procedure (See Protocol step 7).
18. Using toothed forceps, align the skin incision edges and apply 2-3 surgical wound clips to close the incision. Administer analgesia (we use 5-10 mg/kg carprofen using a subcutaneous injection) and place the mouse in the lateral recumbent position in the recovery cage. Ensure maintenance of body temperature with heat from a lamp or heating pad. Observe for full recovery of the mouse, which should occur in less than 15 minutes.
19. Mice should be observed for the next 24 hr for signs of post-operative pain, bleeding and/or other complications. Regularly inspect surgical incision for signs of infection.
20. Remove wound clips with surgical wound clip removal device 7-14 days after surgery.

7. Surgical Procedure: Subcutaneous Pellet Implantation

1. Repeat steps 6.1-6.5 from renal capsule xenografting surgical procedure.
2. Lift the back skin with a pair of skin forceps, and using the coarse scissors make a 1-2 cm dorsal midline incision.
3. Using blunt scissors or a probe, separate the underlying dermis from the body wall in a cranial direction.
4. Using skin forceps, gently lift the cranial aspect of the skin incision, and holding the hormone pellet gently with straight, serrated forceps, insert the pellet and release at the scruff of the neck, in the pocket you created with the blunt probe.
5. Repeat steps 6.18-6.20 from renal capsule xenografting surgical procedure. If performing subcutaneous pellet implantation only, use 1 surgical wound clip to close the surgical incision.

Representative Results

Depending on the hypothesis to be tested, renal capsule xenografting of tissue or cell recombinants can be performed in isolation, or in combination with subcutaneous hormone pellet implantation. A schematic of a hypothetical experiment combining renal capsule xenografting experiment with subcutaneous hormone pellet implantation is illustrated in **Figure 1**. A variety of powdered and/or crystalline substances can be used in the manufacture of compressed pellets with the pellet press. **Figure 2** shows 25 mg, 12 mg and 6 mg compressed hormone pellets.

Figure 3 shows an example of the fire-polished glass pipette used during the surgical procedure. Multiple passes of a glass Pasteur pipette through Bunsen burner flame result in a fire-polished, rounded tip that is used to create a pocket for grafts under the renal capsule and to manipulate grafts under the renal capsule.

As shown in **Figure 4**, primary tissue xenografts from patients with BPH, supplemented with exogenous testosterone (25 mg subcutaneous pellet) to simulate the male human hormonal milieu, survive and the tissue architecture is preserved (**Figure 4A**). Also shown in **Figure 4**, renal capsule grafting can be used to evaluate hormonal carcinogenesis. Cell recombinants containing benign prostate epithelium (BPH-1) and inductive urogenital mesenchyme (UGM) grown in untreated mice form benign growths (**Figure 4B**). The same graft, grown in a host that received subcutaneous hormone pellets of 25 mg T and 2.5 mg E₂ for four months, develops into prostate carcinoma that invades the renal capsule (**Figure 4C**)^{5,14}.

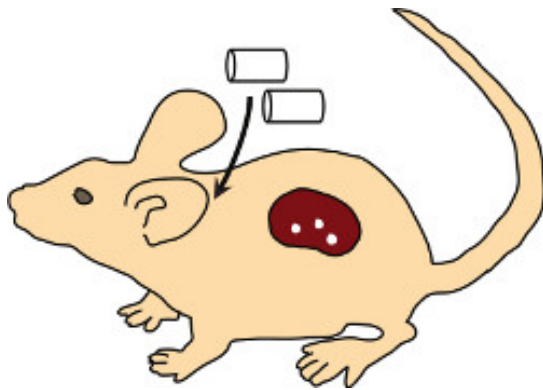


Figure 1. Schematic of renal capsule xenografting and pellet implantation procedure. Renal capsule xenografting is performed via a dorsal midline approach. During the same surgical procedure, the mouse is surgically implanted with slow-releasing subcutaneous compressed hormone and/or drug pellets in the neck scruff region.

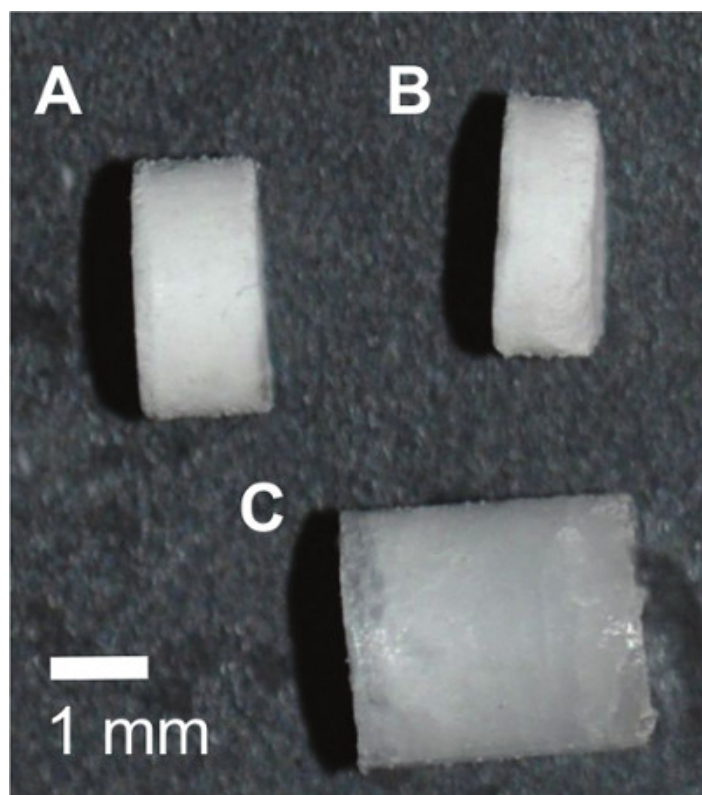


Figure 2. Examples of compressed hormone pellets manufactured with the present protocol. **A.** 12 mg pellet. **B.** 6 mg pellet. **C.** 25 mg pellet.

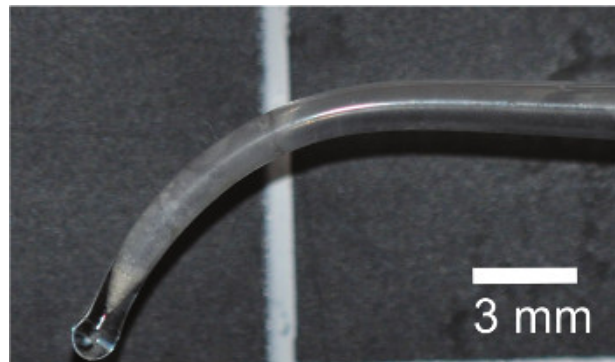


Figure 3. Fire-polished glass Pasteur pipette. The finished pipette is curved, with a smooth rounded tip suitable for creating a pocket for the xenograft under the renal capsule. The pipette is also used to manipulate the graft under the renal capsule.

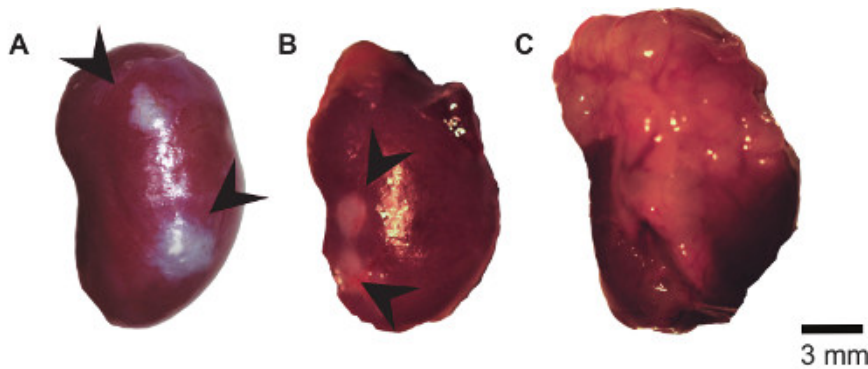


Figure 4. Example of renal capsule xenografts. **A.** Tissue xenografts (arrowheads) of human BPH tissue are grown for one month under the renal capsule of a nude male mouse. The mouse host is supplemented with 25 mg testosterone pellet to simulate the hormonal milieu of the human male. **B.** Cell recombinant xenografts (arrowheads) containing human prostatic epithelial cell line (BPH-1) and inductive urogenital mesenchyme (UGM) grown under the renal capsule of a nude mouse that was not implanted with a subcutaneous hormone pellet for four months. **C.** Cell recombinant xenograft containing BPH-1 and UGM, grown under the renal capsule of a nude mouse that also underwent subcutaneous pellet implantation of 25 mg testosterone and 2.5 mg 17 β -estradiol for four months.

Discussion

This paper and the protocol outlined herein describe the manufacture of compressed hormone and/or drug pellets, and the surgical procedure for subcutaneous pellet implantation of mice. Depending on the research question to be addressed, this technique can be performed singly, or in combination with renal capsule xenografting of prostate cell recombinant grafts or prostate tissue xenografts, which are techniques widely used in prostate research¹⁷⁻¹⁹. Taken together, these techniques offer a powerful approach to study hormonal carcinogenesis and regulation of benign growth in the human and mouse prostate.

Pellet manufacture is simple to perform, easily reproducible, and requires limited equipment. It can be applied to various hormone and drug preparations. It is critical to perform this technique in a chemical safety hood, and wear appropriate personal protective equipment. For experiments assessing prostate carcinogenesis and induction of murine bladder outlet obstruction we use 25 mg T and 2.5 mg E₂^{5,7,15}. These doses are chosen because they produce the desired *in vivo* response and because they create serum hormone levels that are physiologically relevant for human males with the disease process of interest⁶. Pellet molds allow several different sizes of pellets to be manufactured; we typically use 25 mg pellets for evaluating carcinogenesis and benign growth. Choice of the pellet size depends on the hypothesis to be addressed, as well as the production of a desired effect, the production of desired circulating levels and the duration of the experiment. If drug or hormone is combined with a binding agent (such as cholesterol) we recommend a homogenous mixture of hormone and binding agent.

Depending on the study, subcutaneous pellet implantation can be performed following castration of the male host¹⁵. In our experience, implantation of 25 mg of T causes feedback inhibition of the hypothalamic-pituitary-testicular axis, inhibiting endogenous secretion. Therefore we choose to compare mice treated with 25 mg T to simulate the androgenic environment of the human male to intact but untreated males, with endogenous T secretion maintained. We do not routinely utilize a castrate host due to the atrophic response of the prostate and prostate xenografts to androgen withdrawal.

This protocol also describes the creation of a piece of specialized equipment for renal capsule xenografting, the fire-polished glass Pasteur pipette. This can be easily performed with equipment present in most laboratories, resulting in a customized surgical instrument than can be manufactured and sterilized prior to use in the surgical procedure. Wearing personal protective equipment is important to ensure staff safety when creating the fire-polished glass pipette.

Prior to embarking on any of the surgical procedures outlined in this protocol, all techniques must be reviewed and approved by the institutional animal care and use committee. Surgical procedures performed in rodents require training and practice in both the technical aspects of the procedure, as well as the principles and practice of aseptic technique. For an introduction to the equipment and techniques important in these procedures we recommend staff view the video manuscript recently reported by Prichett-Corning *et al.* (2011)²⁰. We also recommend all staff receive hands-on training in rodent surgical procedures and aseptic technique.

In our laboratory, these procedures are typically performed with two staff members. The surgical assistant administers anesthesia, observes animals during recovery, assists the surgeon with non-sterile aspects of the procedure and documents all procedures. The surgeon maintains the sterile field, administers analgesia and performs the surgical procedures outlined in this protocol. With proper planning and training, the surgical procedure of subcutaneous hormone pellet implantation is simple and easy to perform.

There are several experimental design considerations for cell recombinant xenografting. As with any experiments utilizing immortalized cell lines, contamination is important to minimize and detect. For experiments using BPH-1 cell lines, we recommend a low passage number (less than 25). To control for effects of the stroma, we utilize grafts containing only BPH-1 cells for one kidney and grafts containing BPH-1 and UGM on the contralateral kidney. For experiments designed to address hormonal carcinogenesis, we typically have an untreated control condition (mouse is not implanted with a pellet, or is implanted with a cholesterol pellet). Positive controls (host mouse is implanted with T+E₂ pellets) are equally important when evaluating other conditions that may promote carcinogenesis with this model system. Depending on the research question to be addressed, the duration of pellet implantation and/or xenograft growth may range from several weeks to four months; for experiments planned to exceed four months, we recommend additional pellet implantation to maintain exogenous hormone levels. For experiments involving benign

prostate tissue xenografts, it is important to wear proper personal protective equipment and maintain universal precautions for blood borne pathogens. Freshly harvested tissue can be stored prior to xenografting for 12-72 hr.

The surgical technique for renal capsule xenografting requires planning, practice and some manual dexterity. Minimizing the duration of the surgical procedure, maintaining body temperature and careful surgical technique can mitigate perioperative mortality and surgical complications. The chance of perioperative death in our experience increases with the duration of the surgical procedure; for this reason we limit the surgical procedure to no longer than 20-25 min. Use of a heating lamp and warmed wax pad during surgery and recovery is ideal for maintaining body temperature. Routine post-operative care is another important aspect of the success of this technique. Mice should be observed during anesthesia recovery, and the day following surgery for signs of pain and distress. Administer appropriate analgesia to minimize post-procedural pain and distress.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

The authors would like to thank all members of the Ricke lab, past and present. We thank Calvin Patten, Jr., DVM and Brigitte Raabe, DVM, for helpful comments regarding the protocol. We would like to acknowledge the NIDDK, NCI, and NIEHS for their financial support for these studies: R01DK093690, R01CA123199, RC2ES018764. TMN is a trainee in the Medical Scientist Training Program at the University of Rochester funded by NIH T32 GM07356; the NIH under Ruth L. Kirschstein National Research Service Award F30DK093173 also supports this project. CDV is supported by T32CA157322 and ABT is supported by T32ES007015 at the University of Wisconsin-Madison. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or NIH.

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