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Original Article

Genetic background but not prostatic epithelial beta-catenin influences susceptibility of male mice to testosterone and estradiol-induced urinary dysfunction

Kyle A Wegner[1,2,3*], Hannah Ruetten[1,2*], Nicholas M Girardi[1,2*], Chelsea A O'Driscoll[1,2*], Jaskiran K Sandhu[1,2], Anne E Turco[1,2,3], Lisa L Abler[1,2], Peiqing Wang[1,2], Zunyi Wang[1,2], Dale E Bjorling[1,2], Rita Malinowski[1,4], Richard E Peterson[4], Douglas W Strand[1,5], Paul C Marker[1,4], Chad M Vezina[1,2,3]

[1]University of Wisconsin-Madison/UMASS Boston/UT-Southwestern George M. O'Brien Center for Benign Urologic Research, Madison, Wisconsin 53706, USA; [2]School of Veterinary Medicine, University of Wisconsin-Madison, Madison, Wisconsin 53706, USA; [3]Molecular and Environmental Toxicology Center, University of Wisconsin-Madison, Madison, Wisconsin 53706, USA; [4]School of Pharmacy, University of Wisconsin-Madison, Madison, WI, USA; [5]Department of Urology, University of Texas Southwestern Medical Center, Dallas, Texas, USA. [*]Equal contributors.

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Abstract: Urinary voiding dysfunction in aging men can cause bothersome symptoms and irreparable tissue damage. Underlying mechanisms are not fully known. We previously demonstrated that subcutaneous, slow-release testosterone and estradiol implants (T+E2) drive a pattern of urinary voiding dysfunction in male mice that resembles that of aging men. The initial goal of this study was to test the hypothesis that prostatic epithelial beta-catenin (*Ctnnb1*) is required for T+E2-mediated voiding dysfunction. Targeted *Ctnnb1* deletion did not significantly change voiding function in control or T+E2 treated mice but led to the surprising discovery that the C57BL/6J × FVB/NJ × 129S1 mixed genetic background onto which *Ctnnb1* loss of function alleles were maintained is profoundly susceptible to voiding dysfunction. The mixed background mice develop a more rapid T+E2-mediated increase in spontaneous urine spotting, are more impaired in ability to initiate bladder contraction, and develop larger and heavier bladders than T+E2 treated C57BL/6J pure bred mice. To better understand mechanisms, we separately evaluated contributions of T and E2 and found that E2 mediates voiding dysfunction. Our findings that genetic factors serve as modifiers of responsiveness to T and E2 demonstrate the need to control for genetic background in studies of male voiding dysfunction. We also show that genetic factors could control severity of voiding dysfunction. We demonstrate the importance of E2 as a key mediator of voiding impairment, and show that the concentration of E2 in subcutaneous implants determines the severity of voiding dysfunction in mice, demonstrating that the mouse model is tunable, a factor which is important for future pharmacological intervention studies.

Keywords: Bladder outlet obstruction, testosterone, estradiol, beta-catenin, voiding dysfunction

Introduction

Most aging men are bothered by urinary voiding symptoms such as incomplete bladder emptying and frequent urination, especially at night. These symptoms often signify voiding dysfunction, which can worsen with age. If left untreated, urinary voiding dysfunction can cause irreparable bladder and kidney damage [1]. Concentrations of circulating estradiol (E2) and testosterone (T) change with age [2] and these changes have also been implicated in male urinary voiding dysfunction [3-6]. Mice treated with subcutaneous, slow release T+E2 implants, to mimic the hormonal milieu in aging human males, develop progressive voiding dysfunction [7]. T+E2 implants drive prostate cell proliferation and prostatic enlargement, increase spontaneous voiding frequency while also reducing volume per void, promote urinary retention, drive bladder hypertrophy (consistent with bladder outlet obstruction) and cause renal damage [7-9].

The initial goal of this study was to test the requirement for prostatic epithelial beta-catenin (CTNNB1) in T+E2-mediated voiding dysfunction in male mice. The rationale is based on

previous observations that CTNNB1 pathway members are present and more abundant in male versus female pelvic urethra during the period of prostatic development in mice [10]. It has been proposed that signaling pathways mediating prostate development are reawakened to cause prostatic hyperplasia in aging men [11]. Prostatic epithelial expression of a *Ctnnb1* gain-of-function (GOF) allele can also drive prostatic epithelial cell proliferation and expression *Wnt10b*, a putative mediator of prostate epithelial cell stemness [12-15]. Nuclear localized (transcriptionally active) CTNNB1 is more abundant in prostatic epithelial and stromal cells of men with clinically significant non-malignant prostatic disease compared to those of controls [16].

We assessed the requirement for prostatic epithelial *Ctnnb1* in T+E2 induced voiding dysfunction by breeding mice with a knock-in *cre* recombinase allele in the probasin locus (driving expression in prostate luminal epithelial cells) to mice that express a *Ctnnb1* conditional loss of function allele. The resulting mice had reduced expression of CTNNB1 in prostatic luminal epithelial cells and were on a mixed genetic background (C57BL/6J × FVB/NJ × 129S1). There was no significant voiding function difference between *Ctnnb1* conditional loss of function and control mice. However, upon treatment with T+E2, mice maintained on the mixed genetic background had a more rapid progression of voiding dysfunction and bladder decompensation than C57BL/6J purebred mice. We titrated T and E2 concentrations to determine the culpable hormone for voiding dysfunction. We identify E2 as the key hormone mediating T+E2 induced voiding dysfunction. We demonstrate a need to control for genetic background in studies of male urinary voiding dysfunction and suggest that FVB/NJ and/or 129S1 strains harbor alleles that sensitize to T+E2 mediated voiding dysfunction.

Materials and methods

Mice

All procedures were approved by the University of Wisconsin Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were acquired from Jackson Laboratories (Bar Harbor, ME) and included: C57BL/6J pure bred mice (Jackson Laboratories stock number 000664), Tg(Pbsn-cre)4Prb/J (*Pbsn-cre*, Jackson Laboratories stock number 026662) [17], and *B6.129-Ctnnb1*$^{tm2Kem/KnwJ}$ (*Ctnnb1*$^{fl/fl}$ mice, Jackson Laboratories stock number 004152) [18]. The *Pbsn-cre* and *Ctnnb1*$^{fl/fl}$ alleles were maintained on a mixed genetic background consisting of C57BL/6J × FVB/NJ × 129S1. Mouse genetic backgrounds for each experiment are listed in the figure legends. Genotyping for *Pbsn-cre*, and *Ctnnb1*$^{fl/fl}$ alleles was conducted as described by Jackson Laboratories. The genotype of mice described as "*Ctnnb1* LOF" is *Pbsn-cre/+; Ctnnb1*$^{fl/lf}$. The genotype of mice described as "*Ctnnb1* control" is *Ctnnb1*$^{fl/lf}$.

Mice were housed in Udel® Polysulfone microisolator cages; the room was on 12-h light and dark cycles; room temperature was 20.5 ± 5°C; humidity was 30-70%. Mice were fed a 5015 Diet (PMI Nutrition International, Brentwood MO) from conception through weaning (PND 21) and an 8604 Teklad Rodent Diet thereafter (Harlan Laboratories, Madison WI) post weaning. Feed and water were available *ad libitum*, and cages contained corn cob bedding. Six-week-old C57Bl6/J male mice were given subcutaneous hormone implants (25 mg testosterone and 2.5 mg 17-β estradiol unless otherwise stated in the figure legend) or a sham surgery (the experimental control group, labeled as 'sham' in the figure legend) as described previously [7, 9] and then individually housed to prevent fighting.

Urinary function testing

Void spot assay (VSA) and cystometry were performed and analyzed as previously described [19]. We followed the recommended guidelines of reporting VSA data [20-22]. VSA was performed in the vivarium where mice were housed. Whatman grade 540 (Fisher Scientific number 057163-W) filter papers (27 × 16 cm) were placed in the bottom of Udel® Polysulfone microisolator cages. Mice were placed in the cage (singly housed) with food *ad libitum* but no water for four hours starting from 8-12 AM GMT. Filter papers were dried and imaged with an Autochemi AC1 Darkroom ultraviolet imaging cabinet (UVP, Upland, CA) equipped with an Auto Chemi Zoom lens 2UV and an epi-illuminator. Image capture settings were adjusted using UVP VisonWorksLS image acquisition software. Images were captured using an Ethidium Bromide filter set (570-640 nm) and 365 nm epi-

illumination. Void Whizzard was downloaded from http://imagej.net/Void_Whizzard and run according to the user guide [21] to determine total spot count. Statistical analyses for this and other methods were conducted with Statistical analyses were performed with Graph Pad Prism 8.0.2. A two-way ANOVA was used to determine if total spot count changed over time or between groups. Multiple comparisons were performed to compare timepoints within each treatment group (simple effects within a given treatment group). A $P < 0.05$ was considered statistically significant.

Cystometry was performed with minimal alterations to previously published protocols and following best practices [23, 24]. Mice were anesthetized with urethane (1.43 g/kg *s.c.*). Thirty minutes after urethane dosing, an incision was made in the ventral abdomen to expose the bladder. Bladder length and diameter were measured for volume calculation. A purse-string suture was placed in the bladder dome. Polyethylene cystostomy tubing (PE50, outer diameter 0.58 mm, inner diameter 0.28 mm) was inserted into the bladder through the center of the suture and purse-string secured to hold the tubing in place with 2-3 mm of tubing within the bladder. The abdominal wall and skin were closed separately in a simple interrupted pattern. The exterior tubing was secured to the ventral abdominal skin with two simple interrupted sutures. Mice were placed on a heat pad for one hour after the procedure. The exposed cystostomy tube was connected to a three-way stopcock, and the other two arms of the stopcock were connected to an infusion pump (Harvard Apparatus, Holliston, MA) and pressure transducer (Memscap AS, Norway). Intravesical pressure was recorded continuously using a PowerLab data collection system (ADI Instruments, Colorado Springs, CO). Room-temperature sterile saline (0.9%) was infused into the bladder at a rate of 1.5 mL per hour. Mice were placed in lateral recumbency above a force transducer (Model FT03, grass Instruments) attached to a 3D printed urine collection funnel. The force transducer was calibrated with known volumes of saline to create a weight-volume conversion. The mass of voided urine was recorded continuously using PowerLab. At least one hour of voiding activity was recorded. Three to five consecutive voids, occurring after stabilization of micturition cycles, were used for analyses. Whether or not a mouse was able to initiate a void during the one-hour period was noted. Percentage of mice unable to initiate a void was charted for each timepoint and compared between groups.

Gross bladder measurements

All mice were euthanized by CO_2 asphyxiation. Bladder length, width, and height were measured prior to lower urinary tract collection or prior to cystometry surgery and used for bladder volume calculation. Lower urinary tracts were removed and placed in phosphate buffered saline. Bladders were removed, their contents were emptied, and bladders were dried on weigh paper prior to bladder mass measurement. Bladder volume and weight were normalized to mouse body weight. The Shapiro-Wilk test was used to test for normality and transformation was applied to normalize data when necessary. For comparisons of two groups, the F-test was used to test for homogeneity of variance. Welsh's correction was applied when variance was unequal. When variance was equal, comparisons between two groups were made using Student's t-test. If data could not be normalized through transformation, the Mann Whitney test was applied. For comparisons of multiple groups, Bartlett's test was used to test for homogeneity of variance. Welsh's ANOVA was applied when variance was unequal followed by Tamhane's T2 multiple comparisons test. When variance was equal, comparisons between groups were made using ordinary one-way ANOVA followed by Sidak's multiple comparisons test. If data could not be normalized through transformation the Kruskal-Wallis test was applied with Dunn's multiple comparisons test. A $P < 0.05$ was considered statistically significant.

Immunohistochemistry and analysis

Formalin fixed, paraffin embedded dorsal prostate tissue sections (5 μm) were prepared as described previously [25]. Epitope recovery was conducted in microwave-heated 10 mM sodium citrate (pH 6.0) and non-specific sites were blocked with Tris Buffered Saline containing 1% Tween-20, 1% Blocking Reagent (Roche Diagnostics, Indianapolis, IN), 5% normal goat or donkey sera and 1% bovine serum albumin. Primary antibodies included: anti-E-cadherin (ECAD or CDH1, Cell Signaling, 3195S, RRID: AB_2291471, diluted 1:200), and anti-Beta Catenin (CTNNB1, BD Transduction Labs, 610153, RRID: AB_397554, diluted 1:100). For

confocal imaging, secondary antibodies included: Anti-Rabbit 488 (Jackson ImmunoResearch, 111-487-003, RRID: AB_2338046, 1:250), and Anti-Mouse 594 (Jackson ImmunoResearch, 111-585-144, RRID: AB_2307325, 1:250). For compound microscope imaging, secondary antibodies included: Anti-rabbit 647 (Jackson ImmunoResearch, 711-605-152, RRID: AB_2492288, 1:250) and Anti-Mouse Rhodamine Red™-X (Jackson ImmunoResearch, 715-295-151, RRID: AB_2340832, 1:250). DAPI (2-(4-amidinophenyl)-1H-indole-6-carboxamidine, 3:1000) was used to stain nuclei.

Stained tissue sections were imaged either using an SP8 Confocal Microscope (Leica; Wetzlar, Germany) using a 20x oil immersion objective (HC PL Apo CS2 NA = 0.75; Leica) or an Eclipse E600 compound microscope (Nikon Instruments Inc., Melville, NY) fitted with a 20X dry objective (Plan Fluor NA = 0.75; Nikon, Melville, NY) and equipped with a CoolSNAP Dyno CCD camera (Teledyne Photometrics, and NIS elements imaging software (Nikon Instruments Inc.). Fluorophores were detected using the recommended settings for each secondary antibody fluorophore. Multiple non-overlapping images were taken per section to capture minimum 400 epithelial cells per prostate tissue section.

A minimum of 400 epithelial cells were manually counted per hemi lateral prostate lobe section using the ImageJ cell counter macro. A hemi lateral prostate lobe was used as the statistical unit. We assessed 4 hemi lobes obtained from 3-4 mice per treatment group. Shapiro-Wilk's test was used to test for normal distribution, data was normalized using Log transformation, variances were compared using the F test and were not significantly different. Upon meeting all criteria, a two-tailed Student's t-test was applied. A p-value < 0.05 was considered significant.

Results

Ctnnb1 LOF mouse prostates have fewer CTNNB1 protein positive epithelial cells than control mice

We showed previously that nuclear localized (active) CTNNB1 is more abundant in prostate cells of men with clinically significant non-malignant prostatic disease compared to men who had prostatectomy for a different reason (prostate cancer) [16]. Here, we use a genetic approach to test the requirement of epithelial CTNNB1 in mediating prostate related voiding dysfunction in mice. We used the Tg(Pbsn-cre)4Prb transgene, which contains androgen receptor binding sequences from the probasin gene promoter [26], to drive *cre* expression and excise a floxed *Ctnnb1* allele [18] in prostate luminal epithelial cells, creating prostate epithelial cell selective *Ctnnb1* loss of function mutants (*Ctnnb1 LOF*). Mice harboring two copies of the floxed *Ctnnb1* allele and absent of *Pbsn-cre4Prb* transgene were used as controls (*Ctnnb1* control*)*. We used immunostaining to verify CTNNB1 deletion in cadherin 1 (CDH1)-positive epithelial cells. *Ctnnb1 LOF* mouse prostates have significantly fewer CTNNB1 immunopositive epithelial cells than *Ctnnb1* control mice (P = 0.0239) but in histological appearance are otherwise like control mice (**Figure 1**).

Prostatic luminal epithelial cell Ctnnb1 is not required for testosterone and estradiol (T+E2)-mediated urinary voiding dysfunction in male mice

Six-week-old *Ctnnb1 LOF* and *Ctnnb1* control mice were given slow release subcutaneous T+E2 implants to drive voiding dysfunction. The void spot assay (VSA) was used to quantify voiding behavior in a non-invasive manner. Void spot quantity increases in both genotypes as early as two weeks post T+E2 implant and remains increased through at least six weeks post-implant, the terminal endpoint of this study (**Figure 2A**). However, urine spot quantity does not differ between *Ctnnb1 LOF* and controls at any assessment period up to 6 weeks post-pellet implant (**Figure 2A**). Previous studies reported that T+E2 treatment increases bladder volume which is consistent urinary retention [7]. We measured bladder volume at 6 weeks post T+E2 implant and it does not significantly differ between *Ctnnb1 LOF* and controls (P = 0.7195, **Figure 2B**). We conclude from these experiments that *Ctnnb1* in prostatic luminal epithelial cells is not required for T+E2 mediated voiding dysfunction.

Testosterone and estradiol (T+E2) mediated voiding dysfunction escalates more quickly and is more severe in C57BL/6J × FVB/NJ × 129S1 (mixed background) than C57BL/6J pure background mice

Alleles for making *Ctnnb1 LOF* mice were separately maintained on FVB/NJ and 129S1 x

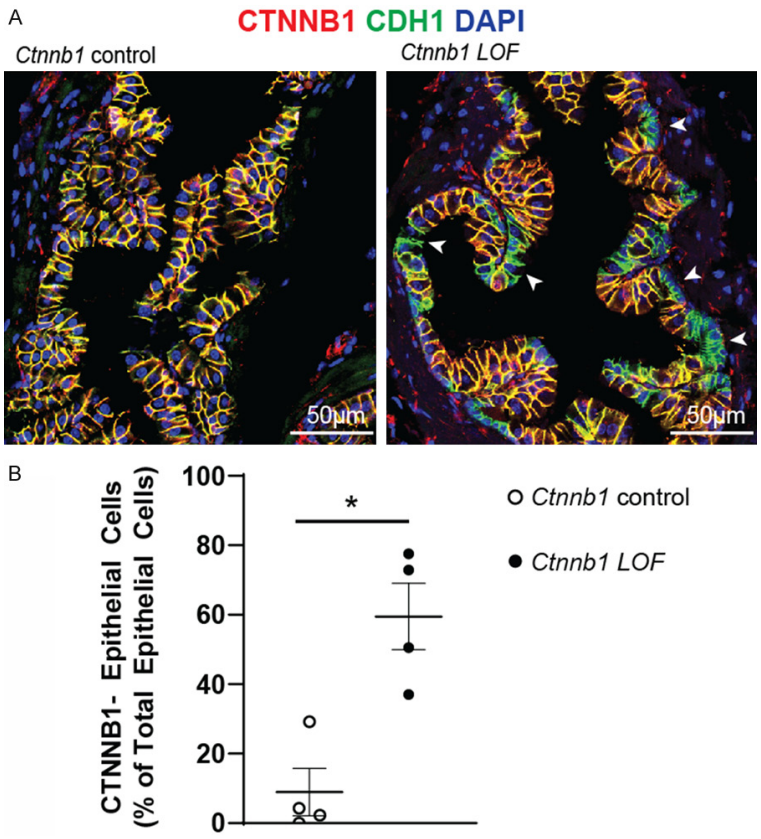

Figure 1. *Ctnnb1* LOF mouse prostates have fewer CTNNB1 protein positive epithelial cells than control mice. Lateral prostate sections from six week old *Ctnnb1 LOF* and control male mice on a C57BL/6J × FVB/NJ × 129S1 (mixed) background were immunostained with antibodies against Beta-Catenin (CTNNB1, red) and E-cadherin 1 (CDH1, green) to (A) visualize and (B) quantify CTNNB1 immunonegative cells. Arrows point to CTNNB1 immunonegative epithelial cells. *Ctnnb1 LOF* mice had a greater percentage of CTNNB1 immunonegative epithelial cells compared to *Ctnnb1 WT* control mice (P = 0.0239, mean ± SE, n = 4 mice per group).

C57BL6/J backgrounds. When combined to make *Ctnnb1 LOF* and *Ctnnb1* control mice, the resulting genetic background is C57BL/6J × FVB/NJ × 129S1, (referred to hereafter as mixed background mice). While testing the requirement for *Ctnnb1*, we observed a more rapid progression of T+E2 mediated voiding dysfunction in the mixed background mice than we have in previous studies with C57BL/6J pure background mice [9, 19]. We evaluated bladder weight and volume at two-weeks post-implantation surgery in mixed strain mice to test for anatomical evidence of voiding dysfunction. T+E2 treated mixed background mouse bladders were filled with urine at the time of necropsy, were significantly larger than sham operated mixed background mice at the same time point, and when drained of urine weighed more than that of sham operated mixed background mice (**Figure 3A**, **3B**).

Because genetic background was recently identified as a modifier of spontaneous voiding activity in male and female mice [24], we next tested whether T+E2 drives urinary dysfunction more rapidly in mixed background than C57BL/6J pure background mice. Mice were given sham surgeries or T+E2 implants and voiding function was measured up to six weeks later. T+E2 implants increased voiding frequency over sham operated controls in mice from all genetic backgrounds. However, urine spotting increases more quickly from baseline (as early as two weeks post implantation surgery) in T+E2 mixed background mice compared to C57BL/6J pure background mice (**Figure 3C**). We used cystometry for longitudinal assessment of bladder pressures in response to filling and emptying. We previously reported that four weeks of T+E2 treatment decreases the percentage of C57BL/6J pure background mice that initiate a voiding contraction during a one hour monitoring period of anesthetized cystometry [9]. We show here that T+E2 treatment more profoundly impairs voiding in mixed background mice compared to C57BL/6J pure background mice. In fact, four weeks of T+E2 treatment is sufficient to eliminate voiding contractions in 75% of mixed background mice and six weeks of treatment eliminates voiding contraction in all mixed background mice during a one hour monitoring period (**Figure 3D**). From these results we conclude that genetic factors influence the rate of urinary voiding dysfunction caused by T+E2 treatment and that C57BL/6J × FVB/NJ × 129S1 mixed background males are more susceptible than purebred C57BL/6J males to hormone driven voiding dysfunction. We also confirm increased bladder mass and volume as

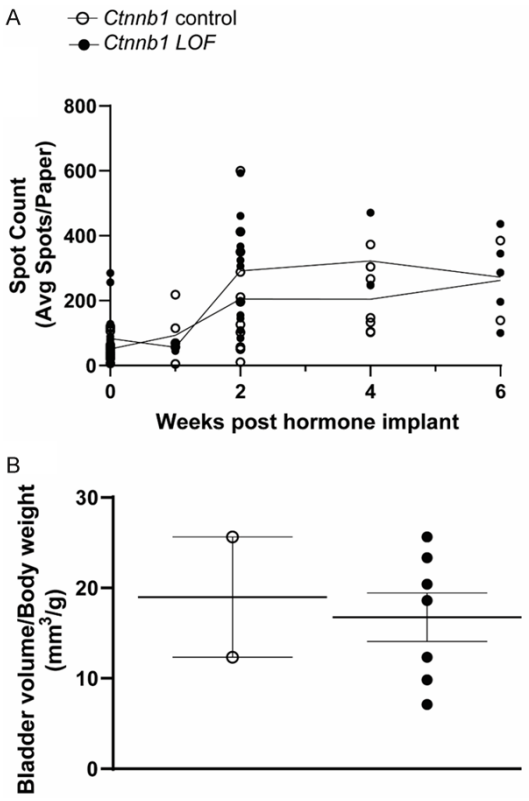

Figure 2. Prostatic luminal epithelial cell *Ctnnb1* is not required for testosterone and estradiol (T+E2)-mediated urinary voiding dysfunction in male mice. Mice were given subcutaneous implants of testosterone (25 mg) and estradiol (2.5 mg) (T+E2) or were sham operated (anesthesia and subcutaneous incision but not hormone implant). A. Spontaneous voiding behavior was examined 1, 2, 4, and 6 weeks post T+E2 implantation surgery (2-17 mice per group per timepoint, using the void spot assay and involving four continuous hours of testing). Urine spot count significantly increased from baseline as early as two weeks post T+E2 implantation surgery in the *Ctnnb1 LOF* ($P < 0.0001$ compared to baseline) and *Ctnnb1* control groups ($P = 0.0059$ compared to baseline) and there were no significant differences between genotypes at any of the time points examined. B. Bladder volume was measured six weeks after hormone implant as an index of urinary retention. There was no difference in bladder volume between genotypes ($P = 0.7195$, mean ± SE, 2-7 mice per group).

sensitive readouts of T+E2-mediated voiding dysfunction.

E2I increases bladder mass and T and E2 in combination increase bladder volume in a dose dependent fashion

We used pure C57BL6/J background mice to test whether voiding dysfunction is mediated by T, E2, or if both hormones are required. Mice were given a T implant (25 mg), an E2 implant (2.5 mg), or the standard T+E2 implants (25 mg + 2.5 mg, respectively) and compared to a sham operated control. Bladder mass increases with the standard implant ($P < 0.0001$) and the 2.5 mg E2 alone implant ($P = 0.0010$) compared to sham operated control (**Figure 4A**). Bladder volume increases in all three implant groups (T+E2, $P < 0.0001$; T, $P = 0.0285$; E2, $P = 0.0026$) compared to the sham operated control group (**Figure 4B**). Because E2 alone is sufficient to increase bladder mass, we implanted mice with E2 (2.5 mg) alone or T (25 mg) plus a second implant with graded amounts of E2 (0.25 µg to 2.5 mg). Implants of E2 alone and implants containing T (25 mg) plus a minimum of 1.25 mg E2 were sufficient to increase bladder mass compared to the sham operated control group ($p = 0.0002$, E2 2.5 mg; $P < 0.0001$, T + E2 2.5 mg; $P < 0.0001$, T + E2 1.25 mg; **Figure 4C**). Implants containing less than 25 µg E2 do not significantly change bladder mass from the sham operated control group (**Figure 4C**). Because both T and E2 alone are sufficient to increase bladder volume from the sham operated control group, next we implanted mice with T (25 mg) + E2 (1.25 mg), T (12.5 mg) + E2 (1.25 mg), T (25 mg) alone, E2 (2.5 mg) alone, and compared bladder volume to that of mice treated with T (25 mg) + E2 (2.5 mg). The T (25 mg) alone and sham operated control groups were the only groups that differ in bladder volume compared to the group receiving the standard size T (25 mg) + E2 (2.5 mg) implants ($P = 0.0451$, T 25 mg; $P < 0.0001$, sham operated control; **Figure 4D**). In contrast, bladder volumes of mice treated with T (25 mg) + E2 (1.25 mg), T (12.5 mg) + E2 (1.25 mg), or E2 (2.5 mg) alone are not significantly different from mice treated with the standard size T (25 mg) + E2 (2.5 mg) implants (**Figure 4D**).

Discussion

Sustained exposure to exogenous T and E2 has been used to model prostate-related voiding dysfunction in dogs, rats and mice [7, 27-34]. The model has gained particular favor in mice, where genetic tractability enables gain and loss of function studies to identify causative genetic factors [8, 35, 36]. We made three new observations relating to T and E2-mediated voiding dysfunction in male mice: (1) mouse genetic

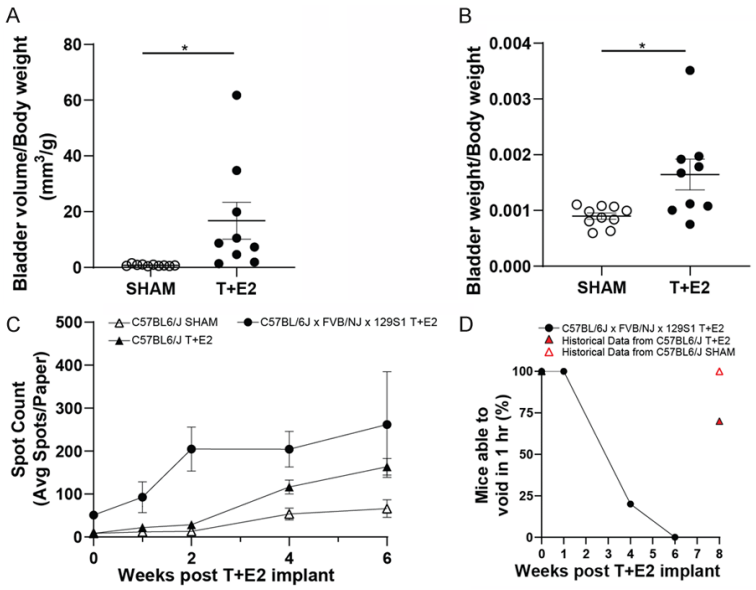

Figure 3. Testosterone and Estradiol (T+E2) mediated voiding dysfunction escalates more quickly and is more severe in C57BL/6J × FVB/NJ × 129S1 (mixed background) than C57BL/6J pure background mice. Six-week-old wild type mixed background and C57BL/6J pure background male mice were treated with testosterone and estradiol (T+E2) implants. The bladders of T+E2 treated mixed background mice were evaluated at two weeks post implantation surgery and were (A) larger ($P = 0.0002$, mean ± SE, 9-10 mice per group) and (B) weighed more ($P = 0.0271$ mean ± SE, 9-10 mice per group) than sham operated mixed background mice. (C) Spontaneous voiding behavior was assessed using the void spot assay. Urine spot quantity significantly increased from baseline as early as two weeks post T+E2 implantation surgery in mixed background mice ($P < 0.0001$ compared to baseline) and remained elevated from baseline until at least six weeks post implantation surgery. Void spot quantity did not increase from baseline until 4 weeks post implantation surgery in C57BL/6J purebred mice ($P < 0.0001$ compared to baseline). 2-30 mice per group per timepoint. (D) Cystometry was used to evaluate bladder pressures during a one-hour monitoring period in response to continuous filling via a cystostomy catheter. T+E2 treatment prevented some mixed background mice from initiating a voiding contraction during the monitoring period, and when compared to previously published data for pure C57BL/6J background sham operated (Historical Data from C57BL/6J SHAM) or treated with T+E2 (Historical Data from C57BL/6J Hormone) [9], a greater percentage of mixed background mice were unable to void beginning as early as four weeks after T+E2 treatment. Values are 3-11 mice per group per timepoint.

background and (2) concentration of hormone in sustained released pellets profoundly influence severity and progression rate of urinary voiding dysfunction, and (3) urinary voiding dysfunction is not prevented by conditional *Ctnnb1* deletion in some prostate luminal epithelial cells. These findings raise the importance of controlling for mouse genetic background in future gain and loss of function genetic studies, demonstrate tunability of the T+E2 treated mouse model, and shed light on the mechanism of T+E2 action.

Although genetic background has been identified as a risk modifier for mouse models of human cancers, it has not been a major focus of benign urinary dysfunction research. In 2015, we evaluated the impact of genetic strains and sex on VSA results [24]. In that study, there was no appreciable difference between male SV.129 and C57BL/6J background mice and analysis of FVB/NJ background was not performed. Here we show that genetic background does not drive differences at baseline but rather influences the severity and rate of progressive voiding dysfunction, specifically that the mixed strain background progress more quickly than the C57BL/6J purebred background in response to T+E2. This important finding highlights the need to report and control for genetic background in all future transgenic mouse studies, not only at baseline, but at all timepoints.

We have revealed that a genetically heterogenous mouse population is likely to be useful for identifying genetic risk modifiers of LUTS sensitivity and severity. Future studies using such mouse models may reveal why LUTS has such a diverse etiology across human populations. A cross-sectional analysis of 1536 men from the Multi-Ethnic Study of Atherosclerosis cohort revealed a higher rate of incontinence, particularly urge incontinence, in non-Hispanic black men compared to non-Hispanic white men [37]. A retrospective cohort study of patients who presented to emergency departments in Florida between 2005 and 2015 indicated that non-Hispanic black men and Hispanic men were more likely than white men to present with acute urinary retention [38]. Additionally, a prospective cohort study of 6 US tertiary care centers showed that bother from LUTS was greater

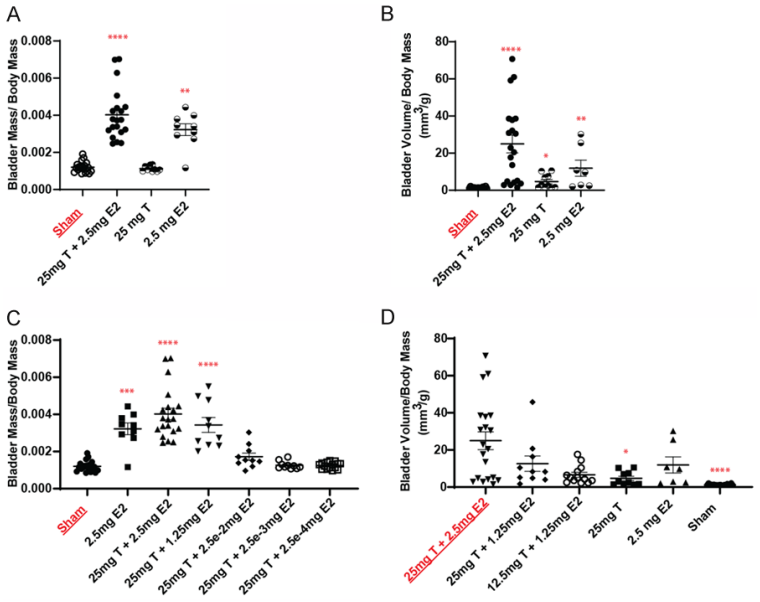

Figure 4. Estradiol alone increases bladder mass and testosterone and estradiol in combination increase bladder volume in a dose dependent fashion. Six-week-old C57BL/6J background mice were implanted with T+E2 pellets of varying amounts or received a sham surgery as described in **Figure 2**. Bladders were dissected from mice two weeks following pellet implantation. The group used for comparison is underlined and colored red in each panel, with (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$, and (****) $P < 0.0001$. A. Treatment with standard T+E2 and E2 alone increase bladder mass compared to sham operated control. B. Treatment with T+E2, T alone, and E2 alone increase bladder volume compared to sham operated control. C. E2 alone or 25 mg testosterone and ≥ 25 µg estradiol led to significantly increased bladder mass compared to sham mice. There is also appears to be a gradual increase in bladder mass with the increasing estradiol dosage. D. Mice treated with T alone or sham had significantly decreased bladder volume compared to standard T+E2 mice. All other treatments, which included minimum 1.25 mg E2, had similar bladder volume. Values are mean ± SE, n = 7-20 per group.

among non-white men than white men [39]. The genetic, epigenetic, and environmental factors that underlie racial and ethnic differences in LUTS risk are not known. Our results will support future forward genetics studies in mice to identify genetic loci that confer sensitivity or resistance. These efforts may reveal genes and signaling pathways that could be examined across the human population.

We found that E2 and not T drives the increase in bladder mass and estrogen is the predominant driver of increased bladder volume in the T+E2 model. Previous studies have shown that estrogen receptor alpha, and not estrogen receptor beta, is required for T+E2 mediated voiding dysfunction in male mice [8]. Here, we show that E2, and not T, is the key driver of voiding dysfunction in mice. Changes in E2 dose moderated the severity of voiding dysfunction, while changes in T dose had little impact. It is likely that the mixed strain mice used in this study exhibit a genetic trait that sensitizes them to E2 either upstream or downstream of the estrogen receptor alpha signaling cascade. Unfortunately, little is known about background strain variation in estrogen sensitivity. One study in 2010 measured variations in estrogen sensitivity during oocyte development in FVB, C57BL/6J, F2 hybrid, and CD-1 strains. They found that C57BL/6J ovaries are more sensitive to E2 than CD-1, FVB, or F2 hybrid ovaries [40]. The strain sensitivity identified by the previous study opposes what we would have predicted for the current study; however, sex- and organ-specific variations in E2 sensitivity are likely involved. Although our finding of a mixed genetic background with sensitivity to E2 does not have a direct translation to clinical medicine, it does hold broad implications for the clinical community. Environmental estrogens are abundant and environmental estrogens can at least partially phenocopy estradiol to mediate LUTS in male mice [41]. If some men with LUTS have a genetic predisposition for estrogen sensitivity, they may be uniquely susceptible to urinary voiding dysfunction driven by environmental estrogens. Quantitative trait loci mapping is already under consideration as a mechanism to identify risk modifiers for female mouse voiding function [42]. There is evidence that environment influences alter risk of benign prostatic disease [41, 43-46]. There are future opportunities to isolate the independent contributions of genetic background and environment, and examine interactions between them, an effect approach that has been fruitful in understanding other biological processes, such as craniofacial malformations [47, 48].

The progressive and reproducible voiding dysfunction in T+E2 treated mice creates an oppor-

tunity for using these mice in the preclinical testing of new LUTS therapies. Essential to these future studies is a clear definition of what it means to improve voiding function in male mice. One possibility is to mimic the Kaplan-Meier estimator approach used to assess therapeutic success in genetically engineered mouse models of human cancers and orthotopic transplant models. We show here that testosterone and estradiol implants progressively increase male mouse urine spot count and bladder mass. However, while progression of many mouse models of human cancers occurs over the course of months and offers a large window for intervention, T+E2 mediated voiding dysfunction progresses far more rapidly. In fact, up to 30% of C57BL/6J purebred mice treated with 2.5 mg E2 and 25 mg T implants progress undergo bladder decompensation within 1 month of treatment [9], offering a narrow window of time to examine the potential benefits of an intervention. By reducing the E2 concentration in sustained release subcutaneous implants, it may be possible to reduce the voiding progression rate, offering more time to observe the impact of interventions. On the other hand, by increasing E2 concentration or using mixed strain mice, it may be possible to examine the impact of interventions on severe voiding disease, or specifically on bladder decompensation due to prostate-mediated outlet obstruction without waiting months for disease progression.

Disclosure of conflict of interest

None.

Address correspondence to: Chad M Vezina, Department of Comparative Biosciences, University of Wisconsin-Madison, 1656 Linden Dr, Madison, WI 53706, USA. Tel: 608-890-3235; E-mail: chad.vezina@wisc.edu

References

[1] Jacobsen SJ, Jacobson DJ, Girman CJ, Roberts RO, Rhodes T, Guess HA and Lieber MM. Natural history of prostatism: risk factors for acute urinary retention. J Urol 1997; 158: 481-487.

[2] Bjornerem A, Straume B, Midtby M, Fonnebo V, Sundsfjord J, Svartberg J, Acharya G, Oian P and Berntsen GK. Endogenous sex hormones in relation to age, sex, lifestyle factors, and chronic diseases in a general population: the Tromso Study. J Clin Endocrinol Metab 2004; 89: 6039-6047.

[3] Broderick GA, Longhurst PA, Juniewicz PE, Wein AJ and Levin RM. A novel canine model of partial outlet obstruction secondary to prostatic hypertrophy. World J Urol 1994; 12: 245-248.

[4] Coffey DS and Walsh PC. Clinical and experimental studies of benign prostatic hyperplasia. Urol Clin North Am 1990; 17: 461-475.

[5] Rohr HP, Bartsch G, Tannenbaum M, Neumann N and Guggenheim R. Hormonal influences on the dog prostate--a correlation study with light microscopy, transmission and scanning electron microscopy. Scan Electron Microsc 1979; 665-671.

[6] Streng TK, Talo A, Andersson KE and Santti R. A dose-dependent dual effect of oestrogen on voiding in the male mouse? BJU Int 2005; 96: 1126-1130.

[7] Nicholson TM, Ricke EA, Marker PC, Miano JM, Mayer RD, Timms BG, vom Saal FS, Wood RW and Ricke WA. Testosterone and 17beta-estradiol induce glandular prostatic growth, bladder outlet obstruction, and voiding dysfunction in male mice. Endocrinology 2012; 153: 5556-5565.

[8] Nicholson TM, Moses MA, Uchtmann KS, Keil KP, Bjorling DE, Vezina CM, Wood RW and Ricke WA. Estrogen receptor-alpha is a key mediator and therapeutic target for bladder complications of benign prostatic hyperplasia. J Urol 2015; 193: 722-729.

[9] Keil KP, Abler LL, Altmann HM, Wang Z, Wang P, Ricke WA, Bjorling DE and Vezina CM. Impact of a folic acid-enriched diet on urinary tract function in mice treated with testosterone and estradiol. Am J Physiol Renal Physiol 2015; 308: F1431-1443.

[10] Mehta V, Abler LL, Keil KP, Schmitz CT, Joshi PS and Vezina CM. Atlas of Wnt and R-spondin gene expression in the developing male mouse lower urogenital tract. Dev Dyn 2011; 240: 2548-2560.

[11] McNeal JE. Origin and evolution of benign prostatic enlargement. Invest Urol 1978; 15: 340-345.

[12] Harada N, Tamai Y, Ishikawa T, Sauer B, Takaku K, Oshima M and Taketo MM. Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. EMBO J 1999; 18: 5931-5942.

[13] Madueke I, Hu WY, Hu D, Swanson SM, Vander Griend D, Abern M and Prins GS. The role of WNT10B in normal prostate gland development and prostate cancer. Prostate 2019; 79: 1692-1704.

[14] Hu WY, Hu DP, Xie L, Li Y, Majumdar S, Nonn L, Hu H, Shioda T and Prins GS. Isolation and functional interrogation of adult human prostate epithelial stem cells at single cell resolution. Stem Cell Res 2017; 23: 1-12.

[15] Mehta V, Schmitz CT, Keil KP, Joshi PS, Abler LL, Lin TM, Taketo MM, Sun X and Vezina CM. Beta-catenin (CTNNB1) induces Bmp expression in urogenital sinus epithelium and participates in prostatic bud initiation and patterning. Dev Biol 2013; 376: 125-135.

[16] Bauman TM, Vezina CM, Huang W, Marker PC, Peterson RE and Ricke WA. Beta-catenin is elevated in human benign prostatic hyperplasia specimens compared to histologically normal prostate tissue. Am J Clin Exp Urol 2014; 2: 313-322.

[17] Wu X, Wu J, Huang J, Powell WC, Zhang J, Matusik RJ, Sangiorgi FO, Maxson RE, Sucov HM and Roy-Burman P. Generation of a prostate epithelial cell-specific Cre transgenic mouse model for tissue-specific gene ablation. Mech Dev 2001; 101: 61-69.

[18] Brault V, Moore R, Kutsch S, Ishibashi M, Rowitch DH, McMahon AP, Sommer L, Boussadia O and Kemler R. Inactivation of the beta-catenin gene by Wnt1-Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development. Development 2001; 128: 1253-1264.

[19] Ruetten H, Wegner KA, Zhang HL, Wang P, Sandhu J, Sandhu S, Mueller B, Wang Z, Macoska J, Peterson RE, Bjorling DE, Ricke WA, Marker PC and Vezina CM. Impact of sex, androgens, and prostate size on C57BL/6J mouse urinary physiology: functional assessment. Am J Physiol Renal Physiol 2019; 317: F996-F1009.

[20] Hill WG, Zeidel ML, Bjorling DE and Vezina CM. Void spot assay: recommendations on the use of a simple micturition assay for mice. Am J Physiol Renal Physiol 2018; 315: F1422-F1429.

[21] Wegner KA, Abler LL, Oakes SR, Mehta GS, Ritter KE, Hill WG, Zwaans BM, Lamb LE, Wang Z, Bjorling DE, Ricke WA, Macoska J, Marker PC, Southard-Smith EM, Eliceiri KW and Vezina CM. Void spot assay procedural optimization and software for rapid and objective quantification of rodent voiding function, including overlapping urine spots. Am J Physiol Renal Physiol 2018; 315: F1067-F1080.

[22] Keil KP, Abler LL, Altmann HM, Bushman W, Marker PC, Li L, Ricke WA, Bjorling DE and Vezina CM. Influence of animal husbandry practices on void spot assay outcomes in C57BL/6J male mice. Neurourol Urodyn 2016; 35: 192-198.

[23] Fraser MO, Smith PP, Sullivan MP, Bjorling DE, Campeau L, Andersson KE and Yoshiyama M. Best practices for cystometric evaluation of lower urinary tract function in muriform rodents. Neurourol Urodyn 2020; 39: 1868-1884.

[24] Bjorling DE, Wang Z, Vezina CM, Ricke WA, Keil KP, Yu W, Guo L, Zeidel ML and Hill WG. Evaluation of voiding assays in mice: impact of genetic strains and sex. Am J Physiol Renal Physiol 2015; 308: F1369-1378.

[25] Wegner KA, Cadena MT, Trevena R, Turco AE, Gottschalk A, Halberg RB, Guo J, McMahon JA, McMahon AP and Vezina CM. An immunohistochemical identification key for cell types in adult mouse prostatic and urethral tissue sections. PLoS One 2017; 12: e0188413.

[26] Birbach A, Casanova E and Schmid JA. A Probasin-MerCreMer BAC allows inducible recombination in the mouse prostate. Genesis 2009; 47: 757-764.

[27] Jeon GS, Won JH, Lee BM, Kim JH, Ahn HS, Lee EJ, Park SI and Park SW. The effect of transarterial prostate embolization in hormone-induced benign prostatic hyperplasia in dogs: a pilot study. J Vasc Interv Radiol 2009; 20: 384-390.

[28] Winter ML and Liehr JG. Possible mechanism of induction of benign prostatic hyperplasia by estradiol and dihydrotestosterone in dogs. Toxicol Appl Pharmacol 1996; 136: 211-219.

[29] Yokota T, Honda K, Tsuruya Y, Nomiya M, Yamaguchi O, Gotanda K and Constantinou CE. Functional and anatomical effects of hormonally induced experimental prostate growth: a urodynamic model of benign prostatic hyperplasia (BPH) in the beagle. Prostate 2004; 58: 156-163.

[30] Ehrlichman RJ, Isaacs JT and Coffey DS. Differences in the effects of estradiol on dihydrotestosterone induced prostatic growth of the castrate dog and rat. Invest Urol 1981; 18: 466-470.

[31] Taylor JA, Jones MB, Besch-Williford CL, Berendzen AF, Ricke WA and S Vom Saal F. Interactive effects of perinatal BPA or DES and adult testosterone and estradiol exposure on adult urethral obstruction and bladder, kidney, and prostate pathology in male mice. Int J Mol Sci 2020; 21: 3902.

[32] Bernoulli J, Yatkin E, Konkol Y, Talvitie EM, Santti R and Streng T. Prostatic inflammation and obstructive voiding in the adult Noble rat: impact of the testosterone to estradiol ratio in serum. Prostate 2008; 68: 1296-1306.

[33] Bernoulli J, Yatkin E, Talvitie EM, Santti R and Streng T. Urodynamic changes in a noble rat model for nonbacterial prostatic inflammation. Prostate 2007; 67: 888-899.

[34] Konkol Y, Vuorikoski H, Streng T, Tuomela J and Bernoulli J. Characterization a model of prostatic diseases and obstructive voiding induced by sex hormone imbalance in the Wistar and Noble rats. Transl Androl Urol 2019; 8: S45-S57.

[35] Streng T, Li X, Lehtoranta M, Makela S, Poutanen M, Talo A, Tekmal RR and Santti R. Infravesical obstruction in aromatase over expressing transgenic male mice with increased ratio of serum estrogen-to-androgen concentration. J Urol 2002; 168: 298-302.

[36] Li X, Nokkala E, Yan W, Streng T, Saarinen N, Warri A, Huhtaniemi I, Santti R, Makela S and Poutanen M. Altered structure and function of reproductive organs in transgenic male mice overexpressing human aromatase. Endocrinology 2001; 142: 2435-2442.

[37] Akbar A, Liu K, Michos ED, Brubaker L, Markossian T, Bancks MP and Kramer H. Racial differences in urinary incontinence prevalence, overactive bladder and associated bother among men: the multi-ethnic study of atherosclerosis. J Urol 2021; 205: 524-531.

[38] Patel PM, Sweigert SE, Nelson M, Gupta G, Baker M, Weaver FM and McVary KT. Disparities in benign prostatic hyperplasia progression: predictors of presentation to the emergency department in urinary retention. J Urol 2020; 204: 332-336.

[39] Liu AB, Liu Q, Yang CC, Griffith JW, Smith AR, Helmuth ME, Lai HH, Amundsen CL, Erickson BA, Jelovsek JE, Agochukwu NQ, Mueller MG, Andreev VP, Weinfurt KP, Kenton KS, Fraser MO, Cameron AP, Kirkali Z and Gore JL; LURN Study Group. Patient characteristics associated with more bother from lower urinary tract symptoms. J Urol 2019; 202: 585-591.

[40] Pepling ME, Sundman EA, Patterson NL, Gephardt GW, Medico L Jr and Wilson KI. Differences in oocyte development and estradiol sensitivity among mouse strains. Reproduction 2010; 139: 349-357.

[41] Nicholson TM, Nguyen JL, Leverson GE, Taylor JA, Vom Saal FS, Wood RW and Ricke WA. Endocrine disruptor bisphenol A is implicated in urinary voiding dysfunction in male mice. Am J Physiol Renal Physiol 2018; 315: F1208-F1216.

[42] Yu W, Ackert-Bicknell C, Larigakis JD, MacIver B, Steers WD, Churchill GA, Hill WG and Zeidel ML. Spontaneous voiding by mice reveals strain-specific lower urinary tract function to be a quantitative genetic trait. Am J Physiol Renal Physiol 2014; 306: F1296-1307.

[43] Turco AE, Thomas S, Crawford LK, Tang W, Peterson RE, Li L, Ricke WA and Vezina CM. In utero and lactational 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure exacerbates urinary dysfunction in hormone-treated C57BL/6J mice through a non-malignant mechanism involving proteomic changes in the prostate that differ from those elicited by testosterone and estradiol. Am J Clin Exp Urol 2020; 8: 59-72.

[44] Ricke WA, Lee CW, Clapper TR, Schneider AJ, Moore RW, Keil KP, Abler LL, Wynder JL, Lopez Alvarado A, Beaubrun I, Vo J, Bauman TM, Ricke EA, Peterson RE and Vezina CM. In utero and lactational TCDD exposure increases susceptibility to lower urinary tract dysfunction in adulthood. Toxicol Sci 2016; 150: 429-440.

[45] Bendinelli M and Friedman H. B and T lymphocyte activation by murine leukemia virus infection. Adv Exp Med Biol 1979; 121B: 91-97.

[46] Gupta A, Ketchum N, Roehrborn CG, Schecter A, Aragaki CC and Michalek JE. Serum dioxin, testosterone, and subsequent risk of benign prostatic hyperplasia: a prospective cohort study of Air Force veterans. Environ Health Perspect 2006; 114: 1649-1654.

[47] Beames TG and Lipinski RJ. Gene-environment interactions: aligning birth defects research with complex etiology. Development 2020; 147: dev191064.

[48] Everson JL, Sun MR, Fink DM, Heyne GW, Melberg CG, Nelson KF, Doroodchi P, Colopy LJ, Ulschmid CM, Martin AA, McLaughlin MT and Lipinski RJ. Developmental toxicity assessment of piperonyl butoxide exposure targeting sonic hedgehog signaling and forebrain and face morphogenesis in the mouse: an in vitro and in vivo study. Environ Health Perspect 2019; 127: 107006.