Soto 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

Dose Groups

Pregnant rats were dosed by gavage with vehicle control (0.3% carboxymethylcellulose), five different doses of BPA (2.5, 25, 250, 2500, and 25000 µg/kg bw/day), and two doses of EE2 (0.05 and 0.5 µg/kg bw/day) from gestation day 6 until the initiation of parturition. The two doses of EE2 were included as a reference estrogen control to determine the sensitivity of the animal model to EE2.

Direct gavage dosing of pups at the same dose level of vehicle, BPA, or EE2 as their dams started on PND 1 (day of birth is PND 0). Each dose group was split into two dosing arms, a continuously dosed group and a stop-dose group, with the latter having treatment terminated at weaning on PND21.

Histological and Morphometric Endpoints

Tissue Collection

Female offspring were euthanized on PND 21, PND 90, and at 6 months of age (Animal Sets # 4, 6, and 8, respectively). The current study used samples from female offspring (n=8-10 per treatment group per time point); only 1 female per litter was used per treatment group. Cycling females were euthanized when predicted to be in estrus based on a vaginal smear from the previous day. Right and left fourth and fifth inguinal mammary glands were collected per animal for evaluation and left and right sides weighed separately. The right 4^{th} and 5^{th} mammary glands were removed as a unit, whole mounted on a glass slide, fixed in 10% neutral-buffered formalin (NBF) for 24h, and bagged individually in 70% ethanol (ETOH) prior to shipping overnight at room temperature from NCTR to Tufts University School of Medicine. The contralateral 4^{th} and 5^{th} mammary glands were fixed in 10% NFB for 24h and placed in 70% ETOH prior to shipping. The whole mounted glands were stained with carmine, as previously described (Murray et al., 2007), and the contralateral glands were processed for paraffin embedding.

The fixed mammary glands were used to assess the time-course of histoarchitectural changes and the emergence of pre-neoplastic and neoplastic lesions. Samples were received from both the stop-dose and continuous dosing arms of the study. All samples were received without knowledge of treatment group, and data were not decoded until data collection of histological and a morphometric analysis was complete and the raw data were locked in the NTP Chemical Effects in Biological Systems (CEBS) database.

Semi-quantitative mammary gland morphometric analysis

PND 21 mammary gland scoring

In a preliminary study using female mammary glands collected from animals from a previous subchronic 90-day BPA study (Delclos et al., 2014), the negative and reference estrogen control samples were identified *a priori* and were evaluated to determine the range of response. A stereomicroscope was used to develop the range of scores reported modified from the criteria reported in Davis and Fenton (2013). The PND21 whole mounted glands were given a morphological developmental score from 1 to 7, which considered 1) the number of terminal end buds (TEBs) relative to the number of duct ends, 2) the degree of ductal branching and/or ductal budding, 3) the number of primary ducts growing from the nipple, 4) the degree of lobule formation, and 5) the lateral and longitudinal growth of the gland (extension) with 7 representing the most well developed. Two individuals independently evaluated all slide sets.

PND 90 and 6 month mammary gland analysis

PND 90 and 6 month mammary glands from both stop-dose and continuous dosing groups were assessed for overall glandular development and density. Whole mounts were assessed and three separate areas of each gland were measured to determine average density of the gland imaged. Image J (NIH) software was used to process and analyze captured images to assess epithelial density of the gland. PND 90 and 6 month glands were visually scored for the following morphological parameters: number of leading edge/internal terminal ends, as well as incidence of lateral branching, lateral budding, alveolar budding, and lobuloalveolar development. Putative lesions were excised for histopathological assessment.

Semi-automatic morphometric analysis of PND 21 mammary glands.

This method is to be published as:

Montévil M, Acevedo N, Schaeberle CM, Bharadwaj M, Fenton SE and Soto AM. Evaluation of the developing mammary gland of rats in the CLARITY-BPA study.

Imaging:

In order to reduce ambiguity in the analysis due to overlapping branches, we obtained optical sections to generate a 3D image instead of a bright field image of the gland. This method is only applicable to PND21 mammary glands due to their smaller size and thickness compared to the later time points.

Samples were imaged with a Zeiss LSM 510 confocal microscope using the auto-fluorescence of carmine as the signal. Due to the large size of the whole glands, the imaging was done on a grid, leading to 150 to

600 partially overlapping stacks. The resolution used was 5µm for the optical plane (x-y) and 3.5µm for the depth (z). The resulting stacks were stitched in Fiji using the method described in (Preibisch et al. 2009).

Identification of epithelium:

Segmentation separates a region of interest from the background. In the mammary gland, the region of interest is the epithelium and the background, including the stroma, blood and lymph vessels. We designed a custom-semiautomatic method because the available algorithms for reconstructing branching structures in the vascular system (Luboz et al. 2005) cannot be used for the mammary gland due to the presence of buds. Additionally, due to optical limitations, the presence of lumen did not provide a consistent pattern that could be used for segmentation. Because of this limitation, we found it easier to segment the stroma first instead of focusing directly on the epithelium. The segmentation algorithm used the following steps:

Step 1: To remove nuclei of stroma cells and noise from image acquisition, we use bilateral filtering (with spatial radius 4 and range 150) followed by the subtraction of local background. The resulting image is then used for the segmentation.

Step 2: The image was inverted, and the stroma segmented as a bright connected region, with a uniform threshold. Then, 3D Gaussian blur (radius 2) was applied to the resulting binary image to remove small structures such as blood vessels, adipocytes, etc. Next, the image was inverted back and the epithelium was obtained as the connected region above a given brightness which included a point in the epithelial tree that had been manually selected. Holes in the epithelium which are due to lumen are filled in and another Gaussian blur is performed. Finally, we perform a second selection of the connected region corresponding to the epithelium and above a given brightness. This second segmentation reduces possible artifacts which mostly stem from small blood vessels and adipocytes.

Step 3: Human intervention was required for comparing the segmented epithelium with the original image. The purpose of this comparison was first to assess whether all the epithelium was accurately segmented. Missing epithelium typically corresponds to a loss of brightness in deeper parts of the sample or particularly thin epithelial structures. Second, the user ensures that structures other than mammary epithelium are not segmented (such as blood, lymph vessels or lymph nodes). If the output is acceptable, segmentation is complete, otherwise human intervention is required to correct the segmentation issues. Intervention corrects the stacks that are used at the beginning of Step 2. Missing epithelial structures should have their brightness enhanced. Wrong structures should be removed either completely or by decreasing the brightness around these structures. After this operation is performed, the program goes back to Step 2, performing the segmentation and subsequent verification again.

Extraction of quantitative morphological features:

The result of segmentation is a 3D reconstruction of the epithelium. This result can then be used to extract several quantitative morphological features of mammary glands. Analyses are performed using ImageJ. Before performing these analyses, we adjust the orientation of the gland so that the axis x, y

and z correspond to the orientation of the gland (i.e., axes of inertia). This provides a standardized meaning to these axes.

We first analyze the properties of the projection of the 3D reconstruction of the epithelial tree on the xy plane which is comparable with assessments performed on bright field microscopy. The analysis includes quantities such as the aspect ratio, the area and the fractal dimension of this projection. The same kind of global analysis is also performed in 3D and includes an evaluation of the surface of the epithelium, of its volume, and of its 3D fractal dimension (based on the box counting method)(Longo and Montévil 2014).

Next, the analysis uses several plugins from ImageJ: the 3D object counter (Bolte and Cordelières 2006), the plugin 3D shape (Sheets et al. 2013), and the bonej plugins (Doube et al. 2010). The latter includes an evaluation of the local thickness which is performed after a normalization of the scales of the three spatial dimensions. The skeletonized epithelium was analyzed by generic methods (counting the number of branches, average branch length, etc.). The analysis was performed both with and without terminal branches since some of the terminal branches may not correspond to actual epithelial structures but may have been artifacts from the process of skeletonization.

Finally, a more specialized approach to reconstructing the epithelial tree was performed by a custom plugin. This plugin starts from the skeleton generated as discussed above and a manual selection of the starting point of the gland (the nipple). The plugin then reconstructs the mammary tree with the main duct as the root. To assess secondary branching, the distance (depth) of the two daughter trees was assessed; if the ratio between these depths was smaller than 0.3, then the branch associated with the smaller tree was identified as a secondary branch. In this case, the parent branch and the main sub-branch were merged since the branching was not assumed to have occurred during the formation of the duct but was instead assumed to have happened later. This reconstruction was then used as the basis for evaluating various properties. For example, the distance from a branch to the nipple counted either in terms of the number of branching points or as the sum of the lengths of the branches that link the two. We also considered branching angles and the tortuosity of the branches (i.e., for a branch, the ratio between its length by the length of a straight line between its extremities: the less straight the branch, the higher the tortuosity). Other quantities such as the local thickness were also determined by considering the average and standard deviation of their values on the skeleton points of every branch.

Statistical methods

The resulting morphological endpoints were analyzed using principal component analysis (PCA), clustering on PCA, non-linear regression, mean comparisons and global analysis of 25-250 µg/kg/day BPA as a breaking point**.**

Genomic DNA Methylome and RNA Transcriptome Endpoints

Tissue Collection

Female offspring (Animal Set # 5) were euthanized on PND 21. The current study used samples from female offspring (n=4 per treatment group); only 1 female per litter was used per treatment group, so no adjustment for litter was needed in the statistical analysis. Right and left fourth and fifth inguinal mammary glands were collected under RNase-free condition as a unit per animal and immediately flash frozen in liquid nitrogen to preserve tissue integrity. The frozen mammary glands were shipped overnight on dry ice from NCTR to Tufts University School of Medicine. All samples were received without knowledge of treatment group, and data were not decoded until data collection of -omics analyses was complete and the raw data were locked in the NTP Chemical Effects in Biological Systems (CEBS) database.

Laser Microdissection

One mammary gland from each animal was cryosectioned in its entirety and all sections stored at -80 ° C until prepared for laser microdissection. The peri-ductal stroma, which corresponded to approximately 100 µm from the outer boundary of the epithelium, and the epithelial ducts were separately captured using the Arcturus Veritas™ Laser Capture Microdissection System (Arcturus Bioscience, Mountain View CA). All the epithelium from one mammary gland was pooled into one tube, while all of the peri-ductal stroma was collected in a second tube for a single sample of each animal.

Deep sequencing analyses of RNA transcriptomes

Total RNA was extracted from laser-captured rat mammary epithelium and peri-ductal stroma using the AllPrep Micro kit (Qiagen; Valencia, CA), following manufacturer's instructions.

RNA transcriptomes were determined by RNA-seq. RNA integrity of the total RNA samples was examined using the Agilent RNA 6000 Nano Kit for Bioanalyzer (Agilent Technologies, Santa Clara, CA). Non-directional RNA-seq deep sequencing libraries for the ABI/SOLiD platform were constructed using the SOLiD Total RNA-seq Kit and EZ Bead (Life Technologies). The XSQ-format data generated by the SOLiD 5500XL deep sequencers (50 nt, single non-directional reads) were directly subjected to analysis by the RNA-seq pipeline of the LifeScope, which generated the RPKM [reads per kilobase exon model and million mappable reads, Mortazavi et al., 2008] values for rat genes annotated in rn5. Uniquely mapped reads generated by LifeScope were examined using fastQC to confirm appropriate read qualities.

Statistical Analysis

Statistical tests for differential means of a single parameter between two independent groups were performed by the unpaired Student's *t*-test under the homoscedastic assumption, which was supported by the *F*-test of variances.

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