Video Article

Formation of Human Prostate Epithelium Using Tissue Recombination of Rodent Urogenital Sinus Mesenchyme and Human Stem Cells

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URL: http://www.jove.com/video/50327
DOI: doi:10.3791/50327

Keywords: Stem Cell Biology, Issue 76, Medicine, Biomedical Engineering, Bioengineering, Cancer Biology, Molecular Biology, Cellular Biology, Anatomy, Physiology, Surgery, Embryonic Stem Cells, ESCs, Disease Models, Animal, Cell Differentiation, Urogenital System, Prostate, Urogenital Sinus, Mesenchyme, Stem Cells, animal model

Date Published: 6/22/2013

Citation: Cai, Y., Kregel, S., Vander Griend, D.J. Formation of Human Prostate Epithelium Using Tissue Recombination of Rodent Urogenital Sinus Mesenchyme and Human Stem Cells. *J. Vis. Exp.* (76), e50327, doi:10.3791/50327 (2013).

Abstract

Progress in prostate cancer research is severely limited by the availability of human-derived and hormone-naïve model systems, which limit our ability to understand genetic and molecular events underlying prostate disease initiation. Toward developing better model systems for studying human prostate carcinogenesis, we and others have taken advantage of the unique pro-prostatic inductive potential of embryonic rodent prostate stroma, termed urogenital sinus mesenchyme (UGSM). When recombined with certain pluripotent cell populations such as embryonic stem cells, UGSM induces the formation of normal human prostate epithelia in a testosterone-dependent manner. Such a human model system can be used to investigate and experimentally test the ability of candidate prostate cancer susceptibility genes at an accelerated pace compared to typical rodent transgenic studies. Since Human embryonic stem cells (hESCs) can be genetically modified in culture using inducible gene expression or siRNA knock-down vectors prior to tissue recombination, such a model facilitates testing the functional consequences of genes, or combinations of genes, which are thought to promote or prevent carcinogenesis.

The technique of isolating pure populations of UGSM cells, however, is challenging and learning often requires someone with previous expertise to personally teach. Moreover, inoculation of cell mixtures under the renal capsule of an immunocompromised host can be technically challenging. Here we outline and illustrate proper isolation of UGSM from rodent embryos and renal capsule implantation of tissue mixtures to form human prostate epithelium. Such an approach, at its current stage, requires *in vivo* xenografting of embryonic stem cells; future applications could potentially include *in vitro* gland formation or the use of induced pluripotent stem cell populations (iPSCs).

Video Link

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Introduction

There is a tremendous need for better human model systems of prostate cancer. In particular, relevant human model systems of normal, non-malignant prostate tissues which can be genetically manipulated to directly discern the role of specific genes in the initiation of prostate cancer would be incredibly informative. The advent of the genomic era has identified numerous genes which may have a role in cancer formation. A lack of experimental human model systems, however, severely impairs our ability to functionally test and characterize candidate prostate cancer susceptibility genes. An ideal model system would facilitate the rapid and more rapid functional analyses of cancer susceptibility genes in combination with appropriate transgenic rodent model systems. Furthermore, such a human model system would enable molecular characterization of the signaling mechanisms of prostate carcinogenesis toward the discovery and validation of novel therapies to prevent prostate cancer formation.

Human embryonic stem cells (hESCs) are capable of forming human prostatic tissues as xenografts. In 2006 Taylor, *et al.* reported that hESCs can be induced to form prostatic epithelia *in vivo* when re-combined with rodent urogenital sinus mesenchyme (UGSM) within a time period of 8-12 weeks.[1] These studies were based upon previous work by the Cunha lab showing that rodent embryonic UGSM can promote prostatic differentiation of stem cells and embryonic epithelial cell populations *in vivo*.[2,3] The prostate develops from an embryonic anlagen termed the urogenital sinus (UGS), and prior to embryonic day 17 (mouse E17; day E18 in the rat) the UGS can be removed and physically divided into epithelium (UGSE) and mesenchyme (UGSM).[4] This tissue recombination approach has significantly enhanced our understanding of prostate development and carcinogenesis, particularly growth factor and hormonal signaling pathways and the molecular relationships between prostate stroma and epithelium.[5-8] This method involves the *ex vivo* combination of UGSM with stem or epithelial cells from the same or distinct species and these cellular/tissue recombinants are implanted and grown and xenografts within mouse host.[4,9] After a period of *in vivo* growth, the implant contains prostate epithelial glandular structures embedded in stromal tissue. Further staining can be conducted to determine whether such structures are truly prostatic and of human origin.[10,11]

Protocol

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the University of Chicago Institutional Animal Care and Use Committee (IACUC, protocol numbers 72066 and 72231). All surgery was performed under anesthesia, and all efforts were made to minimize suffering. The human embryonic stem cell line WA01 (H1; NIH-registration #0043) was acquired from WiCell (Madison, WI) and cultured using the feeder-independent protocol using mTeSR1 media (Stem Cell Technologies; Vancouver, B.C.). ES cells were used within ten passages of thawing.

1. Isolation of the Urogenital Sinus from Mouse or Rat Embryos

1. Euthanize a timed-pregnant mouse (day 17.5) or rat (day 18.5) by inhalation of CO_2 for 5 min. Death is confirmed by cessation of vital signs in the rat. Then break its neck humanely. The rat is euthanized at this point. Spray 70% ethanol to sterilize the abdominal skin and flanks. Cut the abdominal skin and muscle layers and then cut the uterus, separate the rat embryos from the amniotic sac, and cut the umbilical cord. Transfer the embryos into a 50 ml Falcon tube containing ice-cold Hank's Balanced Salt Solution (HBSS) and keep on ice. Fetuses are euthanized by decapitation with surgical scissors.
2. Place the embryo in 100 mm Petri dish. Bisect the embryo with scissors at the level of the umbilicus (**Figure 1A**). Remove the stomach and small intestine. Reveal the ovaries in the female embryo and testis in the male embryo (**Figure 1B** and **1C**). (The ovaries are located at the caudal poles of the kidneys. The testes are approximately at the level of the bladder). At this developmental stage, UGSM isolated from both male and female embryos are capable of inducing a prostatic epithelial lineage in the presence of host testosterone.[4] After this developmental time point, however, prostatic buds begin forming into the UGSM, and isolation of pure UGSM is very challenging.[4]
3. Under the dissection microscope, cut the abdominal wall in the middle line with Vannas scissors to expose the bladder and posterior wall of the abdomen. Free the upper genital tract from attachments (in male embryos, cut the ureter, Wolffian duct and Müllerian duct; in female embryos, cut the ureter and Müllerian duct). Free the bladder from the umbilical cord. Cut pubic bone and bluntly separate from the anterior wall of the urogenital sinus with Dumont fine tip forceps. Finally, separate the posterior wall of the urogenital sinus from the rectum. Cut the urogenital sinus with the bladder at the lower end (from the urethra) and store the urogenital sinus en bloc (with the bladder) in ice cold HBSS.

2. Separation of the Urogenital Sinus Mesenchyme

1. Remove the bladder from the urogenital sinus under the dissection microscope (**Figure 1D**).
2. Transfer the urogenital sinus to a falcon tube containing 1% trypsin in Calcium (Ca^{2+}) and Magnesium (Mg^{2+}) free HBSS. Place the tube in a refrigerator at 4 °C for 75 min.
3. Remove the trypsin and neutralize trypsinization with 10% Fetal Bovin Serum (FBS) in DMEM/F12 medium.
4. Carefully tease the sticky mesenchymal sleeve from the epithelial tube with a needle or Dumont fine tip forceps (maintaining the epithelial tube intact reduces the chances of epithelial cell contamination of the mesenchyme; this can result in rodent glands forming along with your stem cell-derived human glandular epithelium) (**Figure 1D**).[12]
5. Transfer UGSM into a new Falcon tube containing DMEM/F12 medium + 10% FBS + 1% Penicillin/Streptomycin (Pen/Strep; 0.5 mg/ml) + 1% Non-Essential Amino Acids (NEAA) plus 1nM R1881. Place the tube in an incubator at 37 °C with 5% CO_2 overnight.
6. Centrifuge the tissue at 200 x g and discard the supernatant. Wash with Phosphate Buffer Saline (PBS) (do not disturb the tissue pellet).
7. Add 0.2% collagenase (in DMEM/F12) to re-suspend the tissue. Incubate in 37 °C with gentle rocking for 1 hr.
8. Vigorously vortex the digestion tissue three times until it becomes homogenous single-cell mixture and add DMEM/F12 medium + 10% FBS + 1% P/S + 1% NEAA + 1nM R1881 to neutralize the collagenase.
9. Centrifuge at 200 x g and then wash by re-suspending the UGSM cells in HBSS, and repeat three times to fully wash the UGSM. Finally suspend mesenchymal cells in DMEM/F12 and count the cells using a hemocytometer or cell counting device.
10. Dissociate hES cells cultured using feeder-free protocol with mTeSR1 media (Stem Cell Technologies; Vancouver, B.C.) using an Accutase digestion (Millipore; Billerica, MA). Wash the hES cells during the log phase of their growth in warm DMEM/F12 media, then add warm 1x Accutase solution, and incubate at 37 °C for 15-20 min until single cells are uniformly dissociated from colonies. Add an equal amount of 1x defined Trypsin Inhibitor (Invitrogen; Grand Island, NY) to neutralize the Accutase and gently pipette up and down to mix and break up cell clumps. Pipette cells into a centrifuge tube and centrifuge for 3 min at 200 x g, and re-suspend cell pellet in DMEM/F12 media. Centrifuge tube for 3 min at 200 x g again and remove the supernatant, then re-suspend in cold HBSS or DMEM/F12 at desired volume.
11. Add hES cells with 1:2.5 ratio of hES/mesenchymal cells.[3] This will be a cellular composition of $1E^5$ UGSM cells with $4E^4$ hESCs per 10 ml injection (a single rat UGS typically yields about $2E^6$ mesenchymal cells). Centrifuge at 200 x g for 5 min and then discard the supernatant. Re-suspend with 75% Matrigel (BD Biosciences, San Jose, CA; mixed with 25% cold HBSS for easier handling) and place the tube in ice for sub-renal capsule injection.

3. Transplantation of Tissue Recombinant Underneath the Renal Capsule

1. Prepare a sterile surgical area.
2. Anesthetize a male athymic nude mouse with ketamine/xylazine i.p.; use a fresh mix containing a 1:1:8 ratio of ketamine:xylazine:saline. Dosing will be 100 mg/kg Ketamine and 2.5 mg/kg Xylazine. Animals unresponsive to a toe-pinch should be fully under for 20-30 min.
3. Surgically prepare the mouse by shaving the surgical site (if not using a nude mouse), cleansing with three alternating wipes of 70% alcohol followed by betadine solution and applying a surgical drape.
4. Put eye moisture salve (Altalube eye ointment) to the eyes of the mouse to prevent drying during anesthesia.
5. During surgery, respiration rate and core body temperature of the mice are monitored. The respiration rates should be steady and maintained within 40-90 breaths/min. Core body temperature is monitored with observation of the color of the mucous membranes and the pink soles of the feet.

6. Make a 1.0 cm long longitudinal skin incision on the back. Continue to cut the abdominal wall in a 0.5 cm longitudinal incision.
7. Gently squeeze the kidney out of the abdomen and keep the kidney surface moist using drops of sterile saline.
8. Gently puncture the renal capsule using an empty 27-gauge syringe needle. A very shallow puncture is sufficient. This will be where you insert your blunt needle to inject your cellular mixture.
9. Fill a 28-gauge blunt syringe needle with 10 µl of your cell mixture. Slowly insert the puncture site and penetrate the kidney parenchyma to reach an area underneath the renal capsule. Inject 10 µl of the cellular mixture to form a bolus on the kidney underneath the renal capsule. Withdrawal the needle.
10. Return the kidney to the abdominal cavity. Secure the muscular layer of the peritoneum with 4-0 nylon sutures.
11. Close the skin with either suture or staple.
12. Clean blood from the skin using sterile saline.

4. Post-operative Analgesia and Monitoring

1. Inject the animal with buprenorphine (0.1 mg/kg every 12 hr) or ketoprofen (3-5 mg/kg in saline every 12 hr) to manage post-operative pain, and 1 ml of 37 °C saline i.p. to keep the animal warm and prevent dehydration.
2. Put the animal in a clean cage and place it on a mild heating pad.
3. Send the animals back to animal room once they regain consciousness and are moving within the cage.
4. Observe the animal daily for unexpected signs of illness and infection for the following three days. The mice are monitored with observation of activities in cages including accessing food and water 1 day post-operatively. If the mice display fewer activities, Ketoprofen is intraperitoneally administered again once a day.
5. Remove the skin staples or sutures 2 weeks post-surgery.
6. Xenograft tissues can be harvested as early as 8 weeks post-surgery. Xenografts can be imaged *in vivo* using small-animal ultrasound (**Figure 2A**), and fixed tissues can be sectioned and stained for various proteins using immunohistochemistry (**Figure 3**).

Representative Results

Building on the exciting report by Taylor, *et al.*, our lab has developed an engrafting protocol using the commonly used H1 (NIH-designated WA01, genetically male) human embryonic stem cell line.[1] This line has been rigorously tested for quality control and is karyotypically normal.[13] When cultured appropriately, hES cells can be maintained, expanded, and cryopreserved in an undifferentiated and pluripotent state using a feeder-free culture method (feeder-free systems commercially available via Stem Cell Technologies; Vancouver B.C.).[14] Our protocol employs single cell suspensions of hESCs combined with single cell suspensions of E18 rat UGSM and injected under the renal capsule of adult male immunocompromised mice. As important controls, USGM implanted alone yields no discernible growth, while hESCs implanted without UGSM form large teratomas (**Figure 2B**).[15] In our experience, implantation of UGSM without contaminating rat epithelial cells never results in tissue growth, while recombination of UGSM plus hESCs results in robust growth over 80% of the time (after 8 weeks sizes range from 1 mm to 5 mm, with over 80% of the tissue containing glandular epithelium). In recombinants containing both hESCs and UGSM, early glandular formation is observed after 4 weeks, and after 8 weeks fully formed, prostate specific antigen (PSA)-positive human prostate epithelium is formed (**Figure 3**). These glands contain Androgen Receptor (AR)-positive luminal-secretory cells which are not present one week after host castration. Importantly, these glands are positive for the human-specific and prostate-specific protein PSA. Further staining documents that such human glands appear to be non-malignant since they do not express Alpha-methylacyl-CoA racemase (AMACR), which is a prostate cancer-specific marker.[16]

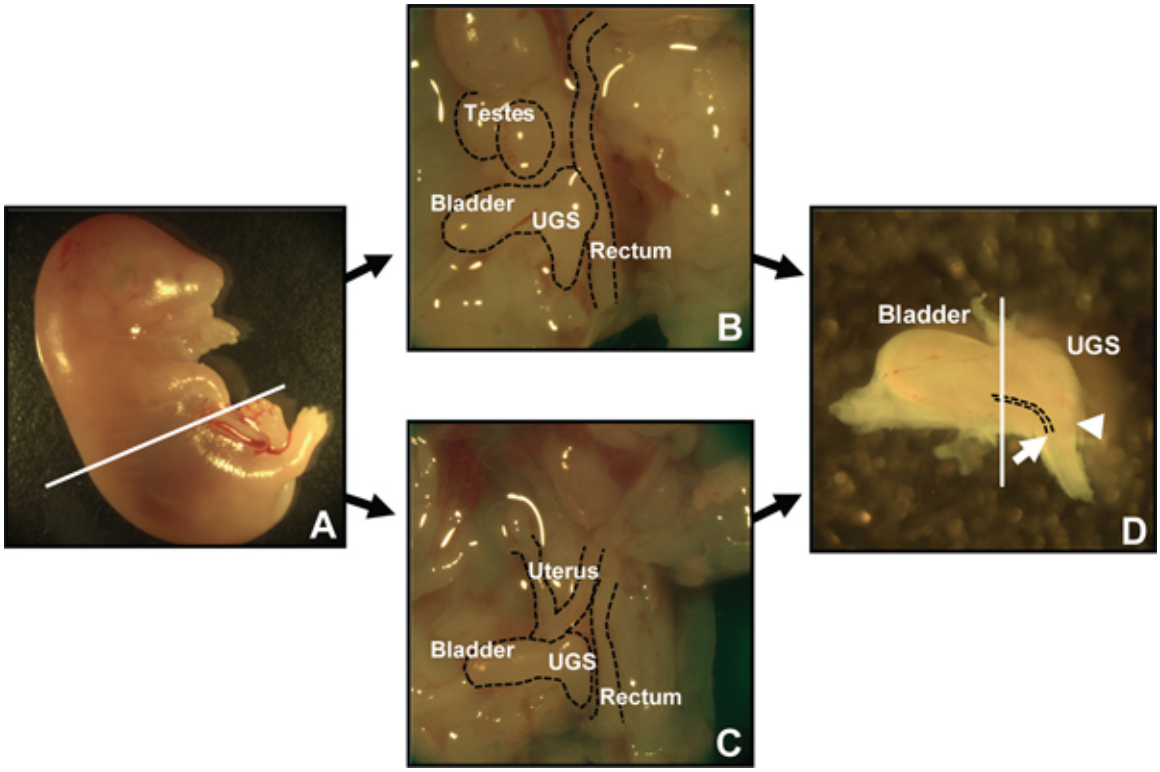

Figure 1. Isolation of the Urogenital Sinus from an E18.5 Rat Embryo. A. E18.5 rat embryo. The white solid line indicates the position to bisect the embryo. **B.** E18.5 male rat embryo. The black dotted lines indicate the bladder, urogenital sinus, rectum, and developing testes. **C.** E18.5 female rat embryo. The black dotted lines indicate the bladder, urogenital sinus and uterus. **D.** The bladder and urogenital sinus removed from the E18.5 rat embryo. The while solid line indicates the position to remove the bladder. The black dotted line and white arrow indicate the epithelial tube. The white arrow head indicates the urogenital sinus mesenchyme.

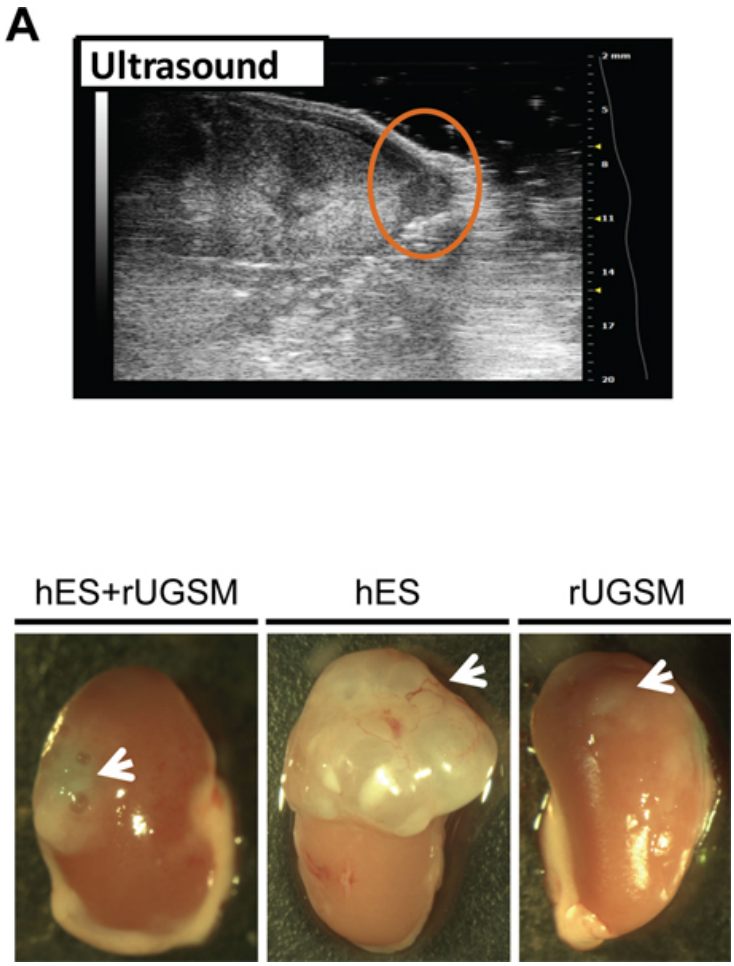

Figure 2. Ultrasound imaging and gross morphology of *in vivo* tissue xenografts derived from UGSM and hESCs. A. Ultrasound imaging of growing xenografts allow live-animal analyses during the course of the experiment. Image was captured 8 weeks post-surgery using a Vevo 2100 small animal ultrasound (Visualsonics; Toronto, ON). Circled area represents the growing tissue xenograft on the kidney surface. **B.** At the experimental endpoint, hES cells combined with rat UGSM (rUGSM) form a visible tissue mass on the kidney surface (*left panel*); hES cells implanted alone form large teratomas as expected (*middle panel*); and UGSM implanted alone fails to form any discernible structure (*right panel*).

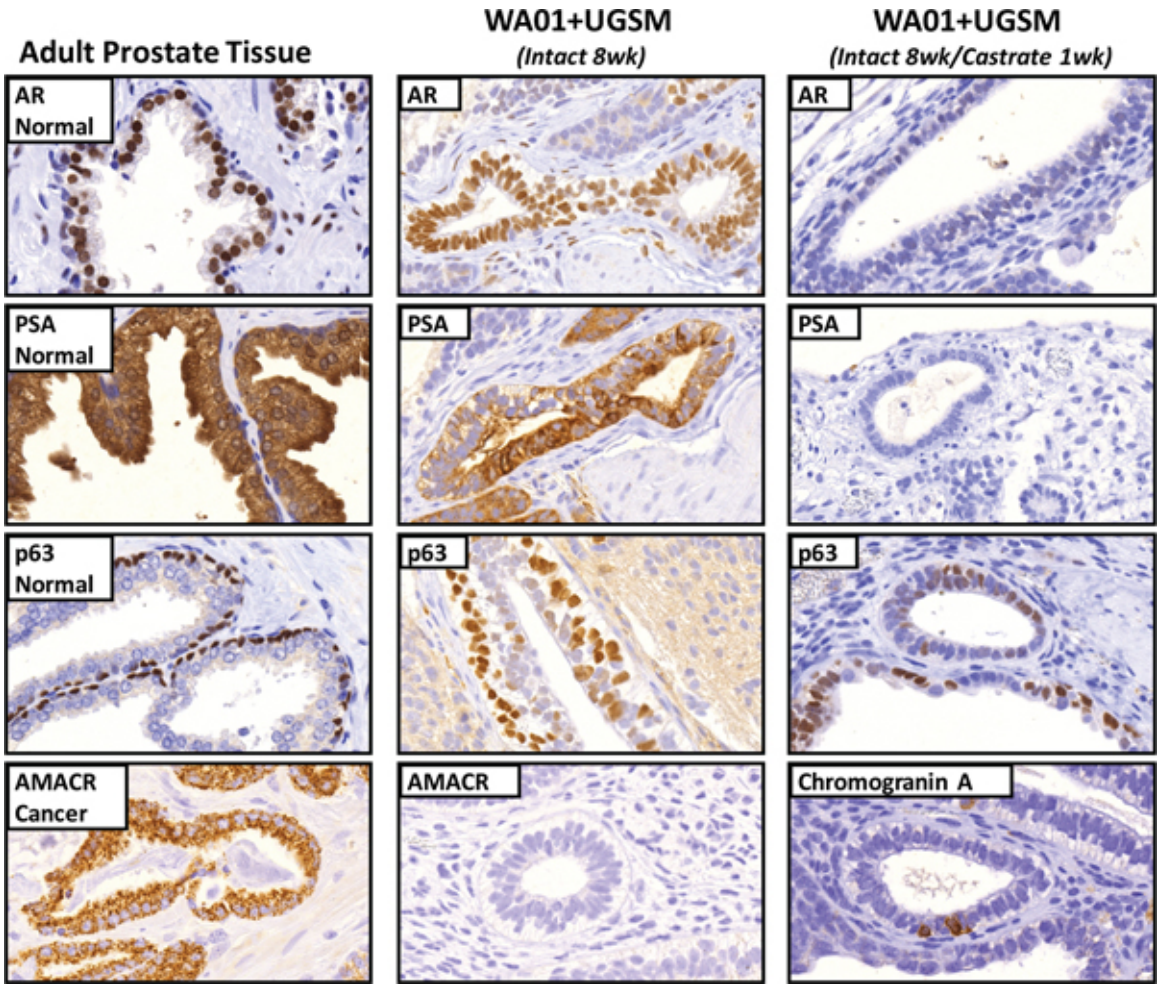

Figure 3. Formation of Human Prostate Epithelia from the Human Embryonic Stem Cell Line WA01(H1). ES cells were recombined with rodent UGSM and implanted under the renal capsule of intact male nude mice. After a growth period of 8 weeks, human prostate glandular epithelium is formed as documented by AR, p63, and human-restricted PSA staining. One week following host castration, the AR-positive, PSA-positive luminal layer is not present, documenting characteristic androgen-dependency. Prostate tissues are non-malignant as demonstrated by a lack of AMACR staining. In addition, ChrA-positive neuroendocrine cells were detectable. Non-malignant human prostate tissue was used as a control for AR, PSA, and p63; a prostate tumor was used as a positive control for cancer-specific AMACR.

Discussion

Tissue recombination using UGSM is an incredibly useful technique to investigate the development of the prostate and the molecular events leading to prostate cancer initiation. The inductive potential of UGSM has been used for numerous applications in prostate research; these include enhancing tumor take of prostate cell lines and tumors, studying stromal-epithelial interactions, and forming cross-species prostate recombinants.[7,17-20] Proper preparation of UGSM, however, is critical to experimental success as contaminating rodent epithelium will result in glandular formation and can confound results. Thus, the separation of the UGSM from the UGSE (Step 2.4) is by far the most critical and also most tricky step in the protocol. To control for such contamination, the use of techniques to discern between species is strongly encouraged, as well as an additional experimental arm using UGSM alone to document no glandular formation from cellular contaminants.[12]

The recent development of feeder-free hES culturing reagents and methods allow pure populations hES cells to be cultured, expanded and cryopreserved.[14] Moreover, the expanded use of lentiviral gene delivery allows the stable genomic incorporation and expression of genes of interest.[21,22] These combined advances allow for the genetic manipulation of pure hES cultures and, when combined with the inductive potential of prostatic embryonic UGSM, will permit the *in vivo* generation of human prostatic glands expressing specific genes of interest. Moreover, this technique would be amenable to induced-pluripotent stem cell lines (iPSCs) derived from adult hosts and from genetically-modified cells.[23,24] Using defined proteins, we show that such hESC-derived human prostate epithelium appear very similar to adult human prostate epithelia, but at a global molecular level there is sure to be some differences in gene expression which should be taken into consideration. To account for this, investigators should use an expanded sample size and consider laser-capture microdissection (LCM)-based approaches and comparative gene expression analyses platforms to validate the molecular profiles of their tissues compared to adult human tissues. Nonetheless, the formation of human prostate glandular epithelium using a serially-cultured and feeder-free stem cell line has the potential to facilitate numerous molecular studies pertaining to prostate function and cancer initiation.

Our technique utilizes single cell suspensions of both UGSM and hESCs, which enables the use of lentiviral infection and flow sorting approaches, as well as more accurate control of cellular quantities. This can, however, diminish the inductive potential of UGSM. An alternate approach has been to use non-dissociated UGSM (after Step 2.5), before collagenase digestion) and either direct incubation with ES cells, or embedding cells within a collagen solution.[4] To implant these tissues under the renal capsule, an alternate technique would be to make an incision in the renal capsule, create a small pocket under the renal capsule on top of the kidney, and physically place the cell mass within that pocket.[4,20]

Disclosures

The authors have no conflict of interest with the work presented.

Acknowledgements

We wish to acknowledge the support of the University Of Chicago Section Of Urology led by Dr. Arieh Shalhav, and the Director of Urologic Research Dr. Carrie Rinker-Schaeffer. We would also like to acknowledge the support of the University of Chicago Comprehensive Cancer Center (UCCCC) led by Dr. Michelle Le Beau. We also with to thank expert technical assistance of the Human Tissue Resource Center core facility led by Dr. Mark Lingen, and the assistance of Leslie Martin and Mary Jo Fekete. We also thank the Immunohistochemistry Core Facility run by Terri Li. This work was funded by the University of Chicago Department of Surgery, the Section of Urology; an American Cancer Society Institutional Research Grant (ACS-IRG, #IRG-58-004); a Cancer Center Support Grant (P30 CA14599); The Brinson Foundation; the Alvin Baum Family Fund; The University of Chicago Cancer Research Foundation Women's Board; S. Kregel is supported by an HHMI: Med-into-Grad Fellowship (56006772) and a Cancer Biology Training Grant (T32-CA09594). Finally, we would like to thank Robert Clark, Dr. VenkateshKrishnan, and Nathan Stadick for their critical evaluation of the manuscript.

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