

## Prins Laboratory - Material and Methods

Description of the test articles used, study material evaluations (diet, drinking water, cage and bedding leachates), general study design, animal treatments, and animal allocations to the 2 year toxicology study conducted at NCTR and the grantee studies can be found in Heindel et al. (2015).

### Animals

*Set #8 (6 month old, Continuous Dose):* SD male rats treated from gestation day (GD) 6 until sacrifice at 6 months of age, from 5 treatment groups: Vehicle (n=12); 2.5 µg BPA/kg BW/day (n=9), 25 µg BPA/kg BW/day (n=9), 250 µg BPA/kg BW/day (n=10), and 0.5 µg EE2/kg BW/day (n=9).

*Set #10 (12 month old, Continuous Dose):* SD male rats treated from GD 6 until sacrifice at 12 months of age, from 7 treatment groups: Vehicle (n=9); 2.5 µg BPA/kg BW/day (n=7), 25 µg BPA/kg BW/day (n=8), 250 µg BPA/kg BW/day (n=10), 2500 µg BPA/kg BW/day (n=8), 25000 µg BPA/kg BW/day (n=9), and 0.5 µg EE2/kg BW/day (n=10).

*Set #10 (12 month old, Stop Dose):* SD male rats treated from GD 6 until 21 days of age and sacrificed at 12 months of age, from 7 treatment groups: Vehicle (n=10); 2.5 µg BPA/kg BW/day (n=8), 25 µg BPA/kg BW/day (n=9), 250 µg BPA/kg BW/day (n=11), 2500 µg BPA/kg BW/day (n=10), 25000 µg BPA/kg BW/day (n=10), and 0.5 µg EE2/kg BW/day (n=9).

*Set #11 (12 month old, Stop Dose):* SD male rats treated from GD 6 until 21 days of age and sacrificed at 12 months of age, from 7 treatment groups: Vehicle (n=19); 2.5 µg BPA/kg BW/day (n=8), 25 µg BPA/kg BW/day (n=5), 250 µg BPA/kg BW/day (n=14), 2500 µg BPA/kg BW/day (n=7), 25000 µg BPA/kg BW/day (n=15), and 0.5 µg EE2/kg BW/day (n=20).

Each rat was from a different litter within each dose group with a few exceptions, as noted in the “Dam Cage” column in the data files (animals with the same dam cage number were littermates).

### Hormone subcutaneous (sc) implant insertion at day 90:

*Set #11 (12 month old, Stop Dose):* At age day 90, rats were implanted sc with hormone implants to drive carcinogenesis. Under sterile conditions, fur was removed from the flank area and the incision site was swabbed with Betadine solution. A small subcutaneous incision was made just above the right flank region and a pocket was created large enough to accept the 3 prepared implants. A small forceps was used to insert Silastic capsules (Dow Corning, Midland, MI; i.d. 1.98 mm, o.d. 3.18 mm) packed with estradiol (one 1-cm tube) and testosterone (two 2-cm tubes) (T+E) to drive prostate carcinogenesis, as described in Ofner et al. (1992).

### Tissue Collection for Culture

*Set #8 (6 month old, Continuous Dose):* Dorsal prostates (DP) were collected and processed for cell culture. Ventral prostates (VP) and bladder were collected and weighed. Tissues were removed under sterile technique. The prostate tissues, including the entire accessory sex gland complex, was removed and immediately immersed *en bloc* in prepared 4C Dissection Media. Dissection Media consisted of 445 mL DMEM (Gibco #11965-092), 50 mL FBS (10% v/v; Gibco #10427-028) and 5 mL 100X Antibiotic-Antimycotic (1x final concentration; Gibco #15240-062) and dispensed into 45 mL aliquots in sterile 50cc conical tubes.

One media tube was used per prostate/accessory sex gland complex per dose group and kept at 4C. Tissues were shipped the same day on ice packs from NCTR to Prins' laboratory for processing.

Upon receipt of the prostate/accessory sex gland complex, the VP, DP, and bladder were excised under sterile conditions. Weights for the left and right sides of the VP lobe and the bladder were measured.

#### Dorsal Prostate Prostasphere Cultures

*Set #8 (6 month old, Continuous Dose):* DP tissues were minced and digested with collagenase for 2hr at 37C to collect DP cells for subsequent 3D prostasphere (PS) cultures, as described in previous publications (Hu et al., 2011; Prins et al., 2014). The second passage of day 7 PS were photographed and analyzed for PS number and size (40-80µm, >80µm, and >40µm). The third passage of day 7 PS were split into 2 treatment groups and cultured with and without 1nM estradiol (E2) and analyzed for PS number and size as above per 10,000 PS cells and for qPCR gene expression. The nine genes of interest analyzed were RPL19, Tbx3, Tacstd2 (Trop2), Sox2, ChgA, Hoxb13, KRT5 (CK5), KRT8 (CK8), and TP63 (p63).

#### Tissue Collection for Histology and RNA-Seq

*Set #10 (12 month old, Continuous Dose):* The VP and dorsolateral prostate (DLP) lobes were collected and processed for paraffin embedding and subsequent hematoxylin eosin (H&E) staining and analysis. Tissues excised from 12 month old male rats included the seminal vesicles, coagulating glands (along with prostate and urethra complex) and were fixed en masse in 10% neutral-buffered formalin (NBF) for 72hr. Three paraffin blocks were cast per animal consisting of Block 1 (both seminal vesicles and coagulating glands), Block 2 (VP lobes) and Block 3 (DLP cut in half transversely, so each half included the DP and lateral prostate (LP)). Blocks were shipped at room temperature from NCTR to Prins' laboratory.

*Set #10 (12 month old, Stop Dose):* One lobe each of VP, DP, and LP from the right side of the prostate were collected, wrapped individually in foil, and snapped frozen in liquid nitrogen. The remaining accessory sex gland complex (including the left side prostatic lobes) were removed and fixed en masse in 10% NBF for 72hr. Three paraffin blocks were cast per animal consisting of Block 1 (both seminal vesicles and coagulating glands), Block 2 (left side VP lobe), and Block 3 (left side DLP cut in half transversely, so each half included the DP and LP). Frozen tissues were shipped from NCTR to Prins' laboratory on dry ice and stored at -20C. Blocks were shipped at room temperature.

*Set #11 (12 month old, Stop Dose):* The VP and DLP lobes from 12 month old male rats were collected and processed for paraffin embedding and subsequent H&E staining and analysis. Tissues excised from 12 month old male rats included the seminal vesicles, coagulating glands (along with prostate and urethra complex) and were fixed en masse in 10% NBF for 72hr. Three paraffin blocks were cast per animal consisting of Block 1 (both seminal vesicles and coagulating glands), Block 2 (VP lobes), and Block 3 (DLP cut in half transversely, so each half include the DP and LP). Blocks were shipped at room temperature from NCTR to Prins' laboratory.

#### Histology

Blocks were sectioned and H&E stained in the UIC Research Histology and Tissue Imaging Core. Pathologist Dr. Maartin Bosland analyzed the slides blinded to treatment.

### RNA-Seq

*Set #10 (12 month old, Stop Dose):* Snap frozen DP tissues from 4 treatment groups: Vehicle (n=4); 25 µg BPA/kg BW/day (n=4), 250 µg BPA/kg BW/day (n=4), and 2500 µg BPA/kg BW/day (n=4) were shipped from Prins' laboratory to Dr. Shuk-mei Ho's laboratory (University of Cincinnati, OH) for RNA-Seq analysis. RNA-Seq methods were previously described in Leung et al. (2017).

### Statistical Analysis

Data were analyzed by ANOVA followed by Post-Hoc Fischer's test.

### **References:**

Heindel JJ, Newbold RR, Bucher JR, Camacho L, Delclos KB, Lewis SM, Vanlandingham M, Churchwell MI, Twaddle NC, McLellen M, Chidambaram M, Bryant M, Woodling K, Gamboa da Costa G, Ferguson SA, Flaws J, Howard PC, Walker NJ, Zoeller RT, Fostel J, Favaro C, Schug TT. NIEHS/FDA CLARITY-BPA research program update. *Reprod Toxicol*. 2015 Dec; 58:33-44.

Hu W-Y, Shi G-B, Lam H-M, Hu D-P, Ho S-M, Madueke-I, Kajdacsy-Balla A, Prins GS. Estrogen-initiated transformation of prostate epithelium derived from normal human prostate stem-progenitor cells. *Endocrinology*. 2011, 152(6): 2150-63. PMID: 21427218.

Leung YK, Govindarajah V, Cheong A, Veevers J, Song D, Gear R, Zhu X, Ying J, Kendler A, Medvedovic M, Belcher S, Ho SM. Gestational high-fat diet and bisphenol A exposure heightens mammary cancer risk. *Endocr Relat Cancer*. 2017, Jul; 24(7): 365-378. PMID: 28487351.

Ofner P, Bosland MC, Vena RL. Differential Effects of Diethylstilbestrol and Estradiol-17β in Combination with Testosterone on Rat Prostate Lobes. *Toxicol Appl Pharmacol*. 1992, 112: 300-309. PMID: 1539166.

Prins GS, Hu W-Y, Shi GB, Hu DP, Majumdar S, Li G, Huang K, Nelles J, Ho SM, Walker CL, Kajdacsy-Balla A, van Breemen RB. Bisphenol A Promotes Human Prostate Stem-Progenitor Cell Self-Renewal and Increases In Vivo Carcinogenesis in Human Prostate Epithelium. *Endocrinology*. 2014, 155(3): 805-17. PMID: 24424067.