Increased Estrogen Levels Altered MicroRNA Expression in Prostate and Plasma of Rats Dosed with Sex Hormones

Noriko Nakamura¹, Kelly Davis², Jian Yan³, Daniel T. Sloper¹, Tao Chen³
¹Division of Systems Biology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, United States
²Toxicologic Pathology Associates, Jefferson, AR 72079, United States
³Division of Genetic and Molecular Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, United States

Running title: MicroRNAs in EB/T/E-dosed rat prostate and plasma

Correspondence to: Noriko Nakamura, Ph.D.
Division of System Biology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, United States
Phone: +1-870-543-7175; Fax: +1-870-543-7662; Email: Noriko.Nakamura@fda.hhs.gov

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Abstract

**Background:** Elevated estrogen (E) levels caused by aging or exposure to endocrine disrupting chemicals are related to prostate disease development. Sixty to seventy percent of prostate cancer or benign prostatic hyperplasia patients are over the age of 65, while prostatitis is likely to occur in men under 45 years. MicroRNAs currently represent a class of distinctive biological indicators to be used for clinical disease diagnosis and treatment monitoring. This study aims to identify microRNAs that could serve as potential biomarkers for prostate disorders induced by elevated E levels according to their altered expression in prostate or plasma.

**Materials and methods:** Groups of Sprague-Dawley rats (offspring) were dosed with estradiol benzoate (EB) on postnatal days 1, 3 and 5, and subcutaneously implanted with tubes containing testosterone (T)/E on postnatal day 90. Expression levels of prostate and plasma microRNAs were evaluated using microRNA microarray and validated via qRT-PCR. The expression levels of the potential targeted genes of a set of identified microRNAs were also examined by qRT-PCR.

**Results:** Postnatal administration of EB, T and E elevated serum E levels with decreased serum T levels in rats. Chronic inflammation was observed in the dorsolateral prostate. Significant changes in expression levels of several microRNAs (rno-miR-146-5p, rno-miR-329-3p, and rno-miR-126a-3p) in the dorsolateral prostate and of a microRNA (rno-miR-329-3p) in the plasma were found in the dosed rats. The target gene expression levels of the altered microRNAs also changed accordingly.

**Conclusion:** Chronic inflammation in the dorsolateral prostate of rats dosed with EB, T and E resulted in deregulated expression in a set of microRNAs whose target genes were related to tumor growth or abnormal proliferation. Our findings suggest the identified microRNAs and their target genes the potential use as biomarkers to predict prostate cancer development. Validation using human samples is warranted.

**Keywords:** prostate, rats, microRNAs, estradiol, testosterone, plasma
Introduction

An imbalance of sex hormones, increased estrogen (E) and decreased testosterone (T) levels, via aging or exposure to endocrine-disrupting chemicals (EDCs) may be factors in developing prostate diseases (i.e., inflammation, hyperplasia, and/or cancer) (Anway & Skinner, 2008; Prins, 2008; Diamanti-Kandarakis et al., 2009; Yao et al., 2011). Sixty percent of prostate cancer patients are over the age of 65 (Salinas et al., 2014), which parallels the prevalence of benign prostatic hyperplasia (BPH), as 70% of men in their 60s have prostate hyperplasia (Wei et al., 2005). Those results support that the imbalance of sex hormones in older men may be related to the development prostate diseases.

Since 1986, prostate-specific antigen (PSA) screening has become a standard, noninvasive test used in diagnosing and monitoring the development of prostate cancer. However, BPH and prostatitis, can elevate PSA levels, leading to misdiagnosis and undue concern for the patient. Although several commercially available methods to improve PSA sensitivity exist, prostate cancer-specific biomarkers are required to provide a higher level of specificity in PSA detection and diagnosis, including metastatic variants of the disease (Kretschmer & Tilki, 2017). Beside the PSA test, there are no prostate cancer biomarkers for the diagnosis, monitoring, and prediction of prostatitis or prostate cancer development and treatment. Therefore, additional biomarkers, especially those specific to early disease development, are desired predict, diagnose, and monitor prostate cancer development at reasonable cost.

MicroRNAs (miRNAs) are noncoding RNAs, approximately 22 nucleotides in length, that regulate gene expression by binding to the 3'-UTR of their target site (Wahid et al., 2010; Gebert & MacRae, 2018). Additionally, miRNAs currently represent a class of distinctive biological indicators/biomarkers used in preclinical toxicity studies for drug development and clinical disease diagnosis, as well as treatment monitoring (Mitchell et al., 2008; Selth et al., 2013; Huang, 2017). The profiling of miRNA expression using prostate tissues from patient (Tong et al., 2009; Nip et al., 2016; Kumar & Lupold, 2016; Fabris et al., 2017; Luu et al., 2017; Zedan et al., 2019) indicated that multiple miRNAs were associated with the development and/or metastasis of prostate cancer. However, it remains uncertain which miRNAs are suitable biomarker candidates specifically diagnosing prostatitis, BHP, or prostate cancer.

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Rats are used as a model for studying prostate cancer and/or BPH (Russell and Voeks, 2003). They are also widely used in studies on gene expression of prostate diseases induced by EDCs and/or testosterone and estradiol dosing (Noble, 1977; Pollard et al., 1982; Bosland et al., 1995). Rat models of prostate disease (neoplasia and inflammation), induced by EDCs supplemented with additional testosterone and estradiol treatments, have been used to examine gene expression profiles during prostate development through adulthood (Gilleran et al., 2003; Huang et al., 2004; Ho et al., 2006). The purpose of this study was to measure miRNAs expression profiles in rat prostate tissue to identify significant changes of miRNA expression induced by EDCs and sex hormone exposure from prepubertal age through adulthood. Sprague-Dawley (SD) rats (Hsd:SD, male offspring) were administrated estradiol benzoate (EB) by subcutaneous injection on postnatal days (PNDs) 1, 3 and 5, and then treated with additional hormones [i.e., E and T] on PNDs 90-200 as described by Ho et al. (2006). We collected the body and organ weights, measured serum hormone levels, and conducted histological of the prostate gland on PNDs 30, 90, 100, 145 and 200. Also, we performed miRNA microarray analysis using prostate tissues and plasma of rats dosed postnatally with EB, T, and E and verified those results by qPCR. A set of miRNAs were identified as potential biomarkers for prostate diseases. Furthermore, gene expression levels of the predicted targets of those miRNAs were also assessed.

2. Materials and Methods

Materials: All reagents were purchased from Fisher Scientific (Pittsburgh, PA, USA) and Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

Animals: To obtain postnatal pups, 11–13-week-old time-mated female SD rats were purchased from Envigo (Indianapolis, IN, USA) and delivered to the National Center for Toxicological Research (NCTR) on gestation day (GD) 3. Pregnant dams were housed individually and maintained under a 12:12-h light-dark cycle with controlled room temperature (23°C ± 3°C) and humidity (50% ± 20%). Dams and male pups were fed low phytoestrogen 5K96 chow (Purina Mills, St. Louis, MO) beginning on GD 3. Water was provided ad libitum. All animal procedures were approved by the NCTR.
Institutional Animal Care and Use Committee and followed the Guidelines set forth by the National Research Council’s Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

**Hormone treatments:** Animal hormone treatments followed the method described by Ho et al. (2006). Male pups were divided into two groups at random: untreated or EB-treated (Table 1). In the EB-treated group, male pups were subcutaneously injected with 2.5 mg/kg body weight (BW) EB (Sigma-Aldrich, E8515) on PNDs 1, 3 and 5 [day of birth = PND 0]. Male pups in the untreated group were injected with a vehicle (tocopherol-stripped corn oil; #0290141584-400; ICN Biomedicals, Inc., Aurora, OH).

On PND 90, the untreated or EB-treated group was divided again (Table 1). All animals were subcutaneously implanted with three silastic tubing inserts (1.47 mm; absorbance, 1.95 mm; Dow Corning, ID); control and EB only groups with three empty inserts (two 2-cm and one 1-cm inserts); T+E only and EB+T+E groups with two 2 cm inserts packed with T powder (Cat#: T-1500, Sigma-Aldrich) and one 1 cm tube packed with 17-β-estradiol (Cat.#: E8875; Sigma-Aldrich) until PND 200. On PND 146 (eight weeks after the first surgery on PND 90), 32 animals underwent a second surgery to add additional hormone-containing silastic tubing implants prior to being sacrificed at PND 200.

**Sample collection:** Animals were euthanized in the morning (8 am -12 pm) on PND 30, 90, 100, 145 or 200 [N = 8/ group except on PND 30 (N = 18)] by carbon dioxide asphyxiation, followed by collection of blood via cardiac puncture into EDTA treated collection tubes for plasma or SST™ Blood collection tubes for serum (BD, Franklin Lakes, NJ, USA). And then, tissue samples were collected. Body and reproductive organ weights (paired testes, paired epididymides, prostates (i.e., dorsolateral and ventral), and anterior prostate with seminal vesicles) were recorded. Liver, paired kidneys, lung, and iliac lymph node weights were also recorded. Plasma and serum were collected by centrifuging the collection tubes at 3,000 × g for 10 min at room temperature.
**Prostate histology:** Dorsolateral prostates were fixed in 4% paraformaldehyde in PBS at 4°C for 24 h. Liver, lung, anterior and ventral prostates, kidney, and iliac lymph nodes were fixed in 10% neutral formalin at 4°C for 24–48 h. After fixation, the tissue specimens were routinely processed, embedded in paraffin, sectioned approximately 4 μm-thick, mounted on glass slides, stained with hematoxylin and eosin and examined by a light microscope. Images were captured using the same exposure time with a Leica/Aperio ScanScope AT2 whole slide scanner and Leica ImageScope software (Leica Biosystems; Buffalo Grove, IL, USA). The severity of microscopic findings in the prostate were graded as 1 (minimal), 2 (mild), 3 (moderate) or 4 (marked) as described by Derelanko (2008) with a minor modification (Supplemental Table 1).

**Measurement of serum T and E levels:** Serum T levels were measured with a testosterone ELISA kit (ALPCO Diagnostics, Salem, NH, USA); plates were read using a microplate reader (Spectra Max 190) and accompanying software (SoftMax Pro 4.3.1 L, both from Molecular Devices, LLC, Sunnyvale, CA, USA). Serum E levels were measured with an estradiol (E2) ELISA kit (Biomatik Corp, Cambridge, Ontario, Canada); the plate was read using a GloMaxR Discover multimode microplate reader (Promega Corp., Madison, WI, USA).

**miRNA microarray analysis:** Representative RNA samples were randomly selected from each time point of the dorsolateral prostate of PND 90, 145 and 200 rats (N = 4/group) or from the plasma of PNDs 145 and 200 (N = 4/group). RNA was extracted using the miRNeasy Kit or miRNeasy Plasma Serum Kit (Qiagen, Valencia CA, USA), respectively. To examine the effect of additional T and E exposure we used samples from PNDs 90, 145 and 200. After extraction, the concentration of each RNA sample was determined using a DS-11 spectrophotometer (DeNovix, Inc., Wilmington, DE, USA).

MiRNA microarray and data analysis were performed by LC Sciences (Houston, TX, USA) using 1 μg total RNA from prostate tissues or 0.2–0.5 μg total RNA from plasma in the control, EB only, and EB+T+E groups.
The LC Sciences µParaflo technology platform was used for miRNA expression profiling. The miRNA microarray can be used to analyze all rat mature miRNAs in the version 22 of the miRBase database (March 2018). Image data were collected after hybridization. The image digitalization was performed using “Array-Pro Analyzer” (MediaCybernetics, Rockville, MD, USA). Locally weighted scatter plot smoothing (LOWESS) was used for normalization (Cleveland, 1979; Cleveland & Devlin, 1988; Bolstad et al., 2003). Significantly differentially expressed miRNAs were selected with log2 (fold change) >1 or log2 (fold change) <-1 (fold changes increased or decreased two times over the control). Statistical analysis was performed using either t-tests (2-group comparison) or ANOVA (multiple group comparison), compared to the control group at PND 90, 145 or 200.

**miRNA cDNA synthesis:** The cDNAs from samples (N = 5/group) were synthesized with a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) using 10 ng total RNA and purified miRNA from the rat prostate and plasma samples.

**miRNA Quantitative PCR (qPCR):** The qPCR analyses were performed using the ABI PRISM 7900HT Fast Real-Time PCR System or AppliedBiosystems ViiA7 Real-Time PCR System (Applied Biosystems, Foster City, CA. USA) with TaqMan Universal Master Mix II and TaqMan small RNA Assays (Applied Biosystems, Foster City, CA. USA) according to the manual procedures provided by the manufacturer. The TaqMan small RNA assays used in this study are shown in Supplemental Table 2. The cDNA, synthesized as described above, served as a template in 10 μl reaction mixtures. The TaqMan Universal Master Mix reaction conditions were as follows: initial denaturation steps at 50°C for 2 min and 95°C for 10 min, followed by 40 amplification cycles (95°C for 15 sec, 60°C for 1 min), respectively. The relative fold changes were calculated using the comparative ΔΔCt value in the following equation: relative quantity = 2^{-ΔΔCt} (Livak & Schmittgen, 2001). The expression levels were normalized using U6 as an internal control for each sample. The relative ratios of transcript levels in each sample were calculated setting the values for controls to one. The qPCR reactions for each sample were performed in triplicate (N = 5/group).
Gene expression profiling (qPCR): We selected high scoring miRNA target genes using an online database for miRNA target prediction and functional annotation: miRDB (Wong & Wang, 2015; Wang, 2016). We measured transcript levels of the predicted target genes using qPCR. The qPCR analysis was performed using an ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific Inc.) per manufacturer’s instructions. The synthesized cDNA served as a template in 10 μl reaction mixtures. The PowerUp SYBR Mix reaction conditions were as follows: initial denaturation steps at 50°C for 2 min and 95°C for 2 min, followed by 40–45 amplification cycles (95°C for 15 sec, 60°C for 1 min) with a dissociation step (95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec). The relative steady state transcript levels were calculated using threshold cycle (Ct) values in the following equation: relative quantity = $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001). Expression levels were normalized using Rn18s as an internal control for each sample. Relative ratios of transcript levels in each sample were calculated setting the values for the control group per collecting time (PNDs 30, 90, 100, 145, or 200) at one. The qPCR reactions for each sample were performed in triplicate ($N = 5$/group). The specific primer pairs used in this study are shown in Supplemental Table 3.

Statistical analysis: Data are presented as the mean ± standard error of the mean. Except for miRNA microarray analysis and qPCR, statistical analyses of the assays were performed using $t$-tests (PNDs 30 and 90) or one-way ANOVA (PNDs 100, 145 and 200) followed by Tukey’s test. The statistical analysis of miRNA microarray analysis was performed using $t$-tests (PND 90) or one-way ANOVA (PNDs 145 and 200) with Bonferroni adjustment (Hochberg, 1988; Shaffer, 1995). qPCR data was analyzed using a $t$-test with Bonferroni adjustment (Hochberg, 1988; Shaffer, 1995). A $p$-value of <0.05 was considered statistically significant.

Results

Body and organ weights:

Body weights: BWs in the EB-treated group were significantly higher compared to the control group on PND 30, while there was no significant difference between the control and EB-treated groups on PND 90 (Figure 1A, Supplemental Table 4A). BWs in the T+E only and EB+T+E groups were
significantly lower than those in the control and EB only groups on PNDs 100, 145 and 200, respectively. There were no significant differences in BWs between the control and the EB only groups at all collection points. No statistical significances in BWs were observed between the T+E only and EB+T+E groups on PNDs 100 and 145; however, there were statistical significances in BWs between those groups on PND 200 (Figure 1A).

**Organ weights:** The absolute and relative prostate weights/ BW (anterior, ventral and dorsolateral regions) were significantly lower in the EB-treated group on PNDs 30 and 90 than in the controls (Figure 1B, Supplemental Table 4A). On PNDs 100, 145 and 200, the absolute and relative prostate weights/BW were significantly higher in the T+E only and EB+T+E groups than in the control and EB only groups (Figure 1B; Supplemental Table 4A). In contrast, absolute and relative testis weight/BW significantly decreased in all treated groups on PNDs 30, 90, 100, 145 and 200 compared to the control group (Supplemental Table 4B). The relative liver to weight/BW was significantly reduced in the EB-treated group compared to the control group on PND 90 (Supplemental Table 4C). There were no significant differences in any absolute organ weight (liver, paired kidneys, lung, and iliac lymph node) or other relative organ weight between the two groups on PNDs 30 and 90. After treatment with additional hormones on PND 90, the absolute and relative iliac lymph node weights/BW in the EB+T+E group were significantly higher than those in the control group on PNDs 100, 145 and 200. The relative kidney and lung weights/BWs were significantly higher in the T+E only and EB+T+E groups than in the control group on PNDs 100 and 145, and it increased on PND 200 compared to the control and EB only groups.

**T and E levels:**
Serum T levels were significantly lower in the EB-treated group compared to the control group on PND 90, while the T levels were reduced with no significant differences between the groups on PND 30 (Figure 2A). After additional dosing of T and E on PND 90, serum T levels in the T+E only (twofold higher) and EB+T+E groups (1.3-fold higher) were significantly elevated compared to the control group on PND 100. The serum T levels in the T+E only group were significantly higher than serum T levels in the EB only group. On PND 145, no significant differences were observed among
any of the groups (Figure 2A). On PND 200, serum T levels in the T+E only and EB+T+E groups (1.3-fold higher) were significantly increased compared to the control and EB only groups (Figure 2A). In the EB+T+E group, serum T levels were significantly higher on PND 200 compared to the serum T levels on PNDs 100 and 145 (Figure 2A). In contrast, changes in the serum E levels were different from those for T on PNDs 90, 100, 145 and 200. Serum E levels were significantly lower in the EB-treated group than the control group on PND 30 (Figure 2B). However, on PND 90, the E levels were increased with no significant differences between the groups (Figure 2B). After administration of additional T+E on PND 90, serum E levels in the T+E only and the EB+T+E groups were higher on PND 100 compared to the control and EB only groups, respectively. On PND 145, the E levels in the T+E only and EB+T+E groups were higher than control group, while on PND 200, the levels were slightly increased in the T+E only, EB only, and EB+T+E groups compared to the control group. The levels in the EB+T+E group on PND 100 were especially elevated, then decreased on PNDs 145 and 200. The serum E levels in the T+E only group peaked on PND 145, and then decreased on PND 200. The serum E levels in the EB only group slightly decreased on PND 145 and then increased on PND 200 when compared to the levels on PND 100. Due to a wide variety of the concentrations among animals, no significant differences were observed in the serum E levels on PNDs 100, 145 and 200.

**Histologic evaluation of prostates from PNDs 30, 90, 100, 145, and 200 male rats:**

On PND 30, hypoplasia was observed in the anterior, ventral, and dorsolateral prostates of rats in the EB-treated group (images of only dorsolateral prostate shown in Figure 3A), with no hypoplasia observed in the control group (Figure 3B). On PND 90, hypoplasia was still observed in anterior, ventral, and dorsolateral prostates of rats in the EB-treated group (Figure 3C, D). No glandular acini were observed in the EB-treated group (Figure 3C, D) compared to the control group (Figure 3E, F). Several animals presented acute inflammation and cellular infiltration of mononuclear cells in the dorsolateral prostates in the control group on PND 90 (arrows; Figure 3E). On PND 145, regenerative hyperplasia and cellular infiltration of mononuclear cells were observed in the dorsolateral prostates in the EB+T+E group. Interestingly, chronic inflammation was observed in only the dorsolateral prostates of EB+T+E group (Figure 3G, H). Cellular infiltration of mononuclear cells was also
observed in the dorsolateral prostates of the T+E only group (arrows; Figure 3I) compared to the control group (Figure 3J).

**Acute/chronic inflammation of rat dorsolateral prostates:** Chronic inflammation was observed in only the dorsolateral prostates of the rat. Table 2 contains a summary of acute/chronic inflammation results of rat dorsolateral prostates. Only data on dorsolateral prostates are shown because chronic inflammation was observed only in dorsolateral prostates. There were no observations of acute/chronic inflammation in rat prostates in the control and EB-treated groups on PND 30 (Figures 3A, B). One of eight animals in the EB-treated group showed acute inflammation in the dorsolateral prostates, while no chronic inflammation was observed in rat prostates from the control group on PND 90. On PND 100, acute inflammation was observed in the dorsolateral prostate of rats in the EB only and EB+T+E groups, while no chronic inflammation was observed in any group (Table 2). Interestingly, acute inflammation was observed in the dorsolateral prostate of rats in the T+E only group on PND 145, while no acute inflammation was observed in the dorsolateral prostate of rats in the EB+T+E group (Table 2). In contrast, chronic inflammation in the rat dorsolateral prostate was observed in the EB+T+E group on PNDs 145 and 200. However, chronic inflammation was observed in the T+E only group on PND 200 only and was not as severe as that seen in the EB+T+E group (Figure 3I, J).

**miRNA microarray analysis:** As shown by histopathology and ELISA, elevated estrogen levels and chronic inflammation were observed only in the dorsolateral prostates of the EB+T+E groups on PND 145 and 200 after additional T and E treatments. We performed miRNA microarray analysis on PNDs 90, 145, and 200 to determine the effect of additional T and E treatments on miRNA expression in the rat prostates and plasma. According to miRNA microarray analysis results, unique miRNAs (e.g., rno-miR-146b-5p, rno-miR-126a-3p) in prostates of PNDs 145 and 200 were identified and the miRNA profiles for these groups were significantly different (Table 3; Supplemental Tables 5-6; Supplemental Figure 1A, B) from the miRNA expression profiling on PND 90 (Supplemental Table 7; Supplemental Figure 1C). Rno-miR-375-3p and rno-miR-24-3p were significantly changed only on PND 145, while rno-329-3p were changed on only PND 200 (Table 3). The rno-miR-329-3p levels
were over 10-fold lower in the EB only and EB+T+E groups than the control group (Table 3). On PND 145, of the top 20 miRNAs, 13 miRNAs were up-regulated and seven miRNAs were downregulated (Supplemental Table 5). Most miRNAs were significantly upregulated in the prostates of PND 200 rats dosed postnatally with EB, T, and E compared to the control group (10 miRNAs of 15 miRNAs that were significantly different; Supplemental Table 6). In addition, rno-miR-146b-5p was almost 10-fold higher in the EB+T+E group than in the EB only group on PNDs 145 and 200 (Table 3; Supplemental Tables 5-6). Only five miRNAs of the 15 miRNAs were down-regulated on PND 200 (Supplemental Table 6).

In plasma, rno-miR-329-3p was downregulated in the rats dosed postnatally with EB, T, and E on both PNDs 145 and 200, despite a lower signal. The miR-329-3p levels were significantly lower in the plasma and prostate tissue of PND 200 rats treated postnatally with EB, T, and E (Supplemental Table 8; Supplemental Figure 3).

According to the results of miRNA microarray analysis, we selected miRNAs that were significantly affected in the prostate tissue and plasma of rats on PNDs 145 and 200 for qPCR validation: rno-miR-146b-5p, rno-miR-130b, rno-miR-126a-3p, rno-miR-145, and rno-miR-375-3p (prostate tissues); rno-miR-329-3p, rno-miR-214-3p, and rno-miR-24-3p (prostate and plasma).

qPCR validation of miRNAs and the expression profiling of their target genes:
1. qPCR validation in prostates of rats dosed postnatally with EB, T, and E

On PND 145, the expression levels of rno-miR-126a-3p, rno-miR-145, rno-miR-214-3p, and rno-miR-24-3p were significantly higher in the EB only group then the control group (Figure 4). The rno-miR-146b-5p levels increased significantly in the EB+T+E group compared to those in the control group, while no significant differences were observed in the T+E only and EB only groups, although the levels in these groups were higher than in the control group.

On PND 200, the expression levels of rno-miR-126a-3p were significantly higher and the expression levels of rno-miR-329-3p in the EB+T+E group was significantly lower in the EB+T+E group compared to those in the control group. The expression of rno-miR-146b-5p significantly increased in
both T+E only and EB+T+E-groups, compared to the control group. The rno-miR-214-3p expression was lower in the T+E only group than in the control group.

2. qPCR validation in the plasma of rats dosed postnatally with EB, T, and E
The expression levels of rno-miR-329-3p and rno-miR-24-3p in plasma were validated by qPCR (Figure 4). Although the qPCR did not detect the expression of miR-329-3p using plasma from PND 145 rats, its expression levels were found lower in T+E only and EB+T+E groups compared to concurrent controls, with a statistical significance in the EB+T+E group on PND 200. The expression levels of miR-24-3p increased in all dosed groups on PNDs 145 and 200 compared to the controls; however, no statistical differences were observed.

3. Transcript levels of target genes of miR-329 in the prostates of rats dosed postnatally with EB, T, and E
Although weaker in signal, miR-329 was detected in prostate tissues on PNDs 145 and 200, and in plasma on PND 200. We searched target genes of miR-329 using the database for miRNA target prediction and functional annotation: miRDB (Wong & Wang, 2015; Wang, 2016). The target genes were selected for examining their transcript levels as following: cyclin-dependent kinase 1 (Cdk1), mitogen-activated protein kinase 1 (Mapk1), phospholipase A2, activating protein (Plaa), prostate transmembrane protein, androgen induced 1 (Pmepa1), estrogen-related receptor gamma (Esrrg), BCL tumor suppressor 7A (Bcl7a), coiled-coil domain containing 71-like (Ccdc71l), bone morphogenetic protein receptor type 2 (Bmpr2), and tumor protein p53 inducible nuclear protein 2 (Tp53inp2). The transcript levels of Esrrg and Tp53inp2 genes significantly decreased in the EB+T+E group compared to the control group on PND 145 and 200 (Figure 5A; Supplemental Figure 3). The transcript levels of Plaa gene were significantly lower in the EB+T+E group compared to the control group on PND 100, while the transcript levels more than fourfold higher in all groups compared to the control group on PND 145. The transcript levels of Bmpr2 gene were significantly lower in the EB+T+E group than in the control group on PND 200. The transcript levels of Bcl7a gene were significantly lower in the T+E only group compared to the control group on PND 200. On PNDs 145 and 200, the transcript levels of Mapk1, Ccdc71l, Pmepa1, and Cdk1 genes were more than five-fold.
higher in the EB+T+E group than in the control group (Supplemental Figure 3). However, there were no significant differences between the control and EB+T+E groups. Although all samples in the EB+T+E group were upregulated, there was a large variance in range (2- through 26-fold), which may have contributed to the lack of statistical significance.

4. Predicted target genes of miR-146b-5p, miR-126a-3p, and miR-375-3p

We also searched the target genes of miR-146b-5p, miR-126a-3p, miR-214-3p, and miR-375-3p. Frizzled class receptor 8 (Fzd8) for miR-375-3p; calmodulin regulated spectrin-associated protein (Camsap1) for miR-126-3p; interleukin-1 receptor associated kinase 1 (Irak1) and potassium channel tetramerization domain containing 9 (Kctd9) for miR-146-5p; sigma non-opioid intracellular receptor 1 (Sigmar1) and leucine zipper protein 1 (Luzp1) for miR-214-3p were selected by the scores obtained with miRDB (Figure 5B). The transcript levels of Fzd8 gene decreased in the T+E only and EB+T+E groups compared to the control, with statistical differences in the T+E group on PNDs 145 and 200. The transcript levels of Camsap1 and Kctd9 genes were significantly lower in the EB+T+E group than in the control groups on PNDs 145 and 200, respectively. Sigmar1 gene expression decreased in the EB+T+E group when compared to the control group on PNDs 100 and 145, respectively. Luzp1 gene expression increased only in the EB group on PND 100.

4. Discussion

Early postnatal exposure to EDCs is known to increase the risk of prostate cancer (Prins, 2008; Diamanti-Kandarakis et al., 2009). An imbalance in the ratio of E to T (elevated estrogen levels due to reduced T levels) is also reported to increase the risk of prostate cancer (Yao et al., 2011). EDC exposure at an early age and subsequent exposure to T and E have previously been reported to affect prostate development and are causative factors in prostate disease (Prins, 2008). Our study has focused on the effects of early postnatal exposure to EB, with additional exposure to T and E, on the developing prostates and occurrence of prostate disease through adulthood. We determined that T and E exposure in rats dosed early postnatally with EB caused prostate disease and the exposure to EB, T, and E affected miRNA expression in the rat prostate and plasma.
**Enlarged prostate weights induced with T and E in rats dosed early postnatally with EB**

EDCs are reported to affect the male reproductive organ (Barlow & Foster, 2003; Prins, 2008; Diamanti-Kandarakis *et al.*, 2009), consistent with our findings: the decreased weights of all lobes of prostates on PNDs 30 and 90 after EB exposure. The significant increases of prostate weights by additional T and E treatments is consistent with those described by Ho *et al.* (2006). Additional T and E treatments do not increase testis size (another male reproductive organ), suggesting the prostate is more sensitive to T and E levels. Moreover, ethinyl estradiol exposure has been reported to increase prostate weight (Prins, 2008; National Toxicology Program, 2010). Estrogen is thought to be related with BPH (Ajayi & Abraham, 2018). Although BPH was not observed in the present study, other studies have suggested that higher levels of estrogen may lead to prostate enlargements.

**Higher estrogen levels cause chronic inflammation of the dorsolateral prostate in rats dosed early postnatally with EB**

Chronic inflammation was observed in rat prostates only in the EB+T+E group on PNDs 145 and 200. No neoplasia was observed in these prostates by histological analysis, consistent with the results reported by Ho *et al.* (2006). Bosland *et al.* (2005) reported that only 33% of SD rats produced neoplasia. We could not confirm why the animals in the EB+T+E group did not produce neoplasia, although they did develop prostate inflammation (Bosland *et al.*, 1995; Prins, 1997; Naslund *et al.*, 1998; Stroker *et al.*, 1999; Prins *et al.*, 2002). This could be a time issue. As chronic inflammation may eventually develop into neoplasia (De Marzo *et al.*, 2007), it is difficult to exclude the possibility that animals in the EB+T+E group may have developed prostate neoplasia with a longer recovery period.

Before observing chronic inflammation in the dorsolateral prostate, we found dramatically higher E levels in the EB+T+E group on PND 100. Due to lower T levels induced by early postnatal exposure to EB, relatively, the estrogen levels are much higher in the EB+T+E group. This phenomenon is similar to the imbalance of the ratio of E levels to T levels in humans as a function of age. Subsequently, many genes were dramatically altered with chronic inflammation in prostates of the EB+T+E group on PNDs 145 and 200. As some studies have described (Straub, 2007; Nelles *et al.*, ...
2011), this imbalance in estrogen levels may be associated with the development of chronic inflammation.

**miRNA expression profiling**

We observed statistical significances in some miRNAs (rno-miR-329-3p and rno-miR-126a-3p) in the dorsolateral prostates of the EB+T+ E group developing chronic inflammation. The underlying mechanism for the miRNA changes is unknown. One possibility is the increased miRNA expression may be associated with miRNAs from invading mononuclear cells (lymphocytes and monocytes or macrophages) in the dorsolateral prostate by chronic inflammation. However, other researchers’ miRNA expression and identification profiling in mononuclear cells in inflammation differed from our current results: miR-155, miR-16-1, miR-15a, miR-181, miR-23b etc (O’Connell et al., 2007; Calin et al., 2008; Hirschberger et al., 2018). Thus, the change of rno-miR-329-3p and rno-126a-3p expression may be prostate specific.

Furthermore, rno-miR-329-3p was found in both the plasma and dorsolateral prostate of rats dosed with EB, T and E. The differential expression of miR-329-3p was found in blood from prostate cancer patients at pretreatment and early treatment stages (Daniel et al., 2017). However, the miR-329-3p expression increased in the patients, different from our results that showed a decrease of the miRNA expression. This difference may be attributable to a different development stages of prostate cancer, prostate inflammation and cancer and different species, rat vs. human. For multiple reasons such as ease of collection and patient comfort, a blood sample would be the best method for screening and measuring prostate cancer biomarkers. (Kretschmer & Tilki, 2017). Thus, rno-miR-329-3p may be a potential biomarker for diagnosing prostate cancer as well as monitoring the development of prostate cancer from chronic inflammation.

However, we have not examined the rno-miR-329-3p expression levels in human blood from diagnosed cancer patients; further study is necessary to determine it.

This study also found the changes of miR-329-3p’s target genes: Esrrg, Tp53inp2 and Bmp2r genes. The ESRRG protein belongs to the estrogen-related receptor (ESRRs) family, whose signaling
pathway is similar to the estrogen receptor pathway and acts on cell growth and differentiation (Misawa & Inoue, 2015). Since the function of Esrrg gene is reported to inhibit tumor growth in prostate cancer cell lines (Yu et al., 2007), the dysregulation of the gene may trigger tumor growth and cell proliferation.

Tp53inp2 gene is related to the onset of malignancy with cell growth in human liposarcoma (Hu et al., 2017). In addition, an LC-MS/MS study found that the significantly downregulated Tp53inp2 gene is present in prostate cancer patients (Haj-Ahmad et al., 2014). The BMPR2 protein encoded to Bmpr2 gene is well known and studied and is a member of the transforming growth factor-β (TGF-β) super family. The complex of BMPR2 and its ligands BMPs (especially, BMP2, 4, 6, and 7) controls many physiological functions (Morrell, 2016) (e.g., cell growth and differentiation, osteogenesis, embryonic/vascular development; Kim et al., 2017). Kim et al. (2004) reported the expression levels of Bmpr2 gene was negatively correlated with the recurrence in prostate cancer. Kobayashi et al. (2017) reported BMP7-BMPR2 complex involved in bone metastasis of prostate cancer.

Thus, miR-329-3p could be related to prostate tumor development according to the functions of its target genes. However, due to the limited plasma sample volume, we were unable to measure the transcript levels of these target genes in plasma. Further study is necessary for verification.

Many studies identified miR-126-3p being related to human prostate cancer progression. It has been suggested to be one of the biomarker candidates for prostate cancer (Song et al., 2016; Hua et al., 2018). In addition, the target gene of miR-126 is involved in inflammation (Tang et al., 2017). MiR-146-5p is associated with inflammation and is upregulated (Sonkoly et al., 2008). MiR-146-5p is activated via NF-κB signaling (Taganov et al., 2006). Our findings show that of miR-146-5p levels in the dorsolateral prostates significantly decreased on PND 200. As chronic inflammation in dorsolateral prostate was observed before PND 200, the upregulation of miRNA was attributed to chronic inflammation.

A summary of these results is provided in Figure 6. Our findings indicated most miRNAs identified in rat dorsolateral prostates in the EB+T+E group were significantly altered and associated with chronic
inflammation. Their target genes were significantly changed, suggesting that they were associated with the altered miRNA expression induced by chronic inflammation and/or elevated estrogen levels. Reduced miR-329-3p levels may lead to reduced *Tp53inp2* and *Essrg* transcript levels, resulting in an activation of malignancy and cell proliferation/tumor growth.

5. Conclusion
In this study we confirmed that EDC exposure are key to elevated estrogen levels and decreased T levels are key to this rat model system and observed chronic inflammation in the prostates of rats. Our results show: (1) multiple miRNAs expression levels (rno-miR-146-5p, rno-miR-329-3p, and rno-miR-126a-3p) were altered in association with chronic inflammation in the dorsolateral prostate of rats dosed postnatally with EB, T and E; (2) the change of rno-miR-329-3p expression levels in occurred the plasma of rats dosed postnatally with EB, T and E; (3) rno-miR-145 expression levels increased in only the EB group; and (4) transcript levels of the target genes of these altered miRNAs were also changed, which may be regulated directly or indirectly. Only chronic inflammation was observed in the dorsolateral prostate of rats treated with EB, T and E in this study. However, the differentially expressed miRNAs and their target genes are not only involved in inflammation, but also in tumor growth and/or abnormal proliferation. These findings suggest that the identified miRNAs and their target genes may have the potential to sever as biomarkers for predicting prostate cancer development and/or may contribute to the development of drugs for its treatment. However, as results were obtained using a rat model, further studies are necessary to validate these findings in humans.

Conflict of interest: The authors declare no conflicts of interest.

Author contributions: N.N. conceived of the presented idea and experimental design. N.N. performed the experiments (animal surgery, interpreting data and qPCR), analyzed all data, and wrote the manuscript. K.D. performed histologic analysis and summarized histological results. D.S. assisted with experiments (animal surgery and miRNA extraction) and manuscript editing. J.Y. and T.C supervised and assisted with qPCR validation of miRNAs and interpreted the results. N.N. finalized
the manuscript incorporated with all co-authors’ comments. D.S., K.D., J. Y. and T.C. provided feedback and critical comments.

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**References**


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Figure 1. Body weights (A) and prostate weight (three regions) (B) of rats dosed postnatally with EB, T and E on PNDs 30, 90, 100, 145 and 200.
Values express mean ± SD. * indicates significantly altered compared to the control group (p < 0.05). 
\(a\) indicates significantly altered compared to the EB only group (p <0.05). \(b\) indicates significantly altered compared to the T+E only group (p < 0.05). CTRL: control.

Figure 2. Serum testosterone (A) and estradiol (B) levels in rats dosed postnatally with EB, T and E on PNDs 30, 90, 100, 145 and 200.
Values express mean ± SD. * indicates significantly altered compared to the control group each collection point (p < 0.05). ** indicates significantly altered compared to the control group (p <0.005). 
\(a\) indicates significantly altered compared to the EB only group (p <0.05). † indicates significantly altered in the EB+T+E group compared to on PNDs 100 and 145. CTRL: control.

Figure 3. Histological analysis of rat dorsolateral prostate dosed postnatally with EB, T and E on PNDs 30 (A, B), 90 (C-F), and 145 (G-J) by hematoxylin and eosin staining. Arrows show cellular infiltration of mononuclear cells. A white box in panel G was enlarged (panel H). CTRL: control. Bars: 50 μm.

Figure 4. Relative expression of miR-126a-3p, miR-329-3p, miR-146b-5p, miR-214-3p, miR-24-3p, miR-145, and miR-375-3p in the dorsolateral prostate and miR-329-3p and miR-24-3p in the plasma of rats dosed postnatally with EB, T, and E
Data are expressed as the mean fold change ± standard error of the mean (\(N = 5\)/group). *\(p < 0.05\) compared to the control group for each endpoint (PND 30, 90, 100, 145, or 200) in rat prostate. N.D.: not detected.
Figure 5. Relative transcript levels of *Esr2*, *Tp53inp2*, *Plaa*, *Bmpr2*, and *Bcl7a* genes, which are potential target genes of miR-329-3p (A), *Fzd8*, *Camsap1*, *Irak1*, *Kctd9*, *Sigmar1*, and *Lupz1* genes, which are potential target genes of miRNAs (miR-126-3p, miR-146-5p, miR-329-3p, and miR-214-3p) (B) in the dorsolateral prostate of rats dosed postnatally with EB, T, and E. Data are expressed as the mean fold change ± standard error of the mean (*N* = 5/group). *p* < 0.05 compared to the control group for each endpoint (PND 30, 90, 100, 145, or 200) in the rat prostate. **p* < 0.01 compared to the control group for each endpoint (PND 30, 90, 100, 145, or 200) in the rat prostate.

Figure 6. Summary of miRNA expression profiling in the prostate tissues of rats dosed postnatally with EB, T, and E.

Elevated estrogen levels induced chronic inflammation in rat dorsolateral prostates. The miR-146-5p, miR-329-3p, miR-126, and miR-214-5p expression levels were altered via chronic inflammation and/or elevated estrogen levels. Their target genes significantly changed in association with the altered miRNA expression. Reduced miR-329-3p levels may lead to reduced *Tp53inp2* and *Esr2* transcripts levels, resulting in activation of malignancy and cell proliferation/tumor growth. Increased miR-146-5p may inactive NK cells via reducing *Kctd9* gene expression levels, so that immune response to tumorigenesis and inflammation may be reduced. *Camsap1* gene expression levels are directly regulated by miR-126. Increased miR-126 levels may negatively affect the *Camsap1* gene, which may cause abnormal cellular structure. *Sigmar1* gene expression levels may be downregulated via miR-214 and/or elevated estrogen levels, resulting in increased ER stress and abnormal Ca$^{2+}$ metabolism.

Red arrows indicate downregulation; green arrows indicate upregulation. Dotted lines show speculated regulation. Solid lines represent known regulation.

Supplemental Figure legends

Supplemental Figure 1. Clustered heat map of genes in the rat prostates on PND 145 (A), 200 (B), and 90 (C)
The differentially expressed miRNAs in the rat prostates dosed with EB and/or T and E were used for the analysis. For a statistical analysis of clustered heat map, a t-test or an ANOVA ($p < 0.05$) was used to perform a 2-group comparison (PND 90, C) or multiple comparisons among three dose groups (i.e., control, EB only and EB+T+E groups) (PNDs 145 and 200, A, B). In clustered heat maps, upregulated genes are represented in red, while downregulated genes are represented in green.

Supplemental Figure 2. Clustered heat map of genes in the rat plasma on PNDs 145 (A) and 200 (B)

The differentially expressed miRNAs in the rat plasma dosed with EB and/or T and E were used for the analysis. For a statistical analysis of clustered heat map, ANOVA ($p < 0.05$) was used to perform multiple comparisons among three dose groups (control, EB only and EB+T+E groups). In clustered heat map, upregulated genes are represented in red, while downregulated genes are represented in blue.

Supplemental Figure 3. Relative transcript levels of Mapk1, Ccde71l, Pmepa1, and Cdk1 genes in the dorsolateral prostate of rats dosed postnatally with EB, T, and E

Data are expressed as the mean fold change ± standard error of the mean ($N = 5$/group). *$p < 0.05$ compared to the control group for each endpoint (PND 30, 90, 100, 145, or 200) in the rat prostate.
Table 1. Group classification

<table>
<thead>
<tr>
<th>Group</th>
<th>EB dosing</th>
<th>Sample collection time (animal number used)</th>
<th>T and E dosing</th>
<th>Sample collection time (animal number used)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>PND 30*</td>
<td>PND 90</td>
<td>PND 100</td>
<td>PND 145</td>
</tr>
<tr>
<td>untreated</td>
<td>-</td>
<td>18</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td>EB-treated</td>
<td>+</td>
<td>18</td>
<td>+</td>
<td>8</td>
</tr>
</tbody>
</table>

N=8 per group per collection point except PND 30. N=18 on PND 30; * Due to the small size of the pups and tissues at PND 30, an increased number of animals was sacrificed. Animals were dosed with 2.5 mg/kg EB on PNDs 1, 3 and 5. Animals were administrated with T and E by subcutaneously implanting tubes on PNDs 90 and 146.

N/A: no applicable; EB: estradiol benzoate; T: testosterone; E: 17-β-estradiol
Table 2. Summary of the severity of chronic inflammation of rat dorsolateral prostates at PNDs 30, 90, 100, 145 and 200.

<table>
<thead>
<tr>
<th>Observation</th>
<th>group</th>
<th>PND 30</th>
<th>PND 90</th>
<th>group</th>
<th>PND 100</th>
<th>PND 145</th>
<th>PND 200</th>
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<td>-</td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>(0/18)</td>
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<td></td>
<td>(0/8)</td>
<td></td>
<td>(1/8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T+E only</td>
<td>-</td>
<td>±</td>
<td>-</td>
<td>+</td>
<td>±</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0/8)</td>
<td>(1/8)</td>
<td>(2/8)</td>
<td>(5/8)</td>
<td>(1/8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EB-treated</td>
<td>-</td>
<td>-</td>
<td>EB only</td>
<td>±</td>
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</tr>
<tr>
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<td>(1/8)</td>
<td>(2/8)</td>
<td>(0/6)</td>
<td>(0/8)</td>
<td>(0/7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EB+T+E</td>
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<tr>
<td>Chronic inflammation</td>
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<td>(0/8)</td>
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<tr>
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<td>-</td>
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<td>-</td>
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<tr>
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<td>EB only</td>
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<td>-</td>
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<tr>
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<td>(0/18)</td>
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<td>(0/8)</td>
<td>(0/6)</td>
<td>(0/8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EB+T+E</td>
<td>-</td>
<td>++++</td>
<td>+++~++++</td>
<td>+++~++++</td>
<td>(7/7)</td>
<td></td>
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</tbody>
</table>

The severity of the observation was categorized into five levels: -, normal; ±, faint; +, minimal; ++, mild; ++++, moderate; ++++, severe/marked

() indicates the number of animals observed acute or chronic inflammation per total number of animals per group.
<table>
<thead>
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<th>PND 200</th>
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</tr>
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<td>EB+T+E</td>
<td>p-Value</td>
<td>EB only</td>
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<td>rno-miR-146b-5p</td>
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<td>N.D.</td>
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<td>1.31</td>
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</tr>
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</table>

N.D.: no detection; N.S. No significances.

Fold changes in the EB only and EB+T+E groups on PND 145 or 200 were calculated setting the signals for the control group per collecting time point (PND 145 or 200) at one (Supplemental Tables 5-6).
Figure 1

A

Body weight

B

Prostate weight
Figure 2

A. Testosterone

B. Estradiol
Figure 3
Figure 6

Estrogen >>> Testosterone

Chronic inflammation

miR-329-3p

Tp53inp2

Esrrg

Malignancy

Cell proliferation

Tumor growth