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Identification of gene expression changes in postnatal rat foreskin after *in utero* anti-androgen exposure

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ABSTRACT

In utero human phthalate exposure has been associated with male reproductive disorders in epidemiological studies, but discovering relationships is hindered by the lack of identifying markers. This study identified gene expression changes following *in utero* dibutyl phthalate (DBP) and flutamide exposures in Sprague-Dawley rat foreskin. Dams were exposed to 100 or 500 mg/kg/day dibutyl phthalate or 5 mg/kg/day flutamide from gestational days 16–20. Microarray analysis was performed on foreskin tissue from gestational day 20 and postnatal day 5. Expression changes found following DBP exposure were not present following flutamide treatment, indicating that expression changes were specific to DBP exposure and not caused by altered androgen signaling. Genes that were expressed at lower levels in tissue from pups treated with the low dose of DBP were reduced more in pups treated with the high dose of DBP, demonstrating a dose response effect of this compound. Changes in expression of *Marcks*, *Pum1*, *Nupr1*, and *Penk* caused by *in utero* phthalate exposure were confirmed by qRT-PCR. Changes in expression of these genes were maintained after birth and consequently their expression could serve as markers of chemical exposure and biological response.

1. Introduction

Rodent and human reproductive masculinization takes place as a direct result of androgen signaling. The male masculinization programming window spans from gestational day (GD) 16–18 in rats (approximating gestational weeks 8–14 in humans) and corresponds to a rise in circulating testosterone that peaks at GD17.5 [1]. *In utero* disruption of testosterone production during the male programming window has been linked to an increased risk of male reproductive disorders including cryptorchidism and hypospadias [2]. It has been estimated that over the past 30–40 years, hypospadias and cryptorchidism rates have doubled [3–5]. In some rare cases, these malformations are brought on by mutations of genes within hormone signaling pathways [6]. For the majority of the cases, the cause of the malformations is unknown. Exposure to environmental antiandrogenic compounds has been proposed as one cause that could explain increased incidence of these malformations [7].

Dibutyl phthalate (DBP), a plasticizing agent used in food packaging and other consumer goods, is implicated in human male reproductive malformations through disruption of testis testosterone production during the male programming window and subsequent decreased androgen signaling [8]. In the rat, fetal exposure to DBP causes feminization of male reproductive development in the form of reduced anogenital distance (AGD), thoracic nipple retention, testicular maldescent, and hypospadias [9–12]. Additionally, *in utero* phthalate exposure leads to the induction of multinucleated gonocytes (MNG) within fetal testis seminiferous cords, regardless of whether testosterone disruption occurs [13,14]. Another compound, flutamide, disrupts androgenic signaling by competitively inhibiting the androgen receptor (AR) [15]. Much like DBP, *in utero* rat exposure to flutamide increases the prevalence of male reproductive malformations [16–18].

Both hypospadias and cryptorchidism are linked to androgen insufficiency during the fetal window of masculinization but sampling of the affected tissue is generally not feasible in humans, so we chose to examine surrogate tissues to measure gene expression changes resulting from disruption of androgen signaling *in utero* [3–5]. In rat development, preputial separation, defined as the separation of the foreskin from the glans penis serves as a pubertal milestone [19,20]. Disruption of testosterone signaling, whether by castration or anti-androgen exposure, causes a delay in preputial separation [19,21,22]. Foreskin is a valuable surrogate tissue for human translational research studies because it expresses the AR

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and can be obtained readily after birth [23]. We therefore chose to examine rat foreskin for expression changes resulting from androgen signaling disruption. By utilizing both DBP and flutamide, this study aimed to identify candidate gene expression changes in the rat foreskin that correspond to levels of *in utero* phthalate exposure, and to determine biological responses to disruption of androgen signaling.

2. Materials and methods

2.1. Materials

Timed-pregnant Sprague Dawley rats (strain code 001) were purchased from Charles River Laboratories (Raleigh, NC), arriving on GD 10 with day of sperm detection denoted GD 0. Animals were acclimated for six days prior to initial gavaging. In postnatal studies, date of parturition was considered postnatal day (PND) 1. Corn oil vehicle (C8267), DBP (524980) and flutamide (F9397) were purchased from Sigma (St. Louis, MO).

2.2. Methods

2.2.1. Animal exposures

All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Alfred I. duPont Hospital for Children. Rats were housed in an on-site facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International in a climate-controlled room with 12 h light–dark cycles. A single dam was housed per polycarbonate cage with pine shaving bedding, fed Lab Diet Rat Chow 5012 (PMI Nutrition International, Brentwood, MO) and provided tap water *ad libitum*. Upon arrival of timed-pregnant animals, maternal body weights were measured and dams were distributed evenly by weight between control and treated groups. Beginning on GD16 and continuing through GD20, dams were administered DBP, flutamide, or corn oil vehicle by oral gavage once each morning between 8 and 10 AM.

A DBP dose level of 100 mg/kg (DBP100) was used because this results in a modest reduction of fetal testis testosterone levels, and a 500 mg/kg dose level (DBP500) was chosen because this leads to maximal testosterone reduction [24]. For the prenatal (GD20) study, we used control dams (6), DBP100 (5), and DBP500 (5). For the postnatal (PND5) study, we used control dams (7), DBP100 (7), and DBP500 (7) dose levels. Animal numbers varied slightly due to variations in supply of timed-pregnant rats. Dams used in flutamide analyses were age and weight matched to those in DBP studies. A flutamide dose level of 5 mg/kg/day by oral gavage was used because this demasculinizes the male reproductive tract without causing systemic toxicity [25]. For the flutamide study we had seven dams in both the treated and control groups.

In prenatal studies, dams were euthanized on GD20 by carbon dioxide asphyxiation 6 h after their final DBP exposure. In postnatal studies, PND5 pups were euthanized by decapitation. AGD was measured post-mortem using a stereomicroscope fitted with a 1 mm ocular micrometer with increments of 0.1 mm. Pups and fetuses were examined for presence of hypospadias *post mortem* by gently pressing on the bladder and observing the location of the urethral opening. Pups in the flutamide study were examined for presence of retained thoracic nipples on PND14. All morphological assessments were done with the examiner blinded to the treatment group.

2.2.2. RNA extraction and processing

Foreskin samples were collected from male rats at GD20 or PND5. Images of rat penis before and after foreskin collection at both GD20 and PND5 are shown in Fig. 1. Two foreskin samples were pooled per litter. Prenatal foreskin was flash frozen and stored at −80 C. Postnatal foreskin was stored in RNAlater (Invitrogen, Carlsbad, CA). RNA was extracted using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA) as per the manufacturer's protocol. RNA quantification was performed using a ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington DE). An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) was used to ensure the integrity of RNA samples, and an RNA Integrity Number above seven was used as a cutoff for use in microarray analyses.

2.2.3. Microarray analysis

For DBP studies, gene expression microarray was performed using the RatRef-12 Expression BeadChip Kit (Illumina, San Diego, CA) at the Northwestern University Genomics Core Facility. Labeling was performed using the Illumina TotalPrep RNA Amplification Kit (Ambion, Carlsbad, CA), and arrays were scanned using iScan (Illumina). Quality control was performed using lumi, a Bioconductor package [26]. Because RatRef-12 arrays were discontinued by the manufacturer, arrays used for the flutamide study were GeneChip Rat Gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA). Foreskin RNA samples from the flutamide study were labeled using the Applause WT-Amp ST System (Nugen, San Carlos, CA) with 50 ng total RNA. After hybridization and washing, Affymetrix microarray chips were scanned using a GeneChip Scanner 3000 (Affymetrix). Quality control was determined with a GeneChip Poly-A RNA Control Kit and a GeneChip Hybridization Control Kit (Affymetrix). Although two different microarray platforms were used, both platforms provide similar microarray data [27–29].

Illumina microarray data were log2 transformed and quantile normalized using lumi, a Bioconductor package. Affymetrix microarray data were normalized by Robust Multiarray Analysis (RMA) within the affylmGUI [30] package using R software. The R package ComBat [31] was used to ameliorate batch effects detected by principal component analysis. Data were analyzed statistically by Linear Models for Microarray Data (LIMMA) and significance analysis of microarray (SAM) using R software (R Development Core Team, 2011) and MultiExperiment Viewer, a part of the TM4 Microarray Software Suite [32,33], respectively. Bioconductor packages for LIMMA analyses included limma [34] as well as supporting packages affy [35] and Biobase [36]. Quality control analyses including principle component analysis and hierarchical clustering were performed using MultiExperiment Viewer [32,33]. A false discovery rate (FDR) of 5% was considered significant.

Microarray data are available through gene expression Omnibus accession number GSE48803.

2.2.4. Filtering of microarray results

Microarray results were filtered and genes were selected for subsequent qRT-PCR analysis. The first cutoff separated genes that reached statistical significance ($p < 0.05$) when compared to the control group. Gene lists were then examined by Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Redwood City, CA). Significant gene lists were mapped in conjunction with key parts of the steroidogenic pathway including the AR, testosterone, and dihydrotestosterone. This determined which genes with significantly altered expression levels interacted either directly or indirectly with the AR or steroidogenic pathway. These lists were then compared to determine which gene expression changes persisted from prenatal to postnatal studies, which genes were identified by both LIMMA and SAM analyses, as well as which changes increased with an increase in DBP dose level. Finally, the fold changes and *p*-values of the remaining lists were examined to select genes for further analysis by qRT-PCR. *Marcks* and *Pum1* were selected for further analysis because they were found to be significant in both prenatal and postnatal studies. Moreover, *Marcks* was found to act downstream of the AR, while *Pum1* acts further downstream from