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Bisphenol A Promotes Human Prostate Stem-Progenitor Cell Self-Renewal and Increases in vivo Carcinogenesis in Human Prostate Epithelium

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Abstract:	Previous studies in rodent models have shown that early-life exposure to bisphenol A (BPA) reprograms the prostate and enhances its susceptibility to hormonal carcinogenesis with aging. To determine whether the human prostate is similarly sensitive to BPA, the current study utilized human prostate epithelial stem-like cells cultured from prostates of young, disease-free donors. Similar to estradiol-17 (E2), BPA increased stem-progenitor cell self-renewal and expression of stem-related genes in a dose-dependent manner. Further, 10 nM BPA and E2 possessed equimolar membrane-initiated signaling with robust induction of p-Akt and p-Erk at 15 min. To assess in vivo carcinogenicity, human prostate stem-progenitor cells combined with rat mesenchyme were grown as renal grafts in nude mice, forming normal human prostate epithelium at 1 month. Developmental BPA exposure was achieved through oral administration of 100 µg or 250 µg BPA/kg BW to hosts for 2 weeks post-grafting, producing free-BPA levels of 0.39 and 1.35 ng/ml serum, respectively. Carcinogenesis

	was driven by testosterone plus E2 treatment for 2-4 months to model rising E2 levels in aging men. The incidence of prostate intraepithelial neoplasia (HG-PIN) and adenocarcinoma markedly increased from 13% in oil-fed controls to 33-36% in grafts exposed in vivo to BPA ($P<0.05$). Continuous developmental BPA exposure through in vitro (200 nM) plus in vivo (250 µg/kg BW) treatments increased HG-PIN/cancer incidence to 45% ($P< 0.01$). Together, the present findings demonstrate that human prostate stem-progenitor cells are direct BPA targets and that developmental exposure to BPA at low-doses increases hormone dependent cancer risk in the human prostate epithelium.

Bisphenol A Promotes Human Prostate Stem-Progenitor Cell Self-Renewal and Increases *in vivo* Carcinogenesis in Human Prostate Epithelium

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ABSTRACT

Previous studies in rodent models have shown that early-life exposure to bisphenol A (BPA) reprograms the prostate and enhances its susceptibility to hormonal carcinogenesis with aging. To determine whether the human prostate is similarly sensitive to BPA, the current study utilized human prostate epithelial stem-like cells cultured from prostates of young, disease-free donors. Similar to estradiol-17β (E_2), BPA increased stem-progenitor cell self-renewal and expression of stem-related genes in a dose-dependent manner. Further, 10 nM BPA and E_2 possessed equimolar membrane-initiated signaling with robust induction of p-Akt and p-Erk at 15 min. To assess *in vivo* carcinogenicity, human prostate stem-progenitor cells combined with rat mesenchyme were grown as renal grafts in nude mice, forming normal human prostate epithelium at 1 month. Developmental BPA exposure was achieved through oral administration of 100 μg or 250 μg BPA/kg BW to hosts for 2 weeks post-grafting, producing free-BPA levels of 0.39 and 1.35 ng/ml serum, respectively. Carcinogenesis was driven by testosterone plus E_2 treatment for 2-4 months to model rising E_2 levels in aging men. The incidence of prostate intraepithelial neoplasia (HG-PIN) and adenocarcinoma markedly increased from 13% in oil-fed controls to 33-36% in grafts exposed *in vivo* to BPA ($P<0.05$). Continuous developmental BPA exposure through *in vitro* (200 nM) plus *in vivo* (250 μg/kg BW) treatments increased HG-PIN/cancer incidence to 45% ($P< 0.01$). Together, the present findings demonstrate that human prostate stem-progenitor cells are direct BPA targets and that developmental exposure to BPA at low-doses increases hormone dependent cancer risk in the human prostate epithelium.

INTRODUCTION

Prostate cancer is the most common non-cutaneous cancer and the second leading cause of cancer-related mortality in men in the United States (1). Despite extensive research, the etiology of prostate cancer remains elusive. Further understanding of factors that contribute to this high disease rate are essential to implement effective tumor prevention and therapeutic strategies. It is well recognized that adult androgens and estrogens play fundamental roles in initiation, promotion and progression of prostate cancer (2). There is also compelling evidence that the developmental hormonal milieu may be linked to the predisposition of this gland to prostate cancer in adult men. While *in utero* prostate morphogenesis is driven by fetal androgens (3), maternal and fetal estrogens also modulate development through estrogen receptors (ER) α and β expressed in the human fetal prostate (4). Importantly, multiple epidemiology studies link elevated estrogen levels during pregnancy to increased risk of prostate cancer in male offspring (5-9). This is supported by extensive laboratory-based research using rodent models which has shown that inappropriate estradiol exposure during development in terms of levels and timing can reprogram the developing prostate gland and increase its susceptibility for prostate cancer during aging (10-13). Together, these findings have led to the hypothesis that an altered steroid balance during prostate gland formation, with a shift favoring estrogen dominance, may predispose the newborn male to prostatic disease including carcinoma later in life.

There is rising concern that exposures to endocrine disrupting chemicals (EDCs) in the environment during sensitive developmental stages may likewise increase susceptibility to prostate cancer in the human population. One ubiquitous EDC with proven estrogenic activity is bisphenol A (BPA), a high production chemical found in thousands of consumer products including polycarbonate bottles, epoxy resins, carbonless paper receipts and dental sealants (14, 15). Importantly, BPA monomers have been shown to leach into food and beverages as well as absorb across the skin (15, 16). In a study of 2500 United States adults, 93% had detectable urine BPA indicating that humans are chronically exposed to this compound during routine daily activity (17). Although adults have a high capacity to rapidly

metabolize and excrete BPA, the fetus and infants have lower hepatic expression of its metabolizing enzyme, UGT2B, and thus are at greater exposure risk to unconjugated (bioactive) BPA than adults (18). While levels of unconjugated or free BPA in adult human serum are typically low (undetectable to ~ 0.5 ng/ml), higher levels have been reported in amniotic fluid, fetal circulation and neonates (19-22). Thus there is considerable potential for BPA to act as an environmental estrogen in the developing prostate.

To address this possibility, our laboratory utilized a rat model and demonstrated that similar to estradiol, a brief, early-life exposure to low-dose BPA markedly increased the incidence of later-life, estrogen-mediated prostate carcinogenesis (23, 24). These findings suggest that developmental BPA exposure can sensitize the prostate to adult estrogenic exposures, thus priming their carcinogenic susceptibility to the relative rise in estrogen levels that occur in aging men. Mechanisms for these life-spanning effects were shown to include permanent epigenetic modifications (23, 25) and reprogrammed prostate stem cells (26, 27) which are both retained throughout life. A major issue that remains to be resolved is whether this reprogramming process may occur in the human prostate gland and whether early-life BPA exposures may increase prostate cancer risk in men as they age, as predicted by animal models. However, direct methods for examining developmental BPA exposures and later life prostate cancer incidence in humans is a particular challenge due to the long latency period between fetal development and age of prostate cancer onset (50-60 years).

Recently, our laboratory established a novel model for *in vivo* development of humanized prostate-like tissues by using epithelial stem-progenitor cells cultured from prostate glands of young, disease-free men (28). Remarkably, we found that the prostate stem-progenitor cells express ERα, ERβ and GPR30 and exhibit a proliferative response to 1 nM estradiol-17β (E_2), implicating them as direct estrogenic targets. When mixed with embryonic rat urogenital sinus mesenchyme (UGM) and grafted under the kidney capsule of nude mice, these progenitor cells formed normal human prostate epithelium that produce prostate specific antigen (PSA) within the prostate-like tissues by one month of age. Treatment of the host mice bearing the humanized prostate-like structures with testosterone and estradiol-

17β (T+E) induced prostate epithelial pathology over a 1-4 month period, progressing from hyperplasia to prostatic intraepithelial neoplasia (PIN) and adenocarcinoma at a relatively low incidence (28). Together, these findings provided the first direct evidence that human prostate stem and progenitor cells are direct estrogen targets and that estrogen, in an androgen-supported milieu, is a carcinogen for human prostate epithelium.

In this context, the present study first assessed whether BPA over a range of doses has similar estrogen-like effects on normal human prostate stem-progenitor cells in terms of self-renewal capacity, differentiation and signaling activity. Next, our *in vivo* humanized prostate system was utilized to determine whether developmental-stage BPA exposures could modulate the incidence of estrogen-mediated carcinogenesis in the human prostate epithelium as the tissues aged. Importantly, circulating levels of free (bioactive) and glucuronidated BPA (BPA-G) in the host mice following oral exposures were directly measured using an UHPLC-MS-MS system and found to be relevant to human exposures. Our findings reveal that BPA mimics estradiol effects on human prostate stem-progenitor cell self-renewal capacity and that brief exposure of the human prostate epithelium to low-dose BPA during a developmental stage significantly increases the incidence of estrogen-mediated PIN and prostate cancer.

MATERIALS AND METHODS

Cell and Prostasphere Cultures

Primary human prostate epithelial cells (PrEC) were obtained from young (19-21 yrs of age) disease-free organ donors (Lonza,Walkersville, MD) and cultured in Prostate Epithelial Cell Growth Medium (PrEGM™; Lonza) on fibronectin-coated flasks. PrEC from four donors were used and all exhibited similar growth and hormone responsive behaviors. Prostaspheres were cultured from PrEC as previously described (28) and confirmed as clonally-derived spheroids of stem/progenitor cells (Supplement Fig 1). Briefly, $1x10^5$ PrEC cells were resuspended in 1:1 Matrigel (BD Biosciences)/PrEGM with 1 ml PrEGM and cultured at 37C in 5% CO_2. Prostaspheres were cultured in the absence or presence of 1 nM - 1μM E_2 (Sigma-Aldrich, Inc., St Louis, MO) or 0.1 nM-1 μM BPA with medium replenished every 48 hr. Crystalline BPA was provided by the National Toxicology Program (NIEHS), dissolved in 100% EtOH with final EtOH at 0.1%. Prostasphere number and size at day 7 were assessed using an automated digital image processing algorithm (See Supplemental methods and Supplement Fig 2).

Flow Cytometry

Analysis of the stem-enriched population in day 7 prostaspheres following E_2 exposure was performed by FACS using the CD49f and Trop2 antibody method (29, 30). Briefly, prostaspheres were dispersed into single cells which were stained with Anti-Human/Mouse CD49f APC and Anti-Human Trop2 Alexa Fluor® 488 (eBioscience, San Diego, CA) antibodies. Dead cells were gated by propidium iodide (PI) staining. Mouse IgG2a K Isotype Control Alexa Fluor® 488 and Rat IgG2a K Isotype Control APC (eBioscience) antibodies were used as negative controls. Cells were sorted using the CyAn™ ADP Analyzer (Beckman Coulter Inc., Brea, CA) and events were analyzed and plotted based on CD49f and Trop2 staining intensity by the Summit Software v4.3 (Beckman Coulter, Indianapolis, IN). Subpopulations of fractionated cells were further delineated using the Summit Software polygon tool.

Stem-like Cell Hoechst Exclusion Assay

A Hoechst dye exclusion assay (31, 32) was used to quantitate the fraction of stem-like cells in primary PrEC cultures following exposure to 1-100 nM E_2 or 10nm-1μM BPA for 72 hr. PrEC cells were pre-incubated for 10 min with or without 50 μM verapamil hydrochloride (Sigma-Aldrich) which inhibits ABCG2 transporter protein expressed at high levels in stem cells, blocking their Hoechst exclusion ability. Cells were next incubated in 0.5 μg/ml Hoechst 33342 (Sigma-Aldrich) in Hanks' balance salt solution, 10% FBS, 1% D-glucose, 20 mM HEPES for 30 min at 37C, washed in PBS and incubated with 1 μg/ml PI for dead cell exclusion. Hoechst-stained cells were separated by single-channel FACS (CyAn^{TM} ADP Analyzer). All results were confirmed by side-population double-channel FACS analysis (MoFlo^{TM} XDP analyzer, Beckman Coulter) using 5 μg/ml Hoechst dye (32). The % prostate stem-like cells were calculated as the difference in Hoechst excluding cells incubated -/+ verapamil.

Quantitative real-time reverse transcription-PCR (RT-PCR)

Total RNA was isolated from prostaspheres using RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNAs synthesized using iScript^{TM} Reverse Transcription Supermix (Bio-Rad, Hercules, CA). PCR reactions in SsoAdvanced^{TM} SYBR Green Supermix (Bio-Rad) were carried out using CFX96 Real-Time System (Bio-Rad). Primer sequences for prostate genes are provided in the Supplement Table S1.The cycling conditions were: 95C for 5 min, followed by 40 cycles of 95C for 15 sec and 60C for 1 min. Data were analyzed by $-\Delta\Delta C_t$ method and individual mRNA levels were normalized to house-keeping gene RPL13.

Estrogen and BPA signaling pathway analysis

To assess rapid, membrane-initiated signaling actions of E_2 and BPA, day 7 prostaspheres (20,000/treatment) were isolated with dispase, resuspended in PrEGM and exposed to 10 nM E_2 or 10 nM BPA for 0, 15, 30, 60 minutes or 6 hr at 37C. Prostaspheres were dounce homogenized and lysed in Cell Lysis Buffer (Cell Signaling, Danvers, MA) for 10 min at 4C as described (33). Lysates were centrifuged (15,000 rpm) and 30 μg supernatant protein was separated via 10% SDS-PAGE gels, transferred to polyvinylidene difluoride membranes and immunoblotted. Primary antibodies against p-Akt (S473), Akt,

p-Erk (thy202/tyr204), and Erk (Cell Signaling) and horseradish peroxidase (HRP)-conjugated secondary antibody were used. Proteins were visualized with Pierce ECL plus and scanned using ImageQuantTM (GE Healthcare).

To assess evidence of E_2 or BPA genomic signaling through an estrogen response element (ERE), prostaspheres were cultured for 5 days, isolated from Matrigel with dispase, plated at 200 prostaspheres/well and incubated in PrEGM for 16h. Spheres were co-transfected with a firefly luciferase reporter construct, ERE2-tk-luc (34), and renilla luciferase reporter as internal control using lipofectamine for 6 hr. Transfected prostaspheres were treated with vehicle, 10nM E_2 or 10 nM-1μM BPA for 16 hr and luciferase activity was measured using Dual-Luciferase® Reporter Assay System (Promega, Madison, WI). The ratio of firefly luciferase activity and renilla activity was used to analyze ERE activation by E_2 or BPA over vehicle control. pGL basic vector and pGL3 control vectors were used as negative and positive controls, respectively.

Animals and formation of humanized prostate-like tissues

Animals were handled according to Principles for Care and Use of Animal Research and studies approved by the Institutional Animal Care Committee. Timed pregnant female Sprague-Dawley Hsd:SD®™ rats were purchased from Harlan (Indianapolis, IN) and fetal pups were collected on gestation day 17 for UGM recovery. Male nude mice were purchased from Harlan at 4-6 weeks of age and acclimated to BPA-controlled conditions prior to their use as renal graft hosts. Care was taken to avoid all polycarbonate and epoxy resin contact to the animals, sera and tissue samples. All materials were screened for BPA content using UHPLC-MS-MS and only products with no detectable BPA levels were utilized. Animals were housed at 21C, on a 14L:10D schedule in polysulfone solid-bottom cages and double-deionized water was supplied from glass bottles. Animals were fed *ad libitum* a phytoestrogen-reduced diet (Teklad 2018; Harlan, Indianapolis, IN). Food lots with < 20 pmol estrogen equivalents/gram, measured by E-SCREEN assay (35) were utilized. *In vivo* formation of humanized prostate-like structures was performed using tissue recombination and renal grafting in host mice as

previously described (28). Briefly, ~3000 human prostate epithelial stem-progenitor cells from dispersed day 7 prostaspheres were mixed with rat UGM in Matrigel and grafted under the renal capsule of host adult male nude mice. Mature chimeric prostate-like structures with normal histology formed by 1 month. PSA immunostaining was used to confirm the human origin of the prostate epithelium.

Developmental in vivo BPA exposure and hormonal carcinogenesis

To recapitulate developmental BPA exposure for the human prostate epithelium, host mice were fed BPA (in 30 μl tocopherol-stripped corn oil, 1% EtOH) daily by mouth for 2 weeks following renal grafting when stem/progenitor cells cytodifferentiate to basal and luminal cells and form glandular structures. Since adult host mice have greater BPA metabolizing capacity than the fetus (36), preliminary studies were conducted using 50-500 BPA μg/kg BW to identify oral doses that produce internal free BPA levels comparable to levels found in human umbilical cord, fetal and neonatal serum. Four treatment groups were subsequently used: 1) vehicle (n=38), 2) 100 μg BPA/kg BW (n=36), 3) 250 μg BPA/kg BW (n=27), and 4) 200 nM BPA during *in vitro* prostasphere culture plus 250 μg BPA/kg BW (n=42). The latter group models BPA exposure from the stem cell stage through prostate morphogenesis *in vivo*. Tail vein blood was collected from host mice 20-30 min after feeding on exposure day 7 for quantitation of internal BPA levels. After 1 month, hormonal carcinogenesis was initiated though subcutaneous T+E pellets (25 mg T, 2.5 mg E_2) which models rising E_2 levels in aging men and promotes progressive carcinogenesis over 4 months (28). Host mice were euthanized at 2 and 4 months and the renal grafts were collected.

Histology and Immunohistochemistry

Grafted tissues were fixed in methacarn, dehydrated and embedded. Serial sections were stained with hematoxylin and eosin (H&E), coded and examined by two board-certified pathologists blinded to treatment. Tissues were classified for human prostate pathology according to criteria established by the Armed Forces Institute of Pathology and categorized as normal, squamous metaplasia (SQM), epithelial hyperplasia, high-grade PIN and adenocarcinoma. While multiple benign (SQM and hyperplasia) and

malignant (HG-PIN and cancer) lesions co-existed in several grafts, the malignancy incidence was ranked based upon the most severe lesion present. Immunohistochemistry was performed for confirmation of human prostate epithelial lesions using PSA, p63, CK14 and CK 8/18 as previously described (28).

Bisphenol A Quantitation

HPLC-grade solvents (acetonitrile, methanol and water) were purchased from Burdick & Jackson (Honeywell, Muskegon, MI) and were determined to be free of BPA contamination. BPA and [*rings*-$^{13}C_{12}$]-BPA-G were purchased from Sigma-Aldrich. Bisphenol A mono-β-D-glucuronide (BPA-G) was obtained from the Midwest Research Institute (Kansas City, MO). [d_6]-BPA was purchased from Cambridge Isotope Laboratories (Andover, MA).

Stock solutions of BPA and BPA-G were prepared in methanol at a final concentration of 1 mg/mL and stored in amber glass vials. Working standards were made by serial dilution from stock solutions. Calibration standards were prepared by mixing 1 µL of each working standard with 24 µL blank mouse serum and vortex mixing. Each unknown serum sample (25 µL) or calibration standard (25 µL) was mixed with 100 µL acetonitrile containing the surrogate standards 5 ng/mL [d_6]-BPA and 5 ng/mL [$^{13}C_{12}$]-BPA-G. The mixture was vortexed for 1 min, centrifuged for 15 min at 13000 x *g* at 4C, and the supernatant was removed and evaporated to dryness. The residue was reconstituted in 25 µL of 50% aqueous methanol and a 5 µL aliquot was injected onto the UHPLC-MS-MS system for analysis.

Chromatographic separations were carried out using a Shimadzu (Kyoto, Japan) LCMS-8050 triple quadrupole mass spectrometer equipped with a Shimadzu Nexera UHPLC system. BPA and BPA-G were separated on a Waters (Milford, MA) Acquity UPLC BEH (2.1 x 50 mm, 1.7 µm) C_{18} column. A 1.5 min linear gradient was used from 10-100% acetonitrile in water followed by a hold at 100% for 0.3 min at a flow rate of 0.4 mL/min. The total run time including equilibration was 3.5 min. The column oven temperature was 45C. Negative ion electrospray mass spectrometry with selected reaction monitoring (SRM) was used for the measurement of each analyte. Two SRM transitions (quantifier and qualifier) were monitored for each analyte as follows: BPA *m/z* 227 to 212 and *m/z* 227 to 133; [d_6]-BPA *m/z* 233 to 215 and *m/z* 233 to 113; BPA-G *m/z* 403 to 227 and *m/z* 403 to 113; and [$^{13}C_{12}$]-BPA-G *m/z* 415 to 239

and *m/z* 415 to 113. The SRM dwell time was 50 ms per transition. The lower limits of quantitation for BPA and BPA-G were 0.5 and 0.2 ng/ml, respectively while the lower limits of detection (LOD) for BPA and BPA-G in mouse serum were 0.2 and 0.1 ng/ml, respectively.

Statistical analysis

For in vitro studies, data were analyzed by Students t-test or ANOVA followed by post-hoc tests (GraphPad Software Inc, San Diego, CA). For *in vivo* studies, power analysis determined an N= 23/group was required for appropriate power, thus a minimum of 25/group was utilized in the present studies. Analysis of prostate lesion incidence between groups was performed using Fisher's Exact Test. Values are expressed as mean ± SEM and $p<0.05$ was considered significant.

RESULTS

Estradiol and BPA stimulate human prostate stem cell self-renewal and progenitor cell amplification

Initial studies had demonstrated that 1 nM E_2 exposure increased prostasphere number and size at day 7, indicative of a stimulation in stem cell self-renewal and progenitor cell proliferation, respectively (28). To determine the dose-response to E_2, prostaspheres were cultured in 1nM-1 μM E_2 for 7 days. As shown in Fig 1A, the maximal stimulatory effect of E_2 on sphere number and size was observed at 1 nM with higher E_2 doses having no further increase in 40-80 μm sphere numbers and a decreasing effect observed on spheroids >80 μm. Next, prostaspheres cultured for 7 days in 1-100 nM E_2 or vehicle were dispersed and FACS sorted for $Trop2^{+}/CD49f^{Hi}$ cells (Fig 1B). The mixed stem and progenitor cells were $CD49f^{+}$ while staining for $Trop2^{+}$ delineated a distinct subset of cells outside of the main population. Polygonal analysis of the $Trop2^{+}$ population demarcated distinct $CD49f^{+}$ subpopulations with $Trop2^{+}/CD49f^{Hi}$ designated as stem-like cells and $Trop2^{+}/CD49f^{Med}$ stained cells classified as early stage progenitor cells. Importantly, exposure to E_2 increased the proportion of $Trop2^{+}/CD49f^{Hi}$ stem-like cells in day 7 prostaspheres in a dose-dependent manner with maximal effects observed at 10 nM E_2 (Fig 1B). For further confirmation, a Hoechst exclusion assay was used to quantitate the stem-like cell side-population in parental primary cultures following E_2 exposure for 72 hr. Similar to the prostasphere assay, 1-10 nM E_2, but not 100 nM, significantly increased the percentage of stem-like cells in primary cultures indicating a stimulation of their self-renewal capacity (Fig 1C). Collectively, these findings show that estrogen stimulates human prostate epithelial stem cell self-renewal and progenitor cell amplification (prostasphere size), with greatest effects observed at lower E_2 doses.

Next, 3-D prostasphere and 2-D primary prostate epithelial cell cultures were exposed to BPA over a 0.1 nM to 1 μM dose range. Similar to E_2, BPA increased prostasphere number and size with significant and maximal effects observed at 10 nM BPA (Fig 1D). While higher BPA levels also increased 40-80 μm sphere numbers, the stimulatory effect was reduced on spheres > 80 μm in size as compared to 10 nM BPA.

Interestingly, FACS analysis of Hoechst-excluding cells in the primary cell cultures showed a marked dose-dependent increase in prostate stem-like cells following 72 hr BPA exposure, with maximal effects observed at 1 μM BPA (Fig 1E). Together, these results provide strong evidence that similar to E_2, BPA increases stem cell self-renewal and progenitor amplification in normal human prostate epithelial cells. Further, since the dose-response patterns for E_2 and BPA exhibit notable differences, the findings also suggest some non-overlapping actions for the two chemicals.

Estradiol and BPA enhance expression of prostasphere stemness genes.

The expression of genes associated with stemness and epithelial cell differentiation was evaluated in day 7 prostaspheres cultured in 1 nM E_2, 10 or 200 nM BPA. Exposure to 1 nM E_2 markedly increased expression of CD49f, TROP2, ABCG2, FOXM1, BMI1, TBX3, SOX2 and NANOG (Fig 2 A&B). In a similar manner, BPA exposure increased expression of some, but not all stemness genes with a significant increase in TBX3 and NANOG in response to 200 nM and a similar trend at 10 nM BPA (Fig 2B). Expression of epithelial cell differentiation genes NKX3.1, HOXB13, CK18 was at the lower limit of detection in day 7 prostaspheres and this was not influenced by E_2 or BPA exposure (Fig2C). Together, these findings provide further support that E_2 and BPA maintain the stem-like state within normal prostate epithelial population and again highlight that subtle differential responses may exist between the separate chemicals.

Estradiol and BPA have equimolar activational capacity for ER rapid signaling pathways but differential genomic ERE capacity in human prostaspheres.

Our previous findings demonstrated that normal prostate stem-progenitor cells within the prostaspheres expressed ERα and ERβ implicating them as direct targets for E_2 and BPA action (28). In the present study, we sought to ascertain whether E_2 and BPA effects were mediated through membrane-initiated ER signaling pathways and/or through classical nuclear genomic signaling mechanisms. To assess rapid signaling at the membrane, day 7 prostaspheres were briefly exposed to 10 nM of E_2 or BPA

for 15, 30, 60 min or 6 hr and assessed for p-Akt and p-Erk, well established downstream targets of membrane-associated ERs (37). Both 10 nM BPA and E_2 markedly increased p-Akt levels within 15 min with sustained levels for 60 minutes and reduction to baseline levels by 6 hr (Fig 3A). A nearly identical pattern was observed for Erk phosphorylation (Fig 3B). Importantly, BPA and E_2 had equimolar capacity for activation of these rapid signaling pathways in human prostaspheres, thus identifying a dynamic and robust signaling pathway initiated by low-dose BPA exposure in prostate stem-progenitor cells.

To identify whether classic genomic signaling (i.e. nuclear receptor-based transcription) is operative in human prostate progenitor cells, an ERE-luciferase reporter was transiently transfected into day 5 prostasphere cells followed by E_2 or BPA exposures. As seen in Figure 3C, 10 nM E_2 stimulated tk-luciferase activity above vehicle treated cells at levels equivalent to the positive control providing direct evidence that genomic ER signaling pathways are intact in the early progenitor cells of the human prostate. Similar E_2 responses were seen with a pS2-luciferase reporter (data not shown). In contrast, BPA at doses up to 1 μM did not elicit an ERE-based responses with the ERE-tk reporter (Fig 3C). Although genomic actions of BPA through EREs could not be confirmed in the present study, this activity has not been excluded as it remains possible that BPA may activate genomic ER signaling on natural promoters or over a different time frame than examined herein. Taken together, these findings identify that both rapid membrane-initiated estrogen action and genomic ER signaling pathways are operative in human prostate progenitor cells. Equimolar rapid actions for BPA and E_2 through membrane-initiated signaling provide a mechanistic framework for similar stimulatory actions on stem/progenitor numbers in the prostate epithelial population. Further, differential genomic-based signaling activities may be responsible for the noted differential dose-responses and stimulation of stemness gene transcription for E_2 and BPA in the present studies.

Developmental-stage, low-dose BPA exposure increases estrogen-driven carcinogenesis of human prostate epithelium

Next, the *in vivo* renal graft model of chimeric human-rat prostate tissues (28) was utilized to test whether developmental exposure to environmentally relevant levels of BPA would influence the susceptibility of the human prostate epithelium to hormone-driven carcinogenesis, as previously shown in the rat prostate gland (23, 38). Murine hosts were given daily oral exposure to BPA for 2 weeks following renal grafting and serum collected 20-30 min after feeding was used to quantitate the biologically relevant, internal BPA levels. The highly sensitive UHPLC-MS-MS system allowed direct quantitation of both free BPA and BPA-G in 25 μl sera, thus permitting measurement in individual mice from tail vein sampling (Fig 4A). As shown in Fig 4B, all vehicle-treated control mice had undetectable levels (<LOD) of BPA and BPA-G, documenting lack of contamination in the system. The circulating free BPA in mice fed 100 μg BPA/kg BW and 250 μg BPA/kg BW, was 0.39 ng/ml and 1.35 ng/ml, respectively. Levels of BPA-G were ~ 10 times higher than free-BPA indicating that ~10% of oral-exposed BPA was bioavailable 20-30 min after ingestion. Together, these results document that levels of bioactive BPA in the present study are similar to levels found in human umbilical cord blood and newborns in the general population (22).

When the chimeric prostate grafts reached maturity at 1 month, hormonal carcinogenesis was initiated/promoted through the administration of T+E pellets for 2-4 months. As previously observed (28), progressive malignancy incidence was noted over time with highest HG-PIN and cancer incidence present at 4 months. Examples of HG-PIN and prostate adenocarcinoma as well as benign lesions of hyperplasia and SQM are shown in Fig 5 with PSA immunostaining to confirm their human origin and lack of basal cell staining to confirm carcinoma. Since the renal graft model is limited to 5-6 months, the combined HG-PIN and adenocarcinoma incidence was used as a surrogate for malignant prostatic growth while combined hyperplasia and SQM was used to quantify overall benign lesion incidence within the prostatic tissues. As shown in Table 1, prostate grafts from animals treated with vehicle during tissue development plus T+E exposure at maturity had an overall incidence of 26% normal grafts, 74% benign lesions and 13% malignant lesions (Note: malignant lesions always co-appeared in grafts containing

benign lesions). Developmental exposure to either 100 or 250 μg BPA/kg BW reduced the normal prostate incidence to 11 % and 0% ($P < 0.01$), respectively, augmented the benign lesion incidence to 89 and 100 % ($P<0.05$), respectively and increased the malignant lesions incidence to 36% and 33%, respectively ($P<0.02$). With the addition of *in vitro* BPA exposure to prostaspheres for 7 days prior to *in vivo* BPA exposure (250 μg/kg BW), to model continuous BPA exposure throughout development, the incidence of malignant lesions further increased to 45% ($P<0.05$). Together, the present findings identify for the first time that *in vivo* exposure of the human prostatic epithelium to low-doses of BPA significantly increases the susceptibility of the human prostate epithelium to hormonal carcinogenesis.

DISCUSSION

Increasing evidence has shown that exposure to low levels of BPA can contribute to multiple adverse health outcomes that include behavioral impairment, infertility, metabolic disorders and increased risk for mammary and prostate cancer (39, 40). The developmental stage has been identified as particularly sensitive to BPA exposures, a phenomenon attributed to tissue organizational processes and molecular programming that occur early in life (23, 41, 42). Additionally, recent findings have shown that stem cells can be reprogrammed by EDCs, which may perpetuate life-long changes in tissue growth and function (32, 43-45). While a growing body of work indicates that humans are equally susceptible to adverse effects of BPA, the majority of research on BPA has been derived in animal models (39). In this context, previous work from our laboratories using a rodent model established a direct link between developmental BPA exposure at environmentally relevant doses and increased susceptibility to estrogen-driven carcinogenesis with aging (23-25). At present, there is a compelling need to assess whether the developing human prostate is similarly reprogrammed by low-dose BPA exposures and whether this may influence prostate cancer risk in men as they age.

The current study provides clear evidence that, similar to E_2, normal human prostate stem and progenitor cells are direct targets for BPA action. Both hormones increased stem-like cell numbers in primary prostate epithelial cultures in a dose-dependent manner and augmented the number and size of 3-D cultured prostaspheres, markers of stem-cell self-renewal and progenitor cell proliferation, respectively. Combined with elevated expression of stem-related genes including SOX2, NANOG and TBX3 in the prostaspheres and a lack of effects on differentiation gene expression, these data indicate that BPA and E_2 have the capacity to amplify the human prostate epithelial stem/progenitor cell populations. This is supported by a recent study using *in vitro* differentiation of human embryonic stem cells into mammospheres which reported that 1-100 nM BPA or 1 nM E_2 increased NANOG and OCT4 levels, suggesting an enhancement of stemness within mammary epithelial cells (46). Although BPA failed to promote adipogenesis in murine mesenchymal stem cells, 10 nM BPA was capable of directing

preadipocytes to adipocytes which suggests a stage-specific or lineage-specific effect of BPA on stem and progenitor cell populations (44). In the present study, a dose-response effect was observed with peak stimulatory effects on prostasphere growth observed at 1 nM E_2 and 10 nM BPA and a declining influence noted on sphere size at higher doses which provides a physiologic and environmentally relevant context to the present data on E_2 and BPA. While a similar bell-shaped dose-response to E_2 levels was found in the stem-like cell population of the parental primary prostate cultures, a linear dose-response was noted with rising BPA, reaching maximal effects at 1 μM BPA. These distinct effects are likely driven by activation of different ERs and/or separate downstream signaling pathways by E_2 and BPA in the stem and progenitor cell populations. An alternate possibility is that BPA may engage non-ER pathways in prostate stem cells, as has been shown in other systems including directed differentiated mammospheres (46-48).

Estrogen action is mediated by ERα and ERβ and may also be initiated by GPR30, all of which are expressed by early-stage human prostate stem-progenitor cells (28). Furthermore, signaling pathways engaged by estrogens through these separate receptors are multiple and complex, including both membrane-initiated signaling as well as genomic activation via ER transcriptional activity (49, 50). Importantly, the current findings demonstrate that BPA and E_2 at 10 nM concentrations have equimolar activational capability in human prostaspheres through membrane-initiated, rapid signaling pathways that include p-Akt and p-Erk. This provides multiple and divergent mechanisms that can be subsequently initiated in prostate stem/progenitor cells by E_2 and BPA through phosphorylation of numerous downstream proteins leading to altered cellular response. One recent example was reported in the rat postnatal prostate where BPA activated PI3K/Akt signaling and rapidly diminished tissue H3K27me3 levels (33) suggesting reduced EZH2 activity, a known downstream action of p-Akt (51). Several studies have previously identified equimolar activational activity between E_2 and BPA at low doses through rapid signaling pathways in a number of cell types including cardiomyocytes (52), pituitary (53), pancreatic (54) and neural cells (55) and the results herein extend this to a stem/progenitor cell population. The

present study also differential capacity of E_2 and BPA for classic genomic actions through ER-ERE activation, with 10 nM E_2 showing strong ERE-luciferase induction whereas no effects were observed on this reporter system by 10 nM-1 μM BPA. It is important to note, however, that these results do not rule out genomic actions of BPA at natural reporters in prostate stem/progenitor cells but rather emphasize differential transcriptional capacities of E_2 and BPA at reporter constructs. The present findings are consistent with early studies which show that BPA has reduced affinity and activational capacity for nuclear ER-ERE signaling (56). In contrast, a recent report demonstrating ERE-luciferase activation by BPA in Ishikawa cells, HeLa cells and HepG2 cells co-transfected with ERα or ERβ at 1 nM-1 μM doses (57) which suggests that ER expression levels, co-activator availability and/or cell specificity may contribute to divergent genomic ER responses by BPA. Differential engagement of signaling pathways by E_2 and BPA in the human prostate stem/progenitor cells are the likely basis for non-overlapping actions of these chemicals in the current work. Ongoing studies are aimed at dissecting downstream pathways activated through p-Akt and p-Erk as well the separate roles played by ERα and ERβ in mediating E_2 and BPA actions on these cell populations.

A primary goal of the current studies was to evaluate potential cancer promoting actions of developmental BPA exposures on the human prostate epithelium *in vivo*. The present findings provide the first evidence that exposure of the developing human prostate epithelium to BPA at relevant human exposure levels markedly increases the incidence of prostate carcinogenesis in the mature epithelium exposed to elevated estradiol. Together, these data contribute to the increasing body of evidence for a link between fetal exposures to EDCs and cancer (58). *In vivo* BPA exposure during the time of stem-progenitor cell lineage commitment, differentiation and formation of glandular epithelium significantly boosted the rate of T+E driven carcinogenesis from 13% in vehicle exposed hosts to 33-36% malignancy. This carcinogenic rate further increased to 45% when developmental BPA exposure was extended to *in vitro* stem cell cultures, implicating the stem-progenitor cells as the direct BPA targets, a hypothesis supported by the *in vitro* studies discussed above. That these malignant lesions arose from normal

prostate epithelium in the relatively short time frame of 2-4 months, as compared to decades in humans, is particularly noteworthy and highlights the potent nature of this EDC on augmenting carcinogenic susceptibility. It is important to emphasize that the stem cells in the present study were cultured from disease-free prostates of young organ donors making it unlikely that the cells had prior initiating events. This implicates BPA exposure as a potential initiating event in human prostate stem-like cells, one that enables promotion by rising estrogens later in life, as occurs in aging men (21). This is similar to our previous findings in a rat model (23, 24) and provides strong evidence that these earlier rodent data are directly relevant to the human disease. The molecular underpinnings of altered prostate memory to estrogenic exposures are likely due to complex reprogramming of the prostate epigenome as shown in our rodent model (23, 25) and studies to identify these specific reprogrammed genes in the human prostate stem-progenitor cells are underway.

The issue of BPA dose levels, route of exposure, pharmacokinetics, potential contamination and methods of quantitation have raised considerable debate in the recent past (39, 59). Consequently strict standards must be adhered to when modeling for human disease. To address these concerns, the present study utilized 2 *in vivo* oral BPA doses in the adult host mice that accurately model internal dose levels of unconjugated or free BPA observed in human fetal cord blood, fetal serum and infants (16, 60-62). Most recently, a study on mid-gestational umbilical cord blood in 85 patients using a new analytical approach that permitted direct measurement of free and glucuronidated BPA by LC-MS-MS reported free BPA levels ranging from <LOD (0.05) to 52 ng/ml with a geometric mean of 0.16 ng/ml in human cord serum (22). Of note, a subset of mid-gestation fetuses was found that had relatively high levels of unconjugated BPA with three fetuses above 18 ng/ml in a system validated as contamination free. Using a similar analytic approach, we herein developed an UHPLC-MS-MS method utilizing labeled BPA and BPA-G standards that permits direct quantitation of free BPA and BPA-G in < 25 μl serum. While all vehicle control mice had BPA < LOD, levels of free BPA were 0.39 and 1.35 ng/ml in our two dosing groups. This permits us to conclude that the BPA exposure levels provided to the humanized prostate during a

two week developmental window are the same as human fetal exposures, making the present findings directly relevant to the daily human experience.

In summary, the present findings demonstrate that the stem-progenitor cells in the normal human prostate gland are direct targets of estrogenic actions and that similar to E_2, BPA can activate downstream signaling pathways that lead to increased self-renewal and maintenance of their stem-like nature. We propose that early-life perturbations in estrogen signaling including inappropriate exposure to BPA, have the potential to amplify and modify the stem-progenitor cell populations within the human prostate gland and, in so doing, alter the normal homeostatic mechanisms that maintain a growth neutral state throughout life. Importantly, the current results indicate that developmental exposure to BPA, at doses routinely found in humans, significantly increases the cancer risk in human prostate epithelium in response to elevated estrogen levels in an androgen-supported milieu. Since relative estrogen levels rise in aging men, we suggest that humans may be susceptible to BPA-driven prostate disease in a manner similar to the rodent models.

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FIGURE LEGENDS

Figure 1. Dose-response effects of E_2 and BPA on prostasphere growth and self-renewal of stem-like cells in primary prostate epithelial cell cultures. **A**: Prostaspheres were grown for 7 days in a 3-D Matrigel culture in the absence (C) or presence of 1 nM-1 μM of E_2. Prostasphere number and size (40-80 μm, >80 μm diameter) were measured by digital imaging (N=6). *P<0.05, **P<0.01 vs vehicle control, $^{+}$P<0.05 vs 1 μM E_2. **B**: Prostaspheres cultured for 7 days with vehicle or 1-100 nM E_2 were dispersed and FACS sorted using CD49f-APC and Trop2-AF488 antibodies. Summit software polygonal analysis of Trop2+ cells identified a Trop2+/CD49f^{Hi} cell subpopulation (red) designated as stem-like cells (sc) and a Trop2+/CD49f^{Med} subpopulation (green), classified as early stage progenitor cells (pc). Representative images of a flow cytometry scatter plots are shown without and with polygonal frames for spheres treated with control vehicle or 1 nM E_2. Exposure to 1-100 nM E_2 increased the % of TROP2+/CD49f^{Hi} stem-like cells in the day 7 prostaspheres. * P<0.05 vs control (N=3). **C:** Parental 2-D PrEC cells (N=6) were treated with 1-100 nM E_2 for 72 hr and % prostate stem-like cells was evaluated by Hoechst-dye exclusion based flow cytometry. 1-10 nM E_2 increased the stem-like population. * P<0.05 vs control (N=6). **D:** Prostaspheres were cultured for 7 days in the absence (C) or presence of 0.1 nM-1 μM BPA and prostasphere number and size were digitally analyzed. A stimulatory effect was first noted at 10 nM BPA. * P<0.05 vs control (N=5). **E:** Parental 2-D PrEC cells were treated with 10, 200 or 1000 nM BPA for 72 hr and Hoechst-dye exclusion assay with FACS was used to determine the % stem-like cells present. Treatment with increasing BPA doses augmented the stem-like fraction suggesting stimulation of stem-cell self-renewal. *P<0.05, **P<0.01 vs vehicle control (N=6).

Figure 2. Estradiol and BPA augment expression of prostasphere stemness genes and do not alter genes associated with cell differentiation. Prostaspheres were grown for 7 days in a 3D Matrigel culture in the absence or presence of 1 nM E_2 or 10-200 nM BPA. **A:** 1 nM E2 treatment significantly increased gene expression of all examined stem cell genes including ABCG2, FOXM1, BMI1, CD49f, TROP2, TBX3 and SOX2. **B:** Similar to 1 nM E_2, 200 nM BPA significantly increased NANOG and

TBX3 expression. **C:** E_2 and BPA exposures did not alter HOXB13, NKX3.1 or CK18 expression, indicating lack of influence on entry into differentiated luminal cell lineage. *P<0.05 vs vehicle control (N=4-6 assays from separate donor cultures).

Figure 3. Estradiol and BPA have equimolar activational capacity for ER rapid signaling pathways but differential genomic ERE capacity in human prostaspheres. A,B: Day 7 prostaspheres grown in the absence or presence of 10 nM BPA or E_2 (n=4) for 15, 30, 60 min or 6 hr were assessed for p-Akt/total Akt (A) and pErk/total Erk (B) by western blot assay. Representative blot shown (top) and graphic representation of densitometry analysis from 3 separate cultures (bottom) are shown. Both 10 nM E_2 and 10 nM BPA markedly increased Akt phosphorylation at 15, 30 and 60 min with a return to baseline activity by 6 hr (A), *P<0.05, **P<0.01 vs vehicle. A similar temporal profile was observed for ERK phosphorylation (B), *P<0.05, *** P<0.001 vs vehicle. **C:** Genomic ERE signaling activity in day 7 prostaspheres was evaluated using an ERE-tk-Luc reporter assay (see Methods). Day 6 prostaspheres pre-transfected with luciferase ERE-tk reporter and a renilla-luciferase control construct were treated with 10 nM E_2 or 1 μM BPA for 16 hr and ERE reporter activity was measured by Dual-Luciferase assay. 10 nM E_2 increased tk-luciferase activity ~2.5 fold compared to the vehicle treated cells (n=3), similar to activity seen in the positive control vector. Treatment with 1 μM BPA did not increase luciferase activity above vehicle treatment .

Figure 4. BPA and BPA-G quantitation in mouse serum 20-30 min following oral exposure. A: Ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS-MS) chromatograms showing BPA and BPA-G in mouse serum from animals receiving 250 μg BPA/kg BW in oil (solid line) or oil vehicle as a control (dashed line). **B:** Levels of free BPA and BPA-G by direct quantitation in sera of individual mice treated with vehicle, 100 or 250 μg BPA/kg BW. Sera was collected 20-30 min after oral exposure on day 7 of daily feeding to adult host mice. LOD= limit of detection. * Total = free BPA + BPA-G.

Figure 5. Hormonal carcinogenesis in human prostate epithelial of chimeric prostate renal grafts induced by T+E. H&E staining and immunofluorescent immunocytochemistry was used to classify and confirm prostate pathology in renal graft tissues following 2-4 months T+E treatment to host mice. **A:** Normal chimeric prostate-like tissue formed from recombination of human prostasphere cells with rat urogenital sinus mesenchyme after 1 month of growth under the renal capsule of host nude mice. **B:** Squamous metaplasia (SQM) was frequently observed in the prostatic grafts after 2-4 months of T+E treatment. **C:** Extensive prostate epithelial hyperplasia with narrowed lumens was observed in most of the tissue grafts after T+E exposure. **D:** Hormonal carcinogenesis induced by T+E was observed in some chimeric grafts as evidenced by areas of HG-PIN with piling and overlapping epithelial cells, nuclear enlargement, hyperchromasia, and prominence nucleoli. **E:** Adenocarcinoma of human prostate epithelium in prostatic grafts following T+E exposure. Heterogeneous small glandular-like structures containing epithelial cells with enlarged nuclei and prominent nucleoli within the underlying stromal region. **F:** Confirmation of prostatic cancer was made using immunofluorescent labeling showing presence of luminal epithelial cell marker CK8/18 (red) and absence of basal cell marker CK14 (green) in the small, glandular-like cancerous regions adjacent to normal ducts (CK14+) in the prostatic grafts. **G,H:** Immunofluorescent labeling for PSA (red) confirms the human identity of the normal prostate epithelium (G) and cancerous areas (H) within the grafted tissues. *Scale bar*, 50 µm.

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Table
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Table 1. Effects of developmental BPA exposure on pathology incidence in human prostate epithelium treated *in vivo* with T+E.

	Oil	**BPA** **100 µg/kg *in vivo***	**BPA** **250 µg/kg *in vivo***	**BPA** **200 nM *in vitro*** **250 µg/kg *in vivo***
N	38	36	27	42
Normal	10 (26%)	4 (11%) *P*=0.093	0 (0%) *P*=0.008[b]	4 (10%) *P*=0.061
Abnormal: Benign Hyperplasia/SQM	28 (74%)[c]	32 (89%)[c] *P*=0.061	27 (100%)[c] *P*=0.035[a]	38 (90%)[c] *P*=0.003[b]
Abnormal: Cancerous HG-PIN/PCa	5 (13%)[c]	12 (36%)[c] *P*=0.016[a]	9 (33%)[c] *P*=0.001[b]	19 (45%)[c] *P*=0.038[a]

[a] $P<0.05$ and [b] $P<0.01$ vs oil using Fisher Exact Test

[c] some specimens contain multiple diagnoses.

E2 and BPA on prostasphere growth

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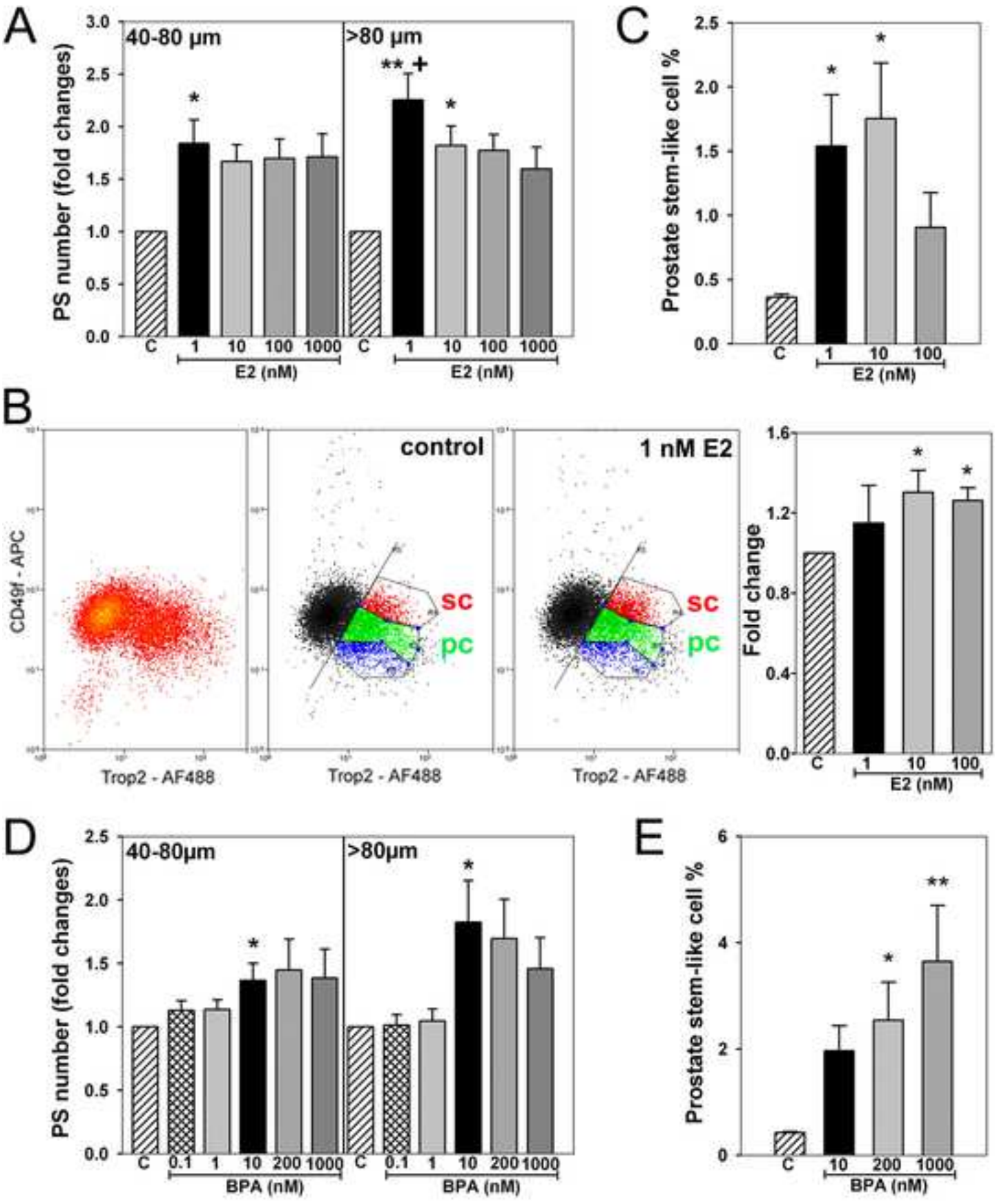

E2 and BPA on gene expression
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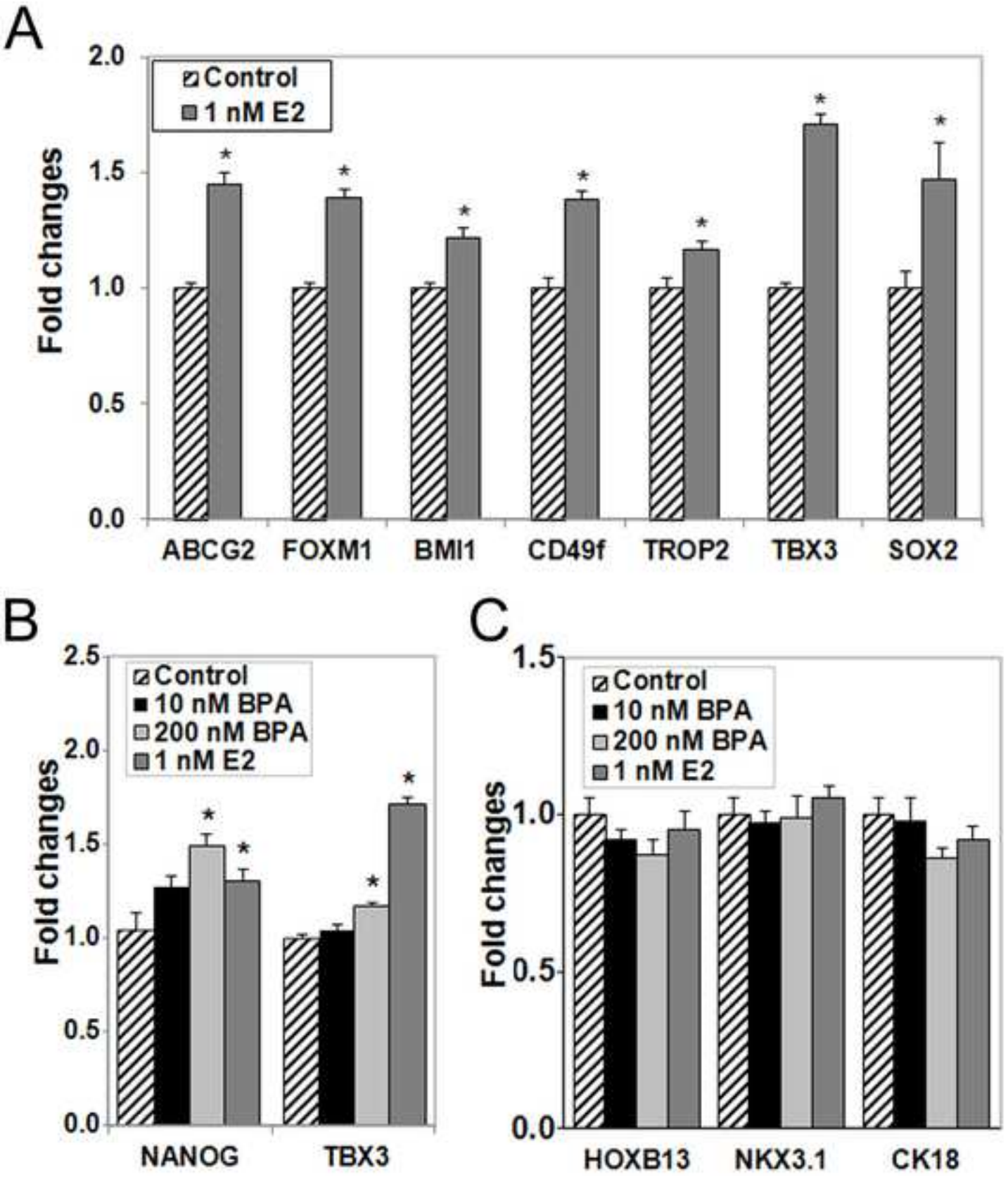

E2 and BPA Signaling Pathways
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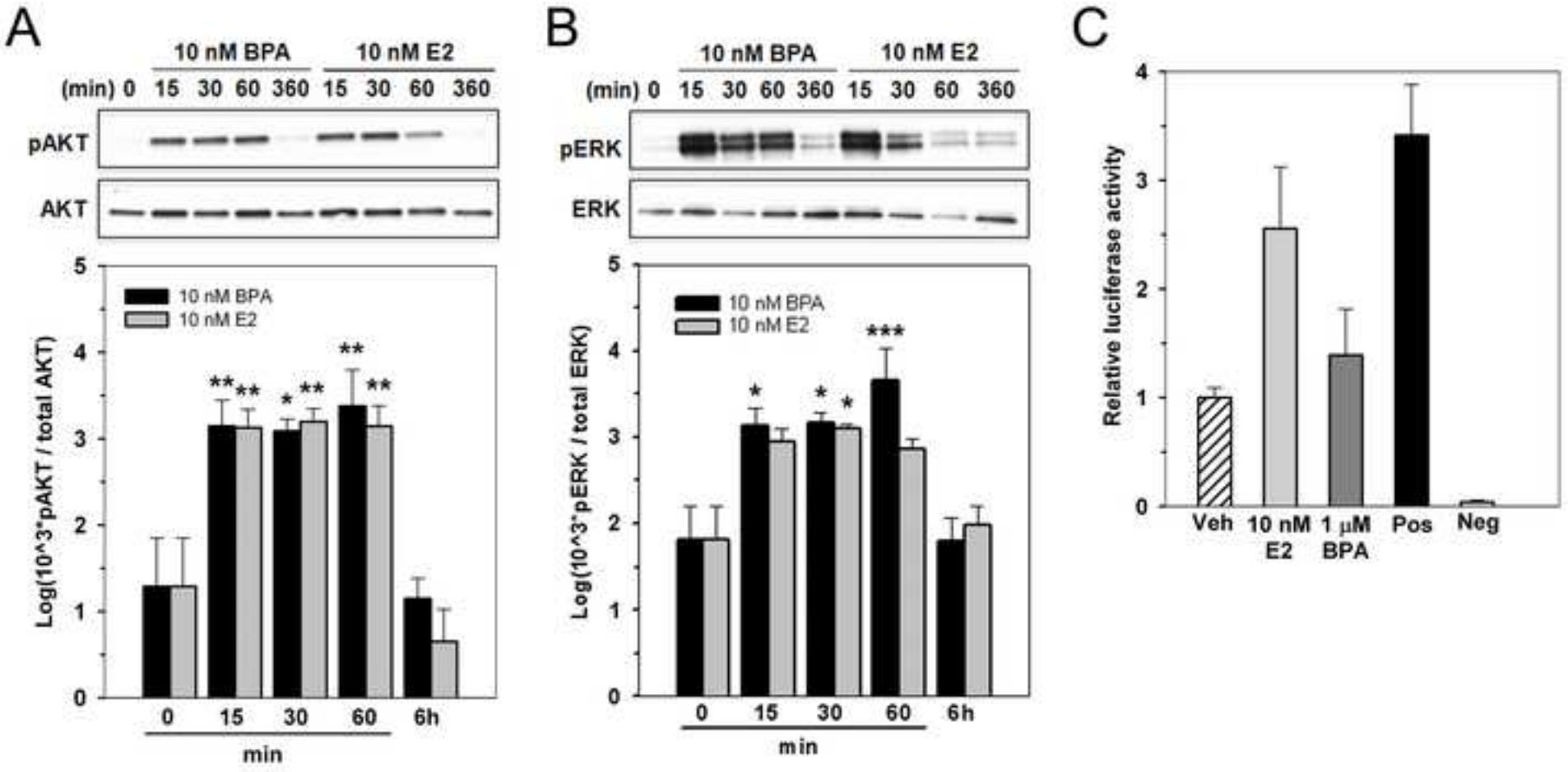

BPA and BPA-G quantitation
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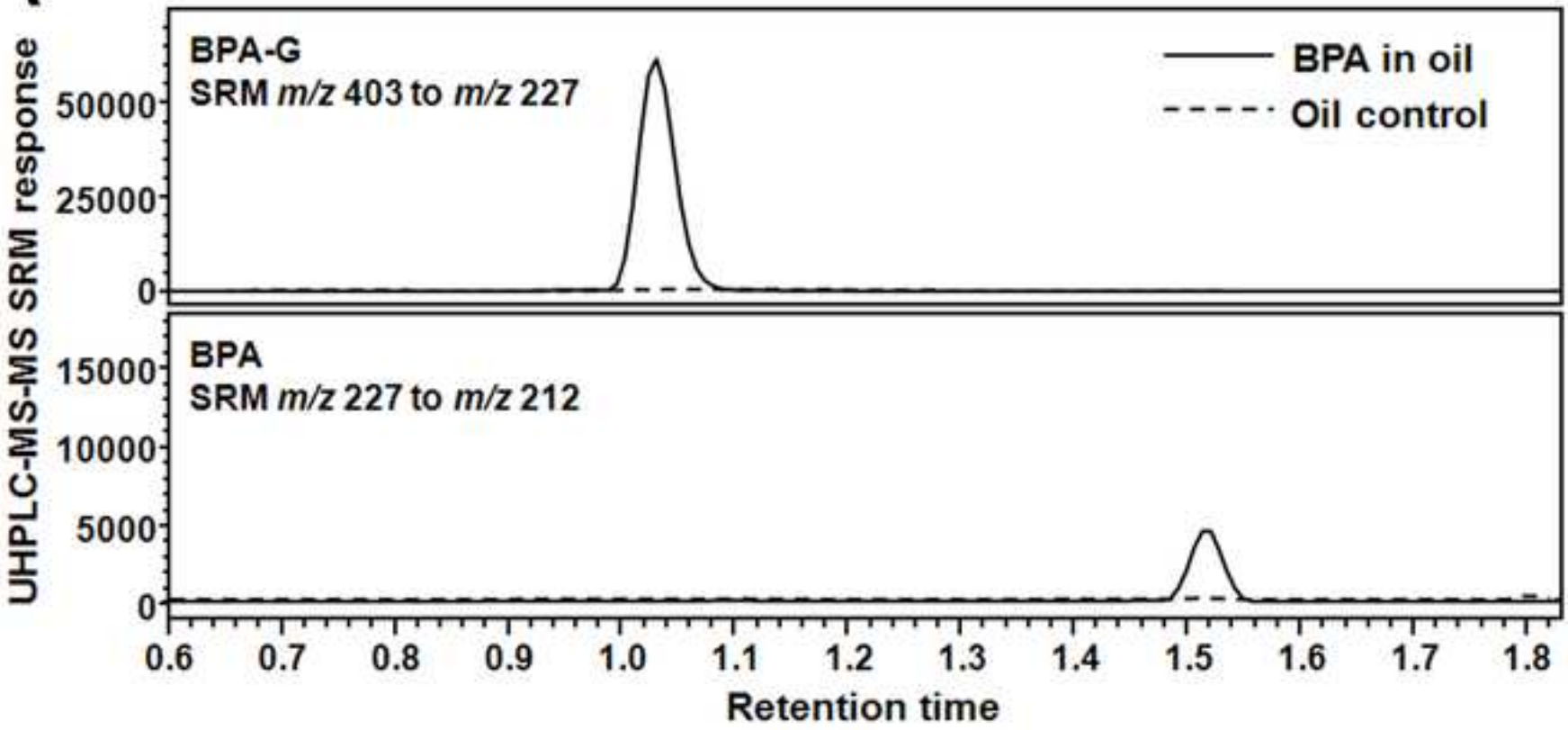
A



B

Free BPA and BPA-G levels in adult host mice sera

Treatment (µg BPA/kg BW)	BPA Concentration (ng/mL)		
	Free	Glucuronidated	Total*
oil control (N=9)	<LOD	<LOD	<LOD
100 (N=12)	0.393 ± 0.167	4.492 ± 0.420	4.884 ± 0.587
250 (N=14)	1.349 ± 0.292	11.779 ± 1.316	13.127 ± 1.608

Hormonal carcinogenesis in renal grafts
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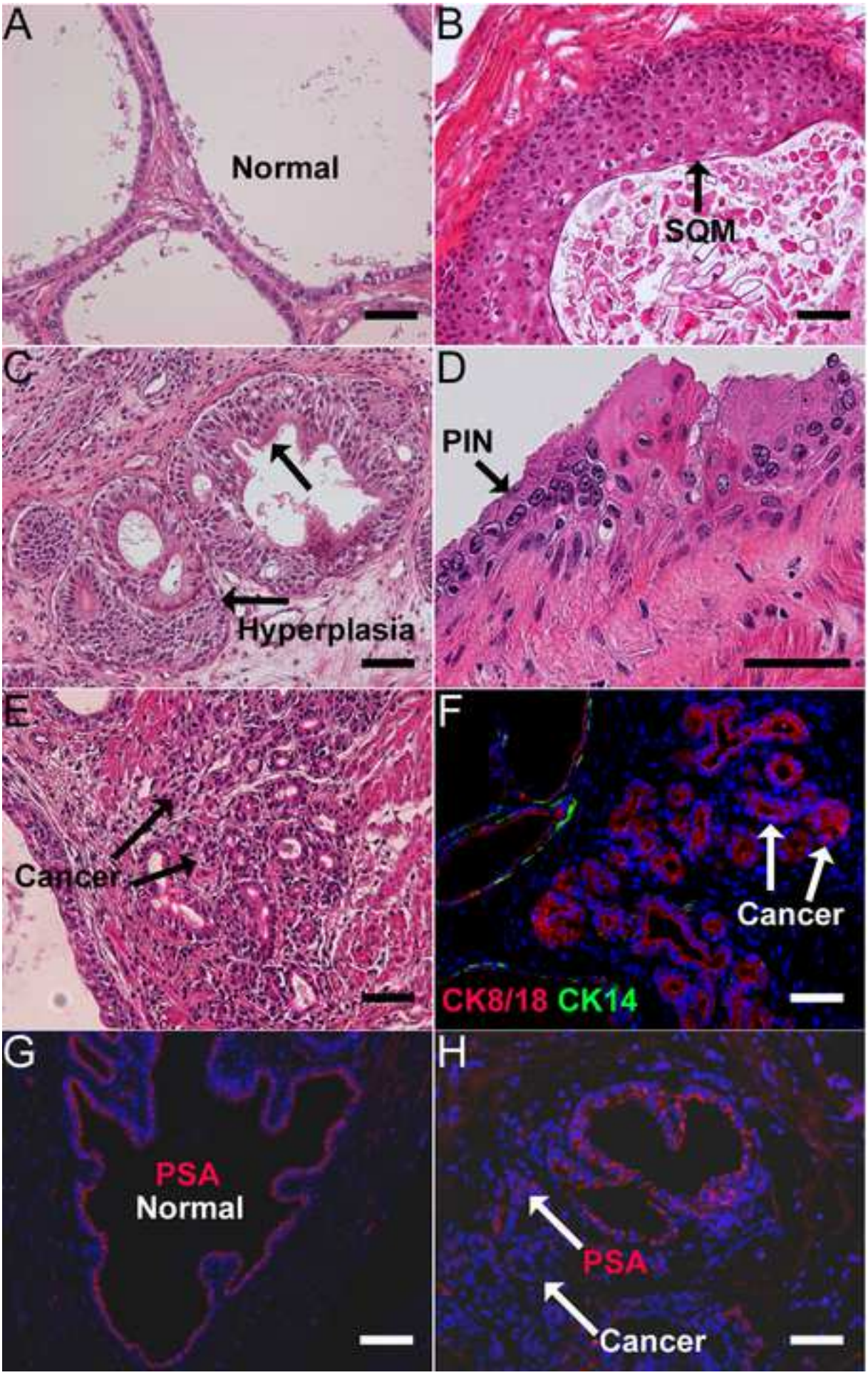

Supplemental Table

Click here to download Supplemental Material: Supplemental table S1.pdf

Supplemental Methods-Figure legends

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Supplemental Figure 1

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Supplemental Figure 2

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Antibody Table
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Peptide/protein target	**Antigen sequence (if known)**
Cytokeratin8/18	
Keratin 14	
Prostate Specific Antigen (PSA)	
CD49f (Integrin alpha 6)	
Trop2 (EGP-1)	
Phospho-Akt	
Phospho-p44/42MAPK (Erk1/2)	
Akt(pan)	
Erk1/2	

Name of Antibody	**Manufacturer, catalog #, and/or name of individual providing the antibody**
Anti-Cytokeratin 8/18	ARP American Research Products, Inc. 03-GP11
Keratin 14	COVANCE, PRB-15P
Anti-Prostate Specific Antigen (PSA)	Millipore
Anti-Human/Mouse CD49f (Integrin alpha 6) APC	Ebioscience; 17-0495-82
Anti-Human Trop2 (EGP-1) Alexa Fluor® 488	Ebioscience; 53-6024-82
Phospho-Akt(Ser473)	Cell signaling, 4060
Phospho-p44/42MAPK (Erk1/2)	Cell Signaling, 4370
Akt (pan)	Cell signaling, 4691
p44/42 MAPK (Erk1/2)	Cell signaling, 4695

Species raised in; monoclonal or polyclonal	**Dilution used**
Guinea Pig; Polyclonal	1:800
Rabbit; Polyclonal	1:1000
Mouse; Monoclonal	1:200
Rat; Monoclonal	0.25ug/ml
Mouse; Monoclonal	0.01ug/ml
Rabbit, Monoclonal	1:1000
Rabbit, Monoclonal	1:1000
Rabbit, Monoclonal	1:1000
Rabbit, Monoclonal	1:1000

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