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Research Article

Exposure to an Environmentally Relevant Phthalate Mixture During Prostate Development Induces MicroRNA Upregulation and Transcriptome Modulation in Rats

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ABSTRACT

Environmental exposure to phthalates during intrauterine development might increase susceptibility to neoplasms in reproductive organs such as the prostate. Although studies have suggested an increase in prostatic lesions in adult animals submitted to perinatal exposure to phthalates, the molecular pathways underlying these alterations remain unclear. Genome-wide levels of mRNAs and miRNAs were monitored with RNA-seq to determine if perinatal exposure to a phthalate mixture in pregnant rats is capable of modifying gene expression during prostate development of the filial generation. The mixture contains diethyl-phthalate, di-(2-ethylhexyl)-phthalate, dibutyl-phthalate, di-isononyl-phthalate, di-isobutyl-phthalate, and benzylbutyl-phthalate. Pregnant females were divided into 4 groups and orally dosed daily from GD10 to PND21 with corn oil (Control: C) or the phthalate mixture at 3 doses (20 μg/kg/day: T1; 200 μg/kg/day: T2; 200 mg/kg/day: T3). The phthalate mixture decreased anogenital distance, prostate weight, and decreased testosterone level at the lowest exposure dose at PND22. The mixture also increased inflammatory foci and focal hyperplasia incidence at PND120. miR-184 was upregulated in all treated groups in relation to control and miR-141-3p was only upregulated at the lowest dose. In addition, 120 genes were deregulated at the lowest dose with several of these genes related to developmental, differentiation, and oncogenesis. The data indicate that phthalate exposure at lower doses can cause greater gene expression modulation as well as other downstream phenotypes than exposure at higher doses. A significant fraction of the downregulated genes were predicted to be targets of miR-141-3p and miR-184, both of which were induced at the lower exposure doses.

Key words: phthalate mixture; prostate development; miRNA; epigenetic; transcriptome.

Phthalates are a family of phthalic acid diesters typically used as plasticizers to soften and increase the flexibility of polyvinyl chloride plastic products (Bosnir *et al.*, 2003). These compounds are not covalently bound to the plastic, and often leach and contaminate foods processed or stored in plastic products (Bosnir *et al.*, 2003). Phthalates have been of special concern due to their antiandrogenic activity (Fisher, 2004) and endocrine disrupting ability (Hannon and Flaws, 2015).

It has been well documented that humans and animals are exposed to mixtures of different phthalates (Ferguson *et al.*, 2014; Meeker and Ferguson, 2014; Watkins *et al.*, 2014). Seven major phthalate metabolites were measured in urine samples from 2540 subjects who participated in the National Health and Nutrition Examination Survey 1999–2000 (Silva *et al.*, 2004). Women had urine phthalate levels 2–4 times higher than men (Silva *et al.*, 2004). In another study, younger children had significantly greater exposure to phthalate as measured in their urine than older children (Koch and Calafat, 2009).

The developmental origins of health and disease (DOHaD) hypothesis proposes that events occurring during early development are key determinants of individual sensitivity or risk of developing disease later in life (Barker, 1990; Lau and Rogers, 2004; Rosenfeld, 2015). Environmental exposures are a major contributor to the chemical environment experienced during embryogenesis. According to Ho *et al.* (2017), the interaction between the epigenome and the exposure profile of an individual during development, contribute to the fate of individual cells and the construction of functional organic systems with differentiated stable tissues. In this sense, cells and organs have various degrees of phenotypic plasticity and epigenetic variation that are manifested even in the absence of genetic variation (Feinberg, 2007).

The prostate develops from the pelvic part of the urogenital sinus (Cunha *et al.*, 2004; Prins and Putz, 2008; Timme *et al.*, 1994). In humans, prostate morphogenesis occurs from the second and third trimesters until the time of birth. In rodents, prostate morphogenesis begins in fetal life and extends through the postnatal period to prepuberty (Prins and Putz, 2008; Vilamaior *et al.*, 2006). Experimental studies have associated fetal and neonatal exposure to endocrine disrupting chemicals (EDCs), such as BPA and phthalates, to an increased susceptibility to prostatic carcinogenesis, increasing the incidence and severity of prostatic lesions and neoplastic alterations (Peixoto *et al.*, 2016; Wang *et al.*, 2017).

MicroRNAs (miRNAs) are small noncoding regulatory RNAs (sncRNAs) that play a fundamental role in the regulation of most mammalian protein codling genes (Berger *et al.*, 2009; Bird, 2007; Goldberg *et al.*, 2007; Zhang and Ho, 2011). miRNAs range in size from 17 to 25 nucleotides and posttranscriptionally repress gene expression. The small RNA recognizes complementary sites of target messenger RNAs (mRNA) and causes its degradation. One hypothesis is that EDCs exert long-term effects in reproductive studies through the action of noncoding miRNAs. Results from a study with rats showed that prenatal exposure to vinclozolin led to the repression of miRNAs such as mir-23b and let-7 gene on embryonic day (E) 13.5 in primordial germ cells (Brieño-Enríquez *et al.*, 2015). The influence of vinclozolin in the expression of miRNAs was observed in 3 successive generations (in the absence of continued exposure), with no prominent changes in DNA methylation (Brieño-Enríquez *et al.*, 2015).

The critical window of early male genital tract development is a sensitive period of exposure to EDCs. Our previous findings showed that perinatal exposure (fetal and lactational periods) to relevant doses of phthalates increased the susceptibility to prostate lesions like prostatic intraepithelial neoplasia, proliferative inflammatory atrophy epithelial reactive atypia, hyperplasia, and chronic inflammation (Peixoto *et al.*, 2016; Scarano *et al.*, 2009). This led us to hypothesize that exposure these tissue anomalies could be mediated by phthalate-induced alterations in the expression of genes and miRNAs during prostatic morphogenesis. This study documents novel results on miRNAome and transcriptome responses to maternal phthalate exposure on the prostate of young rats. We examined a mixture of phthalates at 3 doses during a critical window of gland development.

MATERIALS AND METHODS

Experimental design. This study was carried out in accordance with the Brazilian Council on Animal Experimentation Control (CONCEA) and the protocol was approved by the Ethical Committee on Animal Use of the Institute of Biosciences, São Paulo State University (Protocol: 1040/CEUA). Sprague Dawley rats were supplied by Multidisciplinary Center for Biological Research (CEMIB/UNICAMP) and they were kept during the experimental period in the Bioterium of Small Mammals from Department of Morphology, Institute of Biosciences (UNESP). The rats were bred under controlled temperature (23 ± 2°C) and regulated humidity conditions with periods of light/dark of 12 h and food and water *ad libitum*.

Pregnant female Sprague Dawley rats were exposed to a phthalate mixture by daily oral route (gavage); and rats were euthanized by anesthetic saturation (sodic pentobarbital) followed by cardiac puncture. For this experiment, 4 groups were established: (C) control group (exposed to vehicle; corn oil); (T1) 20 μg of the mixture (20 μg/kg/day); (T2) 200 μg of the mixture (200 μg/kg/day); and (T3) 200 mg of the mixture (200 mg/kg/day). The phthalate mixture was diluted in tocopherol-stripped corn oil (vehicle control). The 2 lower doses were selected to mimic daily human exposure levels based on the amount of DEHP, and our higher dose was selected to compare our results with available single phthalate studies (Zhou *et al.*, 2017a). The animals from groups T1 to T3 received the respective doses of the phthalate's mixture in the following proportion: 21% bis (2-ethylhexyl) phthalate (DEHP), 35% diethyl-phthalate (DEP), 15% di-*n*-butyl-phthalate (DBP), 8% di-isobutyl-phthalate (DiBP), 5% butylbenzyl-phthalate (BBzP), and 15% di-isononyl-phthalate (DiNP). This proportion was based in previous study considering the proportion of phthalates metabolites detected in urine samples from pregnant women as described (Zhou *et al.*, 2017b). The rats received the treatment from gestational day 10 (DG10) to postnatal day 21 (DPN21), a critical period for the development of the urogenital apparatus, and especially of the prostate (Prins and Putz, 2008; Vilamaior *et al.*, 2006).

Pregnant and lactating females treated with the mixture were designated as F0 generation ($n = 8$). The offspring from F0 generation were named as F1 generation. After birth, the number of F1 offspring per litter was reduced to 8 (1:1 ratio between males and females whenever possible), and litters with fewer than 6 pups were euthanized. In order to guarantee the independence of treatments and litters, the experimental unit was the litter and not the individual animal (Brandt *et al.*, 2014).

At PND1, the F1 pups were weighed and sexed through the anogenital distance (AGD). For the analyses, the males with the lowest AGD were selected within the litters. After weaning (PND22), the male rats were weighed and the AGD measurement was performed. AGD adjusted by body weight was calculated as described by Gallavan *et al.* (1999). To investigate both

the immediate and late effects of exposure to the mixture, 2 male rats per litter were selected for the lowest measurement of AGD and they were euthanized at PND22 and PND120. The ventral prostate was collected and weighed in both ages (PND22 and PND120) and designated for molecular analysis (PND22) and histopathological evaluation (PND22 and PND120). The experimental design is summarized in Figure 1.

Histopathological analysis and proliferation index determination. Ventral prostate fragments (PND22 and 120) were removed and fixed by immersion in methacarn (Puchtler *et al.*, 1970) for 3 h and embedded in Paraplast. The histological sections (5 μm) were produced using a rotary microtome and mounted on, silanized slides. Then, the sections were stained with hematoxylin-eosin for histopathological evaluation. For histopathological incidence, lesions were only considered for incidence analysis when they appeared in at least 5 different microscopic fields. Only lesions found with the highest incidence were quantified.

Proliferation index determination was performed as described by Gonçalves *et al.* (2018). To determine the proliferative index, Ki67 positive cells were counted (antibody anti-Ki67; ab16667; Abcam, Cambridge, Massachusetts), and 30 random prostatic areas (6 histological fields by animal; 5 animals by group) from each group were used and were examined using the ×40 objective lens. In each field, the percentage of positive epithelial cells was determined relative to total cells, and the results were analyzed for statistical significance.

Total RNA extraction. RNA extraction was performed using 12 prostate samples (3 samples/group; PND22) with TRIzol (Ambion) containing 1% of β-mercaptoethanol, following manufacturer's instructions. The RNA was quantified by spectrophotometry using a NanoDrop (ThermoScientific). RNA quality, as measured with ribosomal RNAs by the RNA integrity number (RIN), was obtained using the 2100 Bioanalyzer system (Agilent). Only RNA samples with RIN >7 was used for subsequent analysis.

mRNA purification, library construction, and sequencing. RNAs were sequenced using the HiSeq2500 platform (Illumina). An aliquot of total unfractionated RNA was submitted for library construction and sequencing. During library preparation, Ribo-Zero rRNA Removal Kit (Illumina) was used for rRNA depletion. mRNA purification and library construction was carried out with total RNA using the TruSeq Standard mRNA Sample Preparation Kit (Illumina), following the manufacturer's specifications. The sequencing was performed with the HiSeq Sequencing System.

High performance sequencing—sncRNAs. An aliquot of total unfractionated RNA was used for library construction and sequencing of sncRNAs. The construction of the sncRNAs libraries was performed using the TruSeq Small RNA Sample Preparation Kit following the manufacture's protocol. The sequencing was also performed with the NovaSeq Sequencing System.

Sequencing analysis. Quality control of the miRNA raw reads was carried out by FastQC v0.11.5 (Andrews, 2014) to inspect for low quality reads and adapters. The single-end reads were trimmed with trim_galore v0.5 and Cutadapt v1.8.1 (Martin, 2011) using default parameters. The preprocessed reads were aligned to *Rattus norvegicus* pre-RNA sequences, downloaded from miRBase v22 (http://www.mirbase.org/ last acessed on November 10, 2018), using BWA v0.7.15 (Li and Durbin, 2009). Furthermore, a general feature format file containing the coordinates of the miRNAs was created using Gmap (Wu and Watanabe, 2005) and used with HTSeq v0.10 (Anders and Huber, 2010) to combine mapped reads raw counts and generate the count matrix. The mRNA paired-end reads went through the same preprocessing step as the miRNA reads. Later, reads were mapped to the *R. norvegicus* genome (assembly 6.0) using BWA and quantified using HTSeq. We accounted miRNAs and genes to be expressed at a proper level in a sample if each has at least 5 normalized counts.

Read count data normalization and differential expression analysis were performed with DESeq2 v1.20 (Love *et al.*, 2014). For each gene and miRNA, the fold change between treated cases and control was calculated. The differentially expressed miRNAs were determined when the |Log2 FC| is higher than 0.75 and false discovery rate (FDR) corrected *p*-value (Benjamini-Hochberg) is less than 5%. Whereas, the genes that show a fold change above 1 and an FDR below 5% are designed as differentially expressed gene (DEG).

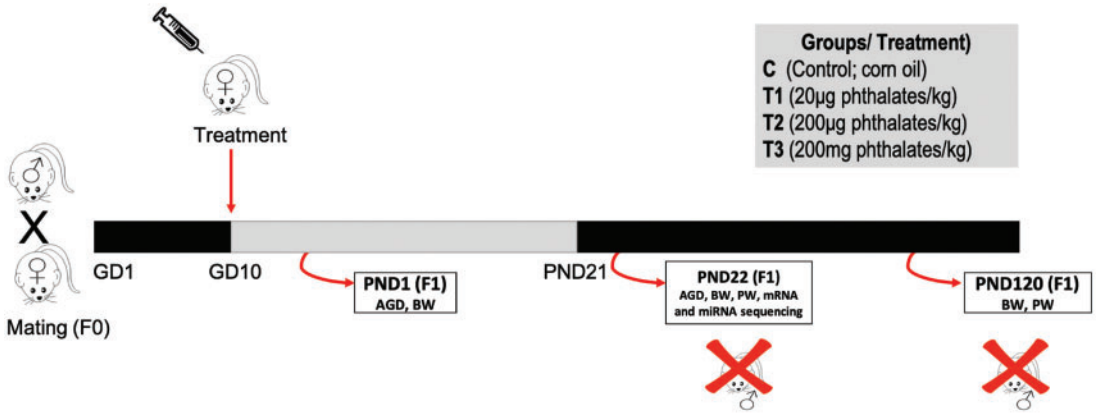

Figure 1. Experimental design. Abbreviations: GD1, first gestation day (positive pregnancy); GD10, 10th gestation day (beginning of mixture treatment); PND1, first postnatal day (phenotypical data collect: body weight [BW]; anogenital distance [AGD]); PND21, 21st postnatal day, weaning (end of treatment); PND22, 22nd postnatal day (phenotypical data collect with euthanasia: body weight [BW]; anogenital distance [AGD]; ventral prostate weight [PW]); PND120, 120th postnatal day (phenotypical data collect with euthanasia: body weight [BW]; ventral prostate weight [PW]). X means euthanasia of male rats.

Sequence data for mRNA and miRNA were inserted and will be available at SRA in bioprojects PRJNA542665 and PRJNA542870, respectively.

miRNAs targets prediction. miRWalk database v3.0 (Sticht *et al.*, 2018) was used, to functionally characterize the differentially expressed miRNAs between phthalate mixture exposure and control, and list their potential targets. The DEGs functional enrichment analysis was performed using GOrilla (Gene Ontology Enrichment Analysis and Visualization tool) (Eden *et al.*, 2009). The significant enrichment threshold was set to *p*-value less than .05.

Statistical analysis. Data analyses for AGD, prostate/body weight, and proliferation index were carried out using GraphPad Prism software (version 5.00; Graph Pad, Inc, San Diego, California). Data were analyzed by one-way analysis of variance (ANOVA) followed by the "Tukey's Multiple" test (parametric data); or "Kruskal-Wallis" and "Dunn's" post hoc test (nonparametric data). Normality was tested by "Shapiro-Wilk" test. For the incidence of histopathological lesions, results were compared by the Fisher's test. Differences were considered statistically significant when the *p*-value was $< .05$.

RESULTS

Biometrical Parameters (Gestational-F0 and Male Parameters-F1)

Phthalate mixture exposure did not change the weight gain of pregnant rats and in the male/female sex ratio in the offspring across experimental groups compared to controls (Figs. 2a and 2b). At PND1, F1 male rats had no change in body weight between the experimental groups (Figure 3a); however, the AGD was significant lower in T3 than in C and T2 (Figure 2c).

At PND22, there was no change in body weight between the experimental groups (Figure 3b). However, there was a significant reduction in testosterone level at PND22 at the lowest exposure dose (T1) compared to control (Figure 3d) and in ventral prostate absolute weight in T1 and T3 related to control (Figure 3e). Additionally, a significant reduction in absolute AGD was observed in all treated groups in relation to C group; and in relation to adjusted AGD, groups T1 and T3 showed a significantly lower AGD than C group (Figure 2d). Histopathological analysis showed no differences in the epithelial and stromal organization among the groups at PND22, however, the predominance of small acini in the prostate of T1 group animals was observed (about 75% of the area of histological sections consisted of small acini in T1, whereas in the other groups, this area was about 40%–50%) (data not shown).

At PND120, there was no difference among the groups in relation to body and prostate weight (Figs. 3c and 3f). Histopathological analysis showed an increase in inflammatory foci incidence in T1 compared to C group (C = 25%; T1* = 75%; T2 = 50%; T3 = 50%, $^*p < .05$; Figure 4e) as well as in focal hyperplasia incidence (C = 37.5%; T1* = 87.5%; T2 = 62.5%; T3 = 62, 5%, $^*p < .05$; Figure 4d). Additionally, the phthalate treatment increased cellularity in all groups at PND120 compared to control as demonstrated by epithelial proliferation index (Figs. 4a–d and 4f).

Transcriptome Responses to Phthalate Mixture Exposure

Transcriptome analysis showed 120 deregulated genes in T1 compared to C; and 3 genes deregulated in T2 in relation to C;

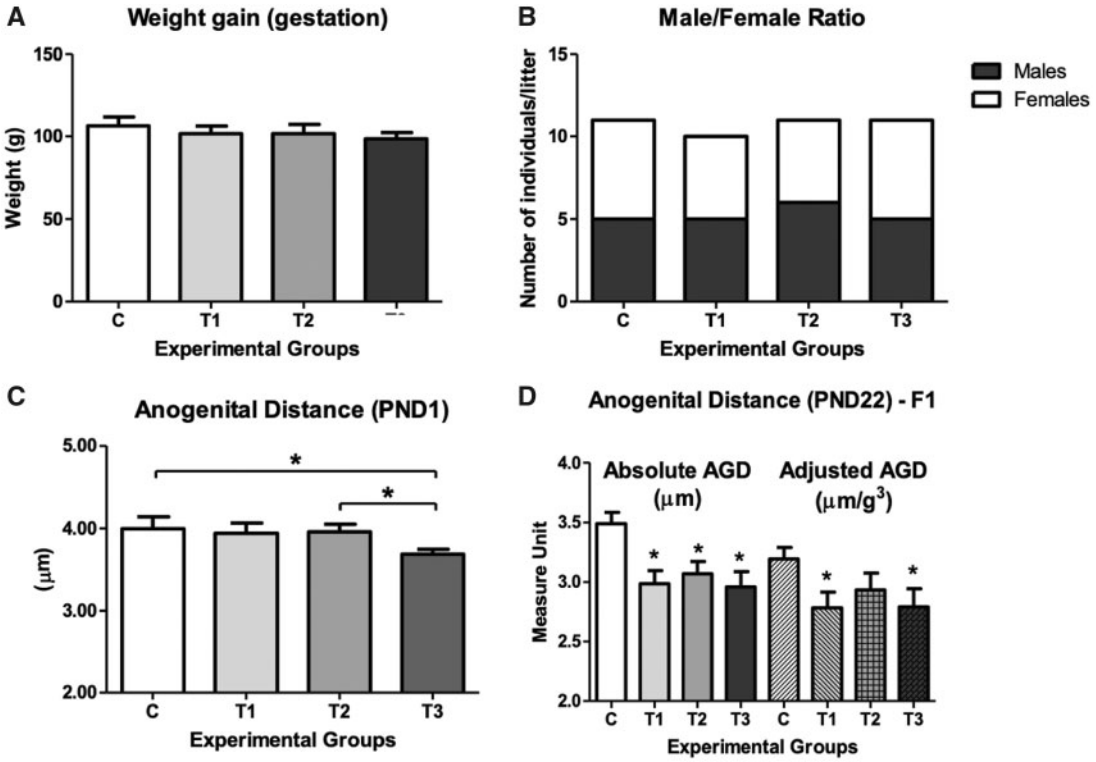

Figure 2. Gestational weight gain from mothers (a); male/female ratio among newborns (b); anogenital distance at PND1 (c); and anogenital distance at PND22, from experimental groups (d). C, control group; T1: 20 μg/kg (low dose); T2: 200 μg/kg (intermediate dose); T3: 200 mg/kg (high dose). For male/female, ratio data were expressed by the average number of males and females in the litters per group. For other parameters data were expressed by mean ± SEM. The asterisk represents significant statistically differences between the groups ($p < .05$), one-way ANOVA followed by the "Tukey's Multiple" test.

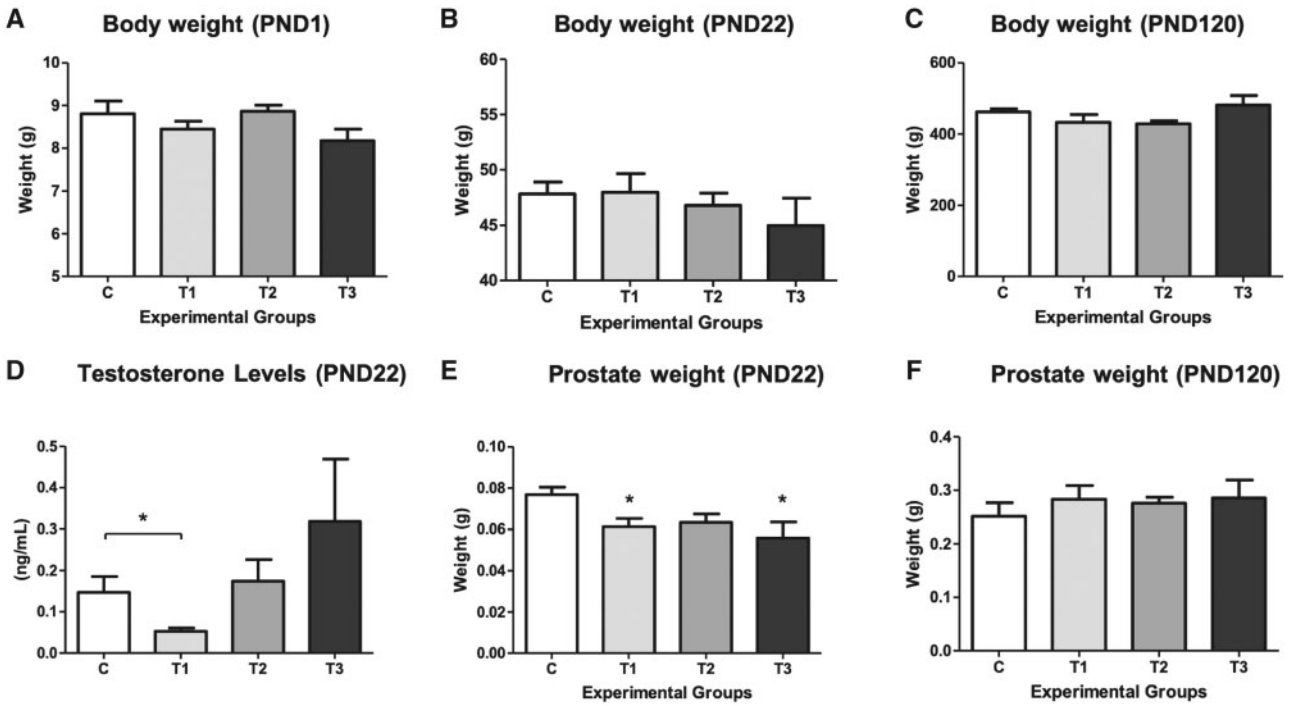

Figure 3. Body weight at PND1 (a), PND22 (b), and PND120 (c); testosterone levels PND22 (d); prostate weight at PND22 (e) and PND120 (f). C, control group; T1: 20 μg/kg (low dose); T2: 200 μg/kg (intermediate dose); T3: 200 mg/kg (high dose). Data were expressed by mean ± SEM. The asterisk represents significant statistically differences between the groups ($p < 0.05$), one-way ANOVA followed by the "Tukey's Multiple" test (weight) and Kruskal-Wallis test, Dunn's post hoc test (testosterone levels).

no deregulated genes were identified in T3 compared to C (Table 1; Supplementary Table 1). Two of the genes deregulated in T2 were also found deregulated in T1. All in all, 121 unique genes were dysregulated in the treated groups relative to the control (Supplementary Table 1). Surprisingly, we observed that 119 of these 121 genes were downregulated in the treatment related to control, with only 2 genes upregulated in the treatments (Rnf183 gene was upregulated in T1 and Zfat gene was upregulated in both T1 and T2 groups in relation to the control; Supplementary Table 1). Among the 119 downregulated genes, 118 genes were downregulated in T1 and 2 genes in T2 (the Tcf23 gene was commonly downregulated in both T1 and T2 groups in relation to the control, and the Wnt6 gene was exclusively downregulated in T2; Supplementary Table 1). The top 10 most significantly downregulated genes (sorted by adjusted p-value $< .05$) in the 118 downregulated genes were Tcf23, Gsta3, Ido2, Ptgr1, Nefh, Slc13a2, Ddc, Wt1, Qsox1, and Tmem130 (Figure 4c; Supplementary Table 1).

Analysis of gene ontology (GO) enrichment in the set of 123 downregulated genes upon treatment revealed processes associated with prostate development. Specifically, we observed that 31 downregulated genes were related to regulation of multicellular organismal development (GO: 2000026, p-value = 1.22E-5), 25 with regulation of cell differentiation (GO: 0045595, p-value = 3.41E-4), 16 with cell secretion (GO: 0032940, p-value = 5.82E-4), and 9 with regulation of developmental growth (GO: 0048638, p-value = 6.24E-4) (Supplementary Table 2).

MicroRNAome Responses to Phthalate Mixture Exposure

Differential expression between the control and treated groups was examined using DESeq2. For miRNA, the results showed a relatively small number of differentially expressed candidates between the control and exposed groups (Table 1; FDR < 0.05). Two miRNAs (miR-30d-5p and miR-30b-5p) were upregulated in both T1 and T2, whereas miR-141-3p and miR-30d-3p were exclusively upregulated in T1 or T2, respectively (Table 2; Supplementary Figure 1). We did not detect upregulated miRNAs in T3, and no miRNA was found to be downregulated in any of the treatment doses. These results emerged under the conservative requirement of a minimum of 5 read counts in all the samples (see Materials and Methods) which yielded 4 miRNAs for analysis. Under a less conservative requirement of a minimum of 1 miRNA count in all samples, we detected 4 miRNAs, with microRNA-184 also emerging as significantly upregulated in all the treated groups compared to the control group (Table 2).

MiRNA-mRNA Relationships

More than 98% of the genes were downregulated in T1 relative to C group, whereas limited deregulation was detected at higher doses. Because miR-141-3p was exclusively upregulated in the T1 compared to C group, whereas other miRNAs, such as miR-30d-5p, were deregulated in other groups with little significant alteration in gene expression, miR-141-3p could be an interesting candidate for downregulating some of those genes downregulated in T1 (Tables 1 and 2; Figs. 5a and 5c). On the other hand, miRNA-184 was upregulated in all treatment groups in relation to the control group in absence of filters (Table 2; Figure 5b); thus, we focused our efforts toward integrating the transcriptome data with both miR-141-3p and miR-184, both upregulated in T1 compared to C group.

The analysis with miRWalk 3.0 revealed 10 202 possible target genes for the miR-184 and 7212 for miR-141-3p (Figure 5d), with 4493 targets common to both miRNAs. Crossing the 120 differentially deregulated genes between T1 and control (Table 1) with the 7212 targets of miR-141-3p, identified 53 possible targets of miR-141-3p (Figure 5d; Supplementary Table 3).

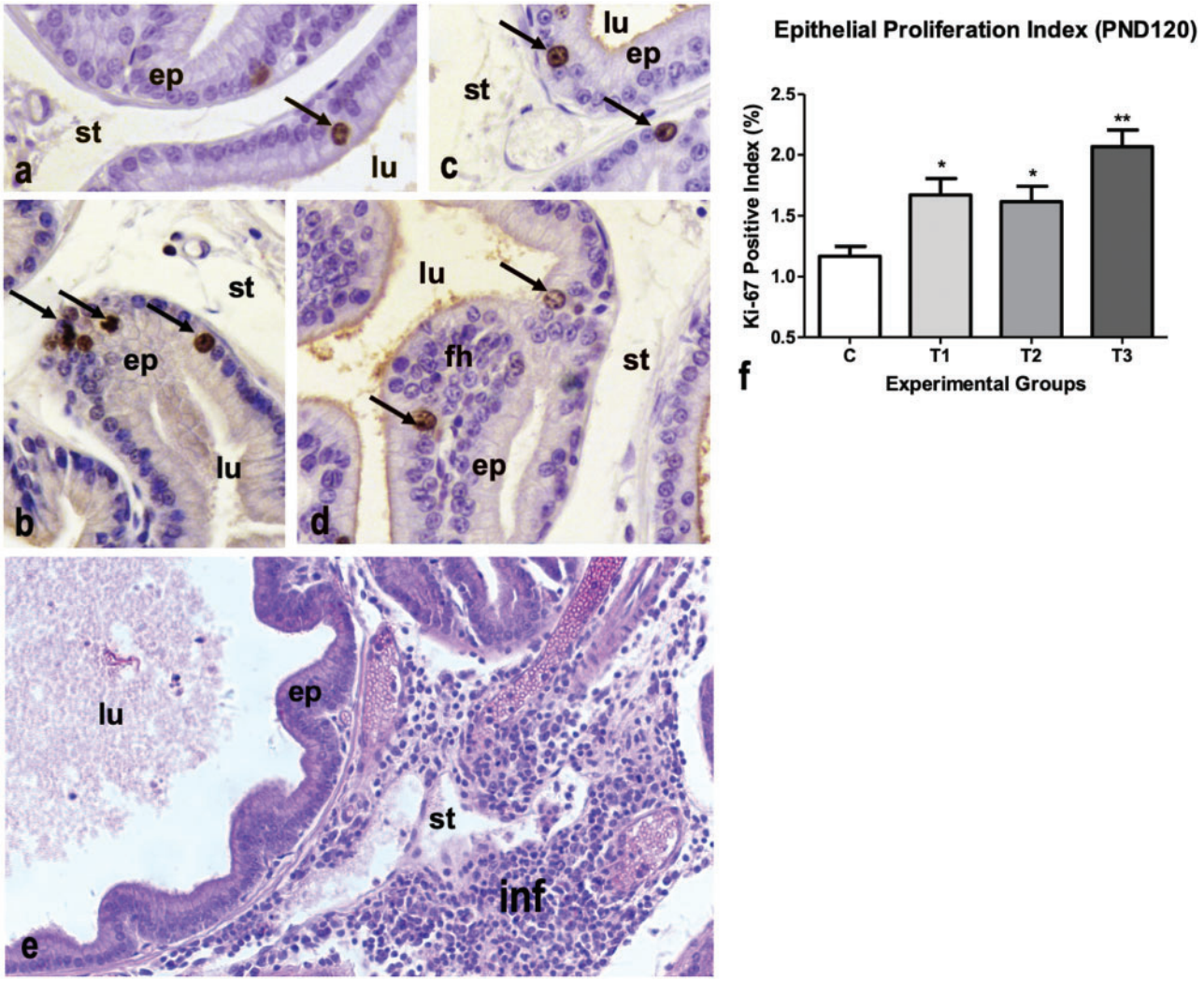

Figure 4. Immunostaining for ki67 (a–d), histological section stained by hematoxylin & eosin (H&E) (e), and epithelial proliferation index (f). Sections (a–d) are representative sections from C, T1, T2, and T3 groups, respectively, and (e) represents a histological section from T1 group. Arrows point to Ki-67 positive cells. Abbreviations: ep, epithelium; st, stroma; lu, lumen; fh, focal hyperplasia; inf, inflammatory focus. In (f), data were expressed by mean ± SEM. The asterisks represent significant statistically differences between the groups (*$p < .05$; **$p < .01$), one-way ANOVA followed Kruskal-Wallis test, Dunn's post hoc test.

Table 1. Overview of Differentially Expressed MiRNAs and MRNAs in Prostate Tissue From Animals Exposed to Different Doses of Phthalates Mixture ($|FC| > 0.75$ and FDR < 5%)

	mRNAs		miRNAs	
	Up	Down	Up	Down
Treated Groups Versus Control				
T1 × C	2	118	3	0
T2 × C	1	2	3	0
T3 × C	0	0	0	0

C, control group; FDR, false discovery rate; T1: 20 μg/kg (low dose); T2: 200 μg/kg (intermediate dose); T3: 200 mg/kg (high dose).

Crossing the 123 DEGs between all treated groups (T1–T3) and C (Table 1) with the 10 202 targets of miR-184 identified 62 possible targets of miR-184 (Figure 5d; Supplementary Table 4).

Among possible targets for miR-141-3p (53 targets), 51 of them were downregulated and could be associated to miR-141-3p modulation in our experiment (Supplementary Table 2); among the 62 possible targets for miR-184, 61 of them were downregulated and could be modulated by miR-184 (Supplementary Table 3). Additionally, among predicted genes for miR-141-3p and miR-184 and differentially expressed, 29 of them are common for both miRNAs (Supplementary Tables 3 and 4).

Next, we conducted a GO enrichment analysis for downregulated targets of miR-141-3p. We observed genes associated with regulation of multicellular organismal process (19 genes; GO: 0051239; p-value = 4.75E-4), regulation of cell development (10 genes; GO: 0060284; p-value = 6.57E-4), regulation of morphogenesis involved in differentiation (6 genes; GO: 0010769; p-value = 4.08E-4), mitotic cell cycle (4 genes; GO: 0000278; p-value = 5.85E-4) (Figure 6, Supplementary Table 5). For downregulated targets of miR-184, we observed genes associated with regulation of developmental process (19 genes; GO: 0050793; p-value = 3.32E-4), regulation of cell differentiation (15 genes; GO: 0045595; p-value = 5.37E-4), and signaling (7 genes; GO: 0023052; p-value = 4.62E-4) were selected (Figure 6, Supplementary Table 6). Since previous findings have shown that exposure to phthalates during the prostate development can increase

Table 2. List of MiRNAs Differentially Expressed in Prostate Tissue From Animals Exposed to Different Doses of Phthalates Mixture (|FC| > 0.75 and FDR< 5%)

	Low-Count Filtering		No Filtering	
	Up	Down	Up	Down
Treated Groups Versus Control				
T1 × C	mir-30b-5p	0	mir-184[b]	0
	mir-30d-5p		mir-30b-5p	
	mir-141-3p[a]		mir-30d-5p	
T2 × C	mir-30b-5p	0	mir-184[b]	0
	mir-30d-5p		mir-30b-5p	
	mir-30d-3p		mir-30d-5p	
			mir-30d-3p	
T3 × C	0	0	mir-184[b]	0

Low-count filtering column includes the miRNAs that are expressed in the all the samples, such as, for each sample, the miRNA is supported by more than 5 normalized read counts. No filter column includes all the miRNAs resulting from the experiment. C, control group; FDR, false discovery rate; T1: 20 μg/kg (low dose); T2: 200 μg/kg (intermediate dose); T3: 200 mg/kg (high dose).

[a]The absence of mir-141-3p from the list of differentially expressed miRNAs in the absence of filters is due to the FDR that is > 0.05.

[b]The absence of mir-184 from the list of differentially expressed while applying filters is due to number of counts in the controls samples that were all less than 5 and did not pass the filters.

susceptibility to carcinogenesis, we selected those biological processes and genes from GO term enrichment analysis that would have more relation with our hypothesis in which the oncogenic process could initiate during prostate development (Table 3).

DISCUSSION

In our previous studies, we showed that exposure to a relevant dose of DBP (100 mg/kg) during the critical window of genital system development reduced AGD at birth and increased the incidence of prostate lesions in adulthood (Peixoto *et al.*, 2016; Scarano *et al.*, 2009). Here we provide evidence that maternal exposure to a mixture of phthalates modulates the expression of protein coding genes and miRNAs, with detectable effects at both low and high concentrations of exposure. Our results showed that the phthalate mixture was able to modify phenotypic parameters such as the AGD on PND1 and PND22, prostate weight and testosterone levels at PND22. RNA sequencing analyses revealed 119 genes downregulated in treatment groups. It is possible that most of genes were downregulated due to different mechanisms associated with developmental period and phthalate exposure, and therefore, the DEGs would be under negative regulation either by epigenetic mechanisms (eg, our upregulated miRNAs) or by the suppression of gene expression by other cell mechanisms.

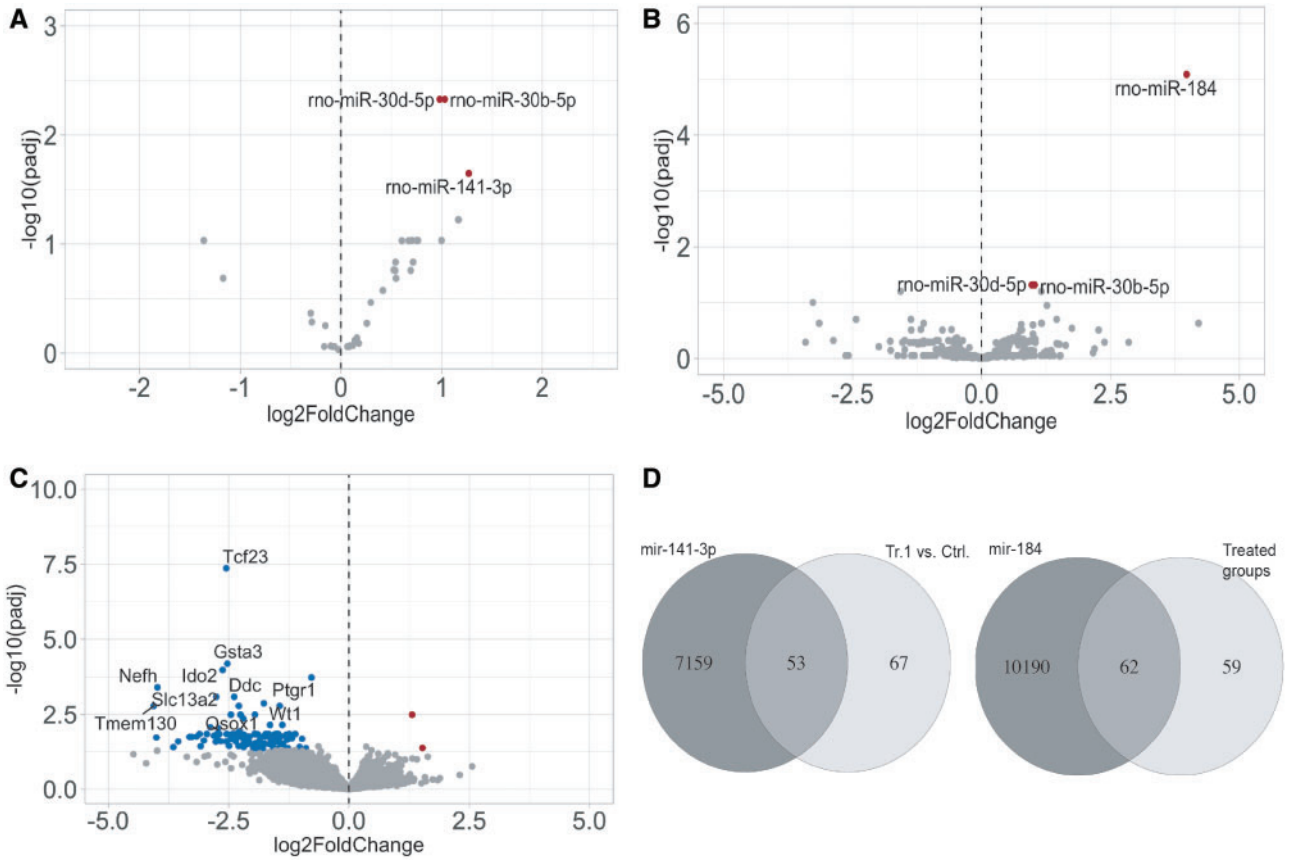

Figure 5. Differential expression of microRNA and mRNA in rats treated with low dose of phthalate mixture versus control. a–c, Volcano plots. Each point represents the difference in expression between treated group (T1) and control plotted against the level of statistical significance. microRNAs (a: counts up to 5 in all the samples and b: counts up to 1 in all the samples) and Genes (c) selected as significantly different are highlighted as red (up) and blue (down) dots. The top 10 genes (sorted by adjusted *p*-value) are labeled with gene symbols. d, Venn diagrams illustrating the overlap of miR-141-3p (left) and miR-184 (right) targets and differentially expressed genes.

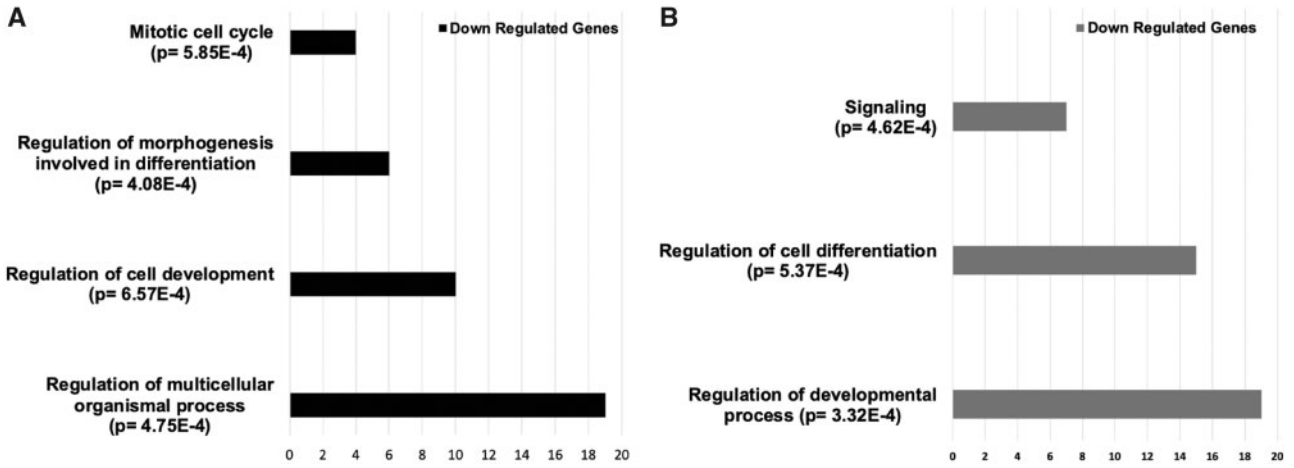

Figure 6. Biological process enrichment analysis of differentially expressed genes in prostate of rats exposed to a phthalate mixture to identify top canonical pathways. Each horizontal bar represents the number of genes presented in the data set for miR-141-3p (a) and miR-184 (b). Differentially expressed genes in each pathway are presented in x-axis (additional information in Supplementary Table 2).

Table 3. Downregulated Predict Genes for MiR-141-3p and MiR-184 Associated With Prostate Development and Oncogenesis

Genes	MiRNA	Function	References
Cited1	miR-141-3p; miR-184	Cited1 are overexpressed in mice prostate with PIN (prostatic intraepithelial neoplasia)-like lesions; probably it has some role during prostate cancer initiation	Thompson *et al.* (2012)
Crmp1	miR-184	Suppressor of tumorigenicity and metastasis in prostate cancer cells	Cai *et al.* (2017)
Fabp5	miR-184	Fabp5 activated expression of metabolic genes via a novel signaling pathway in an ERRα (estrogen-related receptor α)-dependent manner in prostate cancer cell lines	Senga *et al.* (2018)
Fez1	miR-184	Inhibits cancer cell growth through regulation of mitosis	Ishii *et al.* (2001)
Hoxd3	miR-141-3p	Hoxd3 is a frequent hypermethylation target in prostate cancer	Kron *et al.* (2010)
Lcn2	miR-141-3p	Lcn2 could facilitate cell proliferation of castration-resistant prostate cancer via Androgen Receptor (AR) transcriptional activity.	Ding *et al.* (2016)
Ngfr	miR-141-3p	Related to prostatic neuroendocrine differentiation cells	Wan *et al.* (2014)
Pcp4	miR-184	Downregulated in prostate cancer; higher degree correlation with copy number variation (CNV)	Han *et al.* (2017)
Ptgr1	miR-184	Increases apoptosis in prostate cancer cells	Xue *et al.* (2016)
Qsox1	miR-141-3p	High QSOX1 expression correlates with tumor invasiveness, reflects aggressive tumor features, and could be an important biomarker and therapeutic target	Baek *et al.* (2018)
Rtn1	miR-184	Codify a neuroendocrine cell specific protein; enable androgen-independent proliferation of LNCaP cells	Levina *et al.* (2015)
Tmeff2	miR-141-3p; miR-184	Tmeff2 can function as a tumor suppressor by inhibiting cell migration and invasion of prostate cells	Chen *et al.* (2014)
Trpc6	miR-184	Detected in benign and malignant human prostate tissues and prostate cancer cell lines	Yue *et al.* (2009)
Tubb3	miR-141-3p	β-Tubulin III expression was more often seen in high-stage of prostate cancer and more often in metastases than in primary lesions	Egevad *et al.* (2010)
Wnt9b	miR-141-3p; miR-184	Prostate development; Wnt/β catenin pathway; androgen receptor modulation	Mehta *et al.* (2011)
Wt1	miR-184	Prostate development; epithelium-mesenchyme transition (EMT) and mesenchyme-epithelium transition (MET); regulates E-cadherin expression	Brett *et al.* (2013) and Fraizer *et al.* (2016)

Weight gain of pregnant rats during pregnancy and body weight of newborns demonstrate that the different doses, although environmentally relevant, were not overtly disruptive; they agree with previous data in different models of exposure to phthalates during gestation and lactation (Guerra *et al.*, 2010; Scarano *et al.*, 2010). Nevertheless, in animal models, the AGD is a sensitive index of demasculinization of the male reproductive tract (Jiang *et al.*, 2011; Scarano *et al.*, 2010; Zhang *et al.*, 2004). Several epidemiological studies, including prospective cohort studies, showed shortened AGD (suggesting antiandrogenic influence) in boys whose mothers had higher levels of urinary phthalate during pregnancy (Huang *et al.*, 2009; Swan *et al.*,

2015). At PND1, we observed a significant reduction of the AGD only in T3 related to C, however, at PND22 we observed a reduction in the absolute AGD in all treated groups, as well as in T1 and T3 in adjusted AGD compared to C. Because breast milk has been reported as a possible source of exposure to phthalates (Mortensen *et al.*, 2005), probably animals were exposed during lactation which may have interfered with AGD at PND22.

Although AGD is a good antiandrogenic marker for phthalates, it reflects conditions of testosterone availability, responsiveness to androgens or estrogen influence (Fisher *et al.*, 2003; Ge *et al.*, 2007). Several studies have shown that high doses of single phthalates (> 500 mg/kg) are able to reduce testosterone production during the male reproductive tract development (Fisher *et al.*, 2003; Okayama *et al.*, 2017). Buñay *et al.* (2017) showed that a mixture of 3 phthalates (0.3 mg/kg) and 2 alkylphenols (0.05 mg/kg) was not able to alter intratesticular testosterone but reduced intratesticular estrogen. Here, we see that plasma testosterone levels reduced at the lowest dose (T1 group), while T2 and T3 groups were similar to control. In this sense, studies have drawn attention to the biphasic effects of some EDCs, where the outcomes do not follow a simple dose-response behavior (Ge *et al.*, 2007); thus, the phenotypic aspects observed here in animals exposed to low and high doses probably may have different molecular mechanisms in the different groups.

Our results showed that prostate weight at PND22 was sensitive to phthalate mixture treatment (T1 and T3 groups) and T1 animals had an apparent delay in prostate development by increasing small acini proportion; however, we also observed prostate weight recovery in adult animals (PND120). This recovery is known as "catchup growth" in DOHaD models, as postulated by the fetal programing hypothesis (Lapillonne, 2011), and it is related to an adaptive growth after a developmental delay caused by different types of insults, like a toxicological one. Although AGD and prostate weight are very important parameters to evaluate the effects of treatment on male reproductive tract development, they are not sufficient to reflect all the molecular interactions during the genital system development, especially those not related exclusively to the hypoandrogenic environment. In this sense, the epigenetic approach has been important to elucidate molecular mechanisms related to reproductive toxicology and oncotoxicology.

Histopathological and immunostaining analysis showed predominance of small acini in the T1 group compared to C group at PND22. Additionally, we observed increase of diffuse hyperplasia in all treatment groups and focal hyperplasia and inflammatory foci incidence was raised in T1 group compared to the C group at PND120. Klukovich *et al.* (2019) showed that the EDC vinclozolin administered at a critical period of gestation was able to induce epigenetic transgenerational inheritance of susceptibility to prostate disease. They showed a transgenerational increase in susceptibility to prostate pathology (epithelial hyperplasia, metaplasia, and atrophy) and disease in rats ancestrally exposed to vinclozolin. Several reports have showed the association between phthalate exposure and inflammatory and hyperplastic disorders (Kelley *et al.*, 2019; Peixoto *et al.*, 2016; Scarano *et al.*, 2009). Additionally, chronic inflammation plays a crucial role at various stages of prostatic oncogenesis and tumor progression (Tewari *et al.*, 2018). Thus, it seems that the perinatal treatment with the phthalate mixture increased histopathological parameters naturally found in the evolution of prostate oncogenesis.

Despite the phenotypic changes in all treated groups in both ages (T1–T3), molecular alterations at PND22 were more expressive in the T1 group than in the others. It is known that the phenotypic characteristics can represent a large number of genotypic events, and sometimes, to reduce the genotypic variability it is necessary to increase the number of samples, or even, to use isogenic animals or isolated cell types. In addition, in toxicological studies, there is an individual susceptibility that increases the variability within the same treatment (Grandjean, 1992); this type of susceptibility in toxicological studies may be potentiated in developmental toxicologic studies, mainly during fetal and childhood development (Graeter and Mortensen, 1996).

From our 123 deregulated genes, 121 were downregulated; however, 119 of them were downregulated in T1 compared to C. Among the top 10 downregulated genes in T1, 3 of them (Ddc, Ptgr1, and Qsox1) are previously described as involved in prostate carcinogenesis, prostate tumor aggressiveness or as a biomarker (Avgeris *et al.*, 2008; Baek *et al.*, 2018; Poniah *et al.*, 2017; Rychtarcikova *et al.*, 2017; Xue *et al.*, 2016); Wt1 was previously associated with EMT/MET (epithelium-mesenchyme transition/mesenchyme-epithelium transition) during morphogenesis and oncogenesis (Brett *et al.*, 2013; Fraizer *et al.*, 2016); Gsta3 was reported as involved in steroid biosynthesis and the metabolism of some xenobiotics (Tetlow *et al.*, 2004) and Ido2 was expressed during reproductive organs development (Brochez *et al.*, 2017). Additionally, several genes downregulated in our study were classified by functional enrichment as being involved with regulation of multicellular organismal development (31 genes), regulation of cell differentiation (25 genes), regulation of developmental growth (9 genes), and cell secretion (16 genes), and therefore, they could be related to prostate development modulation. Intriguingly, transcriptome analysis identified 2 genes upregulated in T1 and T2 compared to C: Zfat and Rnf183. Zfat have being pointed as an important transcriptional factor associated with cell survival and apoptosis (Fujimoto *et al.*, 2009); likewise, Rnf183 plays an important role in executing programed cell death upon prolonged endoplasmic reticulum stress, likely by inducing apoptosis (Wu *et al.*, 2018). Interestingly, both these 2 genes could participate of tissue remodeling during morphogenesis and, at long term, in oncogenesis process.

Because we verified that T1 group had the largest number of deregulated genes, with most of them downregulated. Upregulation of miRNAs could be a reasonable mechanism accounting for this. Thus, miR-141-3p could be an important candidate to modulate some of these genes, because it was exclusively upregulated in T1 compared to C. Our results also showed that miR-184 was upregulated in all experimental groups compared to control group. Thus, the phthalate mixture was able to increase the expression of this miRNA almost 4 times regardless of the dose. The miR-184 is a single copy gene and evolutionarily conserved at the nucleotide level from flies to humans (Li *et al.*, 2011). According with Li *et al.* (2011), miR-184 may play crucial roles in *Drosophila* development, including tissue fate establishment, differentiation or the maintenance of tissue identity. Furthermore, the miR-184 was shown to be involved in various biological processes, including germline development in the fly (Iovino *et al.*, 2009), neural fate (Liu *et al.*, 2010), cell proliferation, and migration (Yu *et al.*, 2008). This miRNA was reported to be suppressed in prostate carcinoma (Schaefer *et al.*, 2010), neuroblastoma (Foley *et al.*, 2010), epithelial ovarian cancer (Qin *et al.*, 2015), and renal cell carcinoma (Su *et al.*, 2015); and it was observed upregulating pancreatic ductal adenocarcinoma (Li *et al.*, 2015) and hormone refractory prostate adenocarcinoma (Porkka *et al.*, 2007). Thus, the role of this miRNA as

oncogene or tumor suppressor may be context specific (Al-Eryani *et al.*, 2018).

The miR-141-3p belongs to the miR-200 family and it shares an identical seed sequence and differs by only 1 nucleotide with miR-200a (Renthal *et al.*, 2013). Previous studies have shown that miR-141/miR-200 are involved in cancer development and metastasis and miR-141/mirR-200a were associated with ovarian tumorigenesis (Zhang *et al.*, 2010). Furthermore, miR-141/miR-200 family has been reported to inhibit cell proliferation in cancer cells (Du *et al.*, 2009; Yu *et al.*, 2013). From our 53 predicted target genes for miR-141-3p and 62 for miR-184, all of them were downregulated in our study, and therefore are potential targets for these miRNAs as described above. In this sense, the great number of downregulated genes in both cases could reflect a great amount of epigenetic pre- and post-transcriptional events like hypermethylation and miRNAs upregulation (Kim *et al.*, 2012). However, due to the scarcity of results in similar exposure models, it is difficult to make robust comparisons on this aspect.

Among the possible downregulated targets genes for miR-141-3p and miR-184, GO enrichment analysis revealed that for both miRNAs, we have a great number of them associated with developmental processes, differentiation, and cell signaling. Since previous findings have shown that exposure to phthalates during prostate development can increase susceptibility to carcinogenesis (Peixoto *et al.*, 2016; Xia *et al.*, 2018), we have focused our discussion on possible target genes previously described and involved in the development and oncogenesis processes (Table 3).

Among the predicted downregulated genes in the treated groups, 2 of them (Wnt9b and WT1) are very important to urogenital sinus development and, consequently, for prostate morphogenesis. β-Catenin, a multi-functional protein that participates in nuclear signaling and cell adhesion, is a candidate for integrating morphogenetic signals during prostate development (Mehta *et al.*, 2011). WNT/β-catenin signaling pathway seems to be among the most androgen-responsive signaling pathways in the mouse urogenital sinus (Schaeffer *et al.*, 2008). Likewise, WT1 (a predicted target for miR-184) was well studied in urogenital development and its expression was associated as growth suppressive and necessary for cell differentiation, but its role is ambiguous depending on the organ involved and whether epithelial or mesenchymal differentiation occurs (Fraizer *et al.*, 2016). In this sense, the reduction of prostate weight in the mixture exposed groups could be associated with a set of factors, some of them related to the altered expression of 1 or more of these genes.

As it is well known, the oncogenesis process may begin many years before the clinical manifestation of cancer, and hence the stimulation or suppression of oncogenic pathways by epigenetic modulation may be an important factor in susceptibility to prostatic adenocarcinoma. In view of the fact that the genetic and epigenetic changes that modulate oncogenesis are varied and may result in different events as reduction of apoptosis, increase in cell proliferation, alteration in cell differentiation, damage in cell repair mechanisms, etc., several genes may be involved in this process. In the midst of the predicted genes for miR-141-3p, 7 of them were related directly with prostate oncogenesis in different experimental models and/or human cases reports. *Tubb3, Qsox1, Lnc2*, and *Cited1* are associated with increase of aggressive tumor features (Baek *et al.*, 2018; Ding *et al.*, 2016; Egevad *et al.*, 2010; Thompson *et al.*, 2012), whereas *Tmeff2* was reported as a tumor suppressor (Chen *et al.*, 2014) and *Hoxd3* downregulation has been associated with hypermethylation in prostate cancer tumors (Kron *et al.*, 2010). Otherwise, *Ngfr* was reported as being associated with neuroendocrine cells development and differentiation (Wan *et al.*, 2014).

In relation to predicted downregulated genes for miR-184, some of them are deregulated in in prostate cancer like *Rtn1* (Levina *et al.*, 2015) and *Trpc6* (Yue *et al.*, 2009); others act as tumor suppressors like *Crmp1* (Cai *et al.*, 2017) and *Fez1* (Ishii *et al.*, 2001); among others (Table 3). Thus, several pathways miR-141-3p/miR-184-modulated could be related to the preneoplastic and neoplastic alterations observed in our previous studies.

In conclusion, our data indicate that exposure to an environmentally relevant phthalate mixture during the critical window of prostate development induces alteration in reproductive biometric parameters and in the microRNAome and transcriptome profile. Specifically, perinatal exposure to the phthalate mixture affected AGD, prostate weight, and induced deregulation in mRNAs and miRNAs expression profile in the prostate, mainly those that are related to both miR-141-3p and miR-184 upregulation at PND22. Additionally, epithelial hypercellularity and an increase in inflammation incidence were observed at PND120 with phthalate exposure compared to control. Although this was a broad exploratory study, to our knowledge this is the first study using a mixture of phthalates that simulates human exposure on aspects related to prostate development and oncogenesis, including epigenetic aspects. As shown for females (Zhou *et al.*, 2017b), this type of exposure may cause transgenerational effects, and the next step would be to assess if phthalates mixture exposure could transmit some of these epigenetic changes among generations.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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