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Transgenerational Effects of the Endocrine Disruptor Vinclozolin on the Prostate Transcriptome and Adult Onset Disease

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Abstract

PURPOSE—The ability of an endocrine disruptor exposure during gonadal sex determination to promote a transgenerational prostate disease phenotype was investigated in the current study.

METHODS—Exposure of an F0 gestating female rat to the endocrine disruptor vinclozolin during F1 embryo gonadal sex determination promoted a transgenerational adult onset prostate disease phenotype. The prostate disease phenotype and physiological parameters were determined for males from F1 to F4 generations and the prostate transcriptome was assessed in the F3 generation.

RESULTS—Although the prostate in prepubertal animals develops normally, abnormalities involving epithelial cell atrophy, glandular dysgenesis, prostatitis, and hyperplasia of the ventral prostate develop in older animals. The ventral prostate phenotype was transmitted for four generations (F1–F4). Analysis of the ventral prostate transcriptome demonstrated 954 genes had significantly altered expression between control and vinclozolin F3 generation animals. Analysis of isolated ventral prostate epithelial cells identified 259 genes with significantly altered expression between control and vinclozolin F3 generation animals. Characterization of regulated genes demonstrated several cellular pathways were influenced, including calcium and WNT. A number of genes identified have been shown to be associated with prostate disease and cancer, including beta-microseminoprotein (Msp) and tumor necrosis factor receptor superfamily 6 (Fadd).

CONCLUSIONS—The ability of an endocrine disruptor to promote transgenerational prostate abnormalities appears to involve an epigenetic transgenerational alteration in the prostate transcriptome and male germ-line. Potential epigenetic transgenerational alteration of prostate gene expression by environmental compounds may be important to consider in the etiology of adult onset prostate disease.

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Keywords

epigenetic; transgenerational; ventral prostate; endocrine disruptor; vinclozolin; hyperplasia; prostate disease; embryonic exposure

INTRODUCTION

Exposure of a developing embryo to environmental toxicants or nutritional defect (i.e., caloric restriction) results in not only developmental abnormalities, but also an increased propensity for adult onset diseases [1–3]. The mechanism for this fetal basis of adult onset disease is poorly understood [4]. It has been shown that such abnormalities and susceptibility to diseases can be heritable resulting in a transgenerational transmission [1,5,6]. Exposing a pregnant female rat around the time of embryonic sex determination to the environmental endocrine disruptors, vinclozolin, or methoxychlor, resulted in male offspring with reduced spermatogenetic capacity [1]. The testis phenotype was transgenerationally transmitted through the male germ-line for F1–F4 generations by an apparent epigenetic mechanism [1,5]. Sperm were found to have altered DNA methylation patterns transgenerationally. In addition to the testis defects, age related adult onset diseases such as immune abnormalities, kidney disease, prostate lesions, and cancer were also observed in the F1–F4 generations in rats 6–14 months of age [5]. This epigenetic transgenerational disease phenotype suggests one mechanism to consider for the fetal basis of adult onset disease is an epigenetic (e.g., DNA methylation) reprogramming of the male germ-line [1,5,7].

Endocrine disruptors are a class of environmental and therapeutic compounds that can bind to hormone receptors and/or alter hormone signaling (i.e., disrupt) to influence the normal endocrine system [8]. Exposure to these compounds during embryonic or postnatal development can cause abnormalities in male reproductive tissues including the testis, seminal vehicles, and prostate [5,9,10], as well as increased tumor development [5,11]. An interesting example of the actions of an endocrine disruptor on the prostate is the recent observation that the estrogenic compound bisphenol A (BPA) can induce an epigenetic change during pubertal prostate development and result in adult onset prostate disease [12].

The endocrine disruptor vinclozolin is commonly used as a fungicide in the fruit (e.g., wine) industry [13]. Vinclozolin and its two major metabolites (M1 and M2) have antiandrogenic properties [14]. Prenatal exposure to vinclozolin results in developmental defects in the male reproductive tract similar to studies using the antiandrogen flutamide [15–17]. Pubertal exposure to vinclozolin promotes similar morphological and gene expression changes in the ventral prostate as the androgen receptor antagonist flutamide [18]. In addition to the reproductive defects observed following exposure to vinclozolin, behavior and learning abnormalities have also been reported [19–21].

Embryonic testis development prior to and during sex determination (i.e., E8–E14 in the rat) appears to be the critical exposure period to induce a transgenerational phenotype in the adult rat [1,6]. As the vinclozolin generation animals age a transgenerational (F1–F4 generation) adult onset prostate disease phenotype was observed [5]. The frequency of this

prostate disease does not decline with each generation [5], suggesting it could not involve normal Mendalian genetic mechanisms, but instead an epigenetic process [7]. An alteration of the male germ-line epigenome (i.e., DNA methylation) appears to be involved in the transmission of this transgeneratioanl disease phenotype [1,22]. The current study, investigates the transgenerational effects of vinclozolin exposure on the development of age related disease in the ventral prostate. This study suggests an epigenetic transgenerational mechanism may be important in the fetal basis of adult onset prostate disease.

MATERIALS AND METHODS

Animal Model and Exposure Protocol

Timed pregnant rats (F0) were injected intraperitoneally with 100 mg/kg/day vinclozolin (99% pure; ChemService, West Chester, PA) or vehicle (DMSO) as control from embryonic (E) E8–E14 during gestation as previously described [23]. The number of treated F0 mothers was n =7 for control and n =7 for vinclozolin treatment of Sprague–Dawley (SD) rats. Generally, sibling sisters were used as control vehicle treated and vinclozolin treated F0 mothers to maintain similar genetic background for the control and vinclozolin generation animals. Aged animals (P180–P420) were collected from lines (n =3) from previously published studies on young males [6], whereas new lines (n =4) were generated for young (i.e., P15–P120) and other aged (P180–P420) animal collections. Males analyzed from P180 to P420 were pooled for this study due to other adult disease states that develop (i.e., infections, tumors) [5]. Adult males were collected from control and vinclozolin F1, F2, F3, F4 generations, as well as vinclozolin outcross (VOC) and reverse vinclozolin outcross (RVOC) animals. F1 (offspring of F0 mothers) postnatal (P) P60 males and females from different litters of control and vinclozolin treated groups were bred to generate the F2 generation. F2 generation rats were bred to generate the F3 generation. F3 generation rats were bred to generate the F4 generation. Breedings were carefully monitored to eliminate any sibling breedings and potential phenotypes as a result of inbreeding. For the VOC group SD F2 males from the vinclozolin generation were bred to SD wild-type control females. For the RVOC group SD F2 females from the vinclozolin generation were bred with SD wild-type control males. Male SD rats were collected and analyzed at P15, P30, P45, P70–P120 (P120) and P180–P420 (P300). The SD males collected at P15 and P30 were only from the F2 generation. A minimum of three different lines and three different litters were used to collect animals from each generation. The number of adult SD males collected for replicates for each experiment (i.e., n-value) were; F1 (21 control, 26 vinclozolin); F2 (36 control, 39 vinclozolin); F3 (13 control, 30 vinclozolin); F4 (13 control, 10 vinclozolin); and outcross VOC (19 VOC), RVOC (5 RVOC) from ages P70 to P420. The number of SD F2 P15 males collected was four controls and eight vinclozolin generation. The number of SD F2 P30 males collected was 12 controls and 14 vinclozolin generation. The number of SD F3 P45 males collected was 11 controls and 11 vinclozolin generation. The control and vinclozolin generation animals were all housed in the same room and rack in different cages with the same feeding and light conditions. Therefore, the environmental conditions were exactly the same between control and vinclozolin animals. The Washington State University Animal Care and Use Committee approved all animal procedures.

Histology and Cellular Apoptosis

Ventral, dorsal, and lateral prostates were fixed with Bouin's fixative (Sigma, St. Louis, MO) for 6–10 hr then washed with 70% ethanol. Slices of each tissue were embedded in paraffin, and serially sectioned using standard procedures performed by the Center for Reproductive Biology Histology Core Laboratory at Washington State University. All prostate pathologies observed, including prostatitis and hyperplasia, were diagnosed by the Washington Animal Disease Diagnostic Laboratory (WADDL) located at Washington State University. Animal identification and treatment groups were blinded to all pathologists for analyses. All tissues cross-sections were stained with hematoxylin and eosin for analyses.

Apoptotic cells were detected on replicate slides by TUNEL assay using a Fluorescein In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN). The fluorescent cells in each prostate cross-section were counted at 200× magnification. The average number of fluorescent cells per prostate section from duplicate slides from one animal (two sections per slide) was used as a replicate in statistical analysis. The negative control sections where the terminal transferase enzyme was excluded from the assay showed no labeling (data not shown).

Epithelial Cell Preparation

Ventral prostate epithelial cells were isolated from 45-day-old male rats from control (n = 11) and vinclozolin (n =11) F3 generation animals as previously described [24]. Briefly, control and vinclozolin animals were each split into two groups, totaling four individual samples, and ventral prostate tissues were removed and incubated in 675 U collagenase activity/ml of Type II collagenase (Sigma) and 0.04% Dnase (Sigma) at 37°C in HBSS for 4 hr shaking. After digestion, cell mixtures were centrifuged at 30*g* for 4 min to pellet epithelial cells. Epithelial cell pellets were resuspended in HBSS and centrifuged again at 30*g* for 4 min to wash epithelial cells to enrich the purity. This wash was repeated twice. Epithelial cell purities were estimated to be greater than 85% for all samples with the major contaminating cell type being stromal cells as previously described [24]. Freshly isolated epithelial cells were prepared for RNA isolation.

Microarray and Bioinformatics

RNA was collected from freshly isolated epithelial cells from 45-day-old males and whole ventral prostate tissue from 180-day-old males from control and vinclozolin F3 generation animals. RNA was hybridized to the Affymetrix (Affymetrix, Santa Clara, CA) rat 230 2.0 gene chip. The Genomics Core in the Center for Reproductive Biology at Washington State University performed the analysis as previously described [25,26]. Briefly, RNA as reverse transcribed into cDNA and cDNA was transcribed into biotin-labeled RNA. Biotin-labeled RNA was then hybridized to the Affymetrix rat 230 2.0 gene chips. Biotinylated RNA was then visualized by labeling with phycoerythrin-coupled avidin. The microarray chip was scanned on an Affymetrix Gene Chip Scanner 3000 (Affymetrix). The microarray image data were converted to numerical data with GeneChip Operating Software (GCOS version 1.1; Affymetrix) using a probe set scaling factor of 125. An absolute analysis was performed with GCOS to assess the relative abundance of the transcripts based on signal and detection calls (present, absent, or marginal). This information was imported into Gene-spring

software (Silicon Genetics, Redwood City, CA) and normalized using the recommended defaults. This includes setting signal values below 0.01 to a value of 0.01, total chip normalization to the 50th percentile, and normalization of each chip to the median. Unless otherwise indicated, in order for a transcript to be considered present it had to be both flagged as present in the GCOS present/absent call, and have an expression level greater than 75. Briefly, the 16 sets of oligonucleotides for a specific gene were used to make comparisons of a signal to statistically determine a present call using a one-sided Wilcoxon's signed rank test. In order for a transcript to be considered changed between treatment groups it had to exhibit at least a twofold change between the means of the treatments and have a Student's *t*-test *P*-value $P \leq 0.05$ between treatments. The raw signal cut off was 75 and was used to avoid false positives associated with a signal cut-off of 50 and false negatives with a signal of 100. Therefore, the data presented are for genes that were determined to be statistically present and found to be statistically different with a given treatment.

Two different experiments were performed involving two different sets of animals, RNA sample preparations and microarray chips. Therefore, two control and vinclozolin F3 generation samples were analyzed on two different chips. This allowed a 2 × 2 factorial comparison with all present/absent calls and changes in expression to be statistically significant for further analysis. The R^2 for the comparison between microarray chips was found to be $R^2 > 0.94$ indicating negligible variability between chips, experiments, and samples. This statistical analysis indicated the chip number used was appropriate. The number of chips required for specific experiments has been previously reviewed [27]. Previous studies have demonstrated that microarray data are validated with quantative PCR data [28,29]. Due to the presence of 16 different oligonucleotide sets for each specific gene being used on the microarray versus only a single primer set for a gene in a quantative PCR, the microarray is more effective at eliminating false positive or negative data and provides a more robust quantitation of changes in gene expression. However, validation of microarray data was examined with selected genes using a semi-quantative PCR procedure previously described [28]. The genes selected for semi-quantative PCR confirmation in the current study are *fos* and beta-microseminoprotein (*Msp*), with L19 as a constitutively expressed control gene. As stated in the Results section, similar data was obtained with the semi-quantative PCR analysis and the microarray analysis.

Radioimmunoassays

Serum samples were collected from trunk blood and stored at −80°C until assay for testosterone. The serum testosterone was determined by testosterone radioimmunoassay double antibody RIA kit (Diagnostic Systems, Webster, TX) by the Center for Reproductive Biology Assay Core Laboratory at Washington State University. The sensitivity of the assay was 10 pg/tube and sample values were within the linear range of the assay.

Statistical Analysis

The data from prostate weights, apoptotic cell counts, and testosterone assays were analyzed using the SAS software program. The values were expressed as the mean ± SEM. Statistical analysis was performed and the difference between the means of treatments and respective

controls were determined using a paired Student's *t*-test or Fisher's Exact test. Experiments were repeated with 5–23 rats per experimental group. A statistically significant difference was confirmed at $P < 0.05$.

RESULTS

The transient embryonic exposure to vinclozolin had no effect on the litter size, male/female sex ratios, or neonate mortality in any of the generations analyzed [6]. As previously reported [1,6], the young adult animals had no significant differences in body or testes weights between the control and vinclozolin generations, but vinclozolin generation males had reduced epididymal sperm motility and concentration [6]. In the young adults (i.e., P70–P120), the ventral prostate weights were not changed in any vinclozolin generation, Table IA. The prostate/body weight ratios in the F1, F2, and F3 generations were not statistically different, Table IA. There was no difference in serum testosterone levels between the control and vinclozolin generations, Table IA. Therefore, any effects observed are not due to altered serum androgen levels.

The vinclozolin generation males develop age related diseases when they become older than P180 [5]. The vinclozolin F2 and F3 generation males had a statistically significant increase in body weight compared to their controls. The physiological significance of this increase in body is unclear since the F4 generation had no difference in body weight. The ventral prostate weights in the F1, F2, and F4 vinclozolin generation males tended to increase compared to the controls, but only the F2 vinclozolin generation was statistically significant, Table IB. Interestingly, the F3 vinclozolin generation had a statistically significant decrease in ventral prostate weight compared to the control. The prostate/body weight ratios demonstrated the same trends as the prostate weights with the F1, F2, and F4 tending to increase from the control values. Interestingly, the F3 generation was the only one having a statistically significant decrease in prostate body weight ratio from the control, Table IB. It is unclear whether the differences observed in the ventral prostate are physiologically significant, since there was no correlation with weight nor to any observed abnormalities. Furthermore, the VOC ventral prostate weight was also not changed compared to the RVOC value. The serum testosterone was similar in the control and vinclozolin generations except for the VOC generation, which was significantly lower than the RVOC level. As with the young animals, the observed effects do not appear to be due to altered serum androgen levels in the aged males.

To determine if there were any developmental defects in the ventral prostate, ventral prostates from P15 and P30 F2 generation males were collected for morphological analyses. Analyses of the morphology of the ventral prostate at P15 showed no structural abnormalities and were very similar to controls, Figure 1A and B. However, analyses of the ventral prostates from P30 animals showed some degeneration of the prostatic ducts, Figure 1C–F. The ventral prostates in the vinclozolin generation animals appeared to have atrophy of the secretory epithelial cells in some of the prostatic ducts. This abnormality in the morphology of the tissue occurred at a low frequency in approximately 15% of the P30 animals, Figure 2. TUNEL analyses showed no increase in apoptosis in the ventral prostate at P30, suggesting no wide spread death of cells in the tissue (data not shown). Furthermore,

ventral prostate morphology of VOC animals showed similar abnormalities (data not shown).

The ventral prostate abnormality (i.e., epithelial cell atrophy) became more frequent and was regionally specific in the young adult (i.e., P70–P120) males, Figure 3. Analysis of the ventral prostate showed that the degeneration (i.e., lack of secretory epithelial cells) in the prostatic ducts was observed in approximately 30% of the vinclozolin generation F2 animals compared to less than 5% of the F2 controls, Figure 2. The degenerated ducts appeared in the distal region of the ventral prostate, Figure 3A and B. There was no degeneration observed in the intermediate and proximal region of the ventral prostate, Figure 3C–F. This degeneration of the ventral prostatic ducts, Figure 3B, was seen at similar frequencies (i.e., approximately 30%) in all vinclozolin generation animals examined, F1–F4.

In the aged adult (i.e., P180–P420) males, the ventral prostate abnormality was much more frequent and severe, Figure 4. The degeneration of the prostatic ducts (e.g., abnormalities) was observed in all regions of the ventral prostate, unlike the young adult, which showed only the degeneration in the distal region. This abnormality was observed in approximately 90% of the F2 vinclozolin generation animals compared to less than 40% in the control generation, Figure 2. The abnormality observed in at least 30% of the ventral prostate ducts, occurred in approximately 45–55% of the F1–F4 vinclozolin generation animals, with 15% having a severe phenotype. The severe phenotype consisted of more than 80% of the ducts being degenerated/atrophic in a given cross-section, Figure 4C. Prostatic ducts from the vinclozolin generations contained regions of complete loss of epithelial cells, with vacuoles/spaces in the epithelial cell layer, Figure 4E. Approximately 15% of the aged animals developed prostatitis [5]. The RVOC males were very similar to the control animals and contained a very low level of atrophic ducts and no prostatitis or hyperplasia was detected. The VOC samples exhibited a high frequency of atrophic ducts [5] very similar to the frequency of the F3 treated animals. However, the VOC animals did not have any prostatitis or hyperplasia observed. Several of the vinclozolin generation aged animals developed hyperplasia, 4/51 vinclozolin (2 F2, 1 F3, and 1 F4 generation) and 1/45 F2 control generation animals had epithelial cell hyperplasia, Figure 4F.

The ventral prostate of the vinclozolin generation animals contained degenerating secretory epithelial cells. However, the morphology of the lateral and dorsal lobes of the prostate from aged vinclozolin and control generations, showed no gross abnormalities, Figure 5. Observations suggest the transgenerational phenotype induced by the vinclozolin exposure only affected the ventral prostate.

The prostate transcriptome was investigated to examine the potential causal factors in the epigenetic transgenerational ventral prostate disease observed. Since the F1 embryo and F2 generation germ-line have direct exposure to the vinclozolin, the F3 generation is the first to be unequivocally transgenerational [22]. RNA was collected from F3 generation whole ventral prostate tissues and isolated ventral prostate epithelial cells for microarray analyses. The analyses of the arrays of the ventral prostate tissue yielded 954 genes with a raw signal expression over 75 and at least a 1.5-fold change from the control with a confidence of $P < 0.05$, supplemental Table S1. The dendrogram of the genes with altered (>1.5-fold change)

expression demonstrated that approximately 50% of the genes are increased in expression, Figure 6A. Microarray analyses from the isolated epithelial cells resulted in 259 genes with a raw expression signal over 75 and at least a 1.5-fold change from the control with a confidence of $P<0.05$, supplemental Table S2. The dendrogram of the genes with altered expression also shows that approximately 50% of the genes are increased from the control levels, Figure 6B. The F3 generation prostate 954 and epithelial 259 regulated genes were compared to identify genes present on both gene lists. The comparison demonstrated 55 genes are similar between each list, and these are identified in bold on the gene list in supplemental Table S2. Therefore, observations demonstrate a transgenerational impact on the transcriptomes of both the whole prostate and isolated epithelial cells. Genes *fos* and *Msp* from the ventral prostate gene list were further analyzed by semi-quantitative RT-PCR. Both *fos* and *Msp* expression went down in the array analysis and the PCR supported these findings using different RNA preparations from different F3 control and vinclozolin animals (data not shown).

The regulated genes were functionally categorized to identify what cellular pathways and processes were affected. Related gene category classification of the 954 regulated gene list from the whole ventral prostate tissue array demonstrated many of the genes are unclassified express sequence tags (EST), Supplemental Table S1. A graphical representation of the break down of the 954 regulated gene list is shown in Figure 7A with the Supplemental Table S1 showing the specific genes in each category. The transcription, metabolism, cytoskeleton, signaling, and immune response were the most highly represented gene categories, all having at least 50 genes, Figure 7 and Supplemental Table S1. Interestingly, the category of immune response had 51 genes of which 49 were up-regulated. Additionally, the growth factor family had nine genes, seven of which were up-regulated. All other categories had a mixture of up- and down-regulated genes with no difinitive trends present. The gene category classification for the 259 regulated gene list from the prostate epithelial cells demonstrated fewer EST then the whole prostate and both up- and down-regulated genes for all categories, Figure 7B and Supplemental Table S2. The transcription, metabolism and signaling categories are predominant with the epithelial cell list, Figure 7B and Supplemental Table S2.

The 55 genes in common between the whole ventral prostate and epithelial cell lists are presented in Supplemental Table S2 as bolded genes. The connectivity for this list of common regulated genes demonstrates that five cellular processes (i.e., proliferation, maturation/differentiation, cell survival, secretion, and assembly) are predominantly influenced, Figure 8. Analysis of cellular signaling pathways with the GeneSpring Kegg pathway for the 954 regulated genes in the whole prostate demonstrates two pathways with greater than 10 regulated genes present. These signaling pathways are the calcium signaling pathway, Supplemental Figure S1, and WNT signaling pathway, Supplemental Figure S2. The down-(blue) and up-regulated (red/orange) are shown, Figures S1 and S2, and suggests these pathways may be involved in the transgenerational adult onset prostate disease phenotype.

The genes affected within the prostate transcriptome and associated with the epigenetic transgenerational prostate disease phenotype were compared with a list of genes previously

shown to be associated or linked to prostate disease. A list of these prostate disease associated genes is shown in Table II. For example, the *Msp* gene was found to have a decreased expression in the F3 vinclozolin generation whole prostate by both the microarray and semi-quantitative PCR procedures. Therefore, a number of the regulated genes in the transgenerational prostate transcriptome identified have been previously correlated to prostate disease.

DISCUSSION

After a transient exposure to vinclozolin during gonadal sex determination a number of adult onset disease states develop in aged rats that are transmitted to subsequent generations in a transgenerational manner [5]. These transgenerational adult onset diseases included prostate disease, kidney disease, increased tumor formation, and immune abnormalities [5]. The current study demonstrates that the adult onset disease in the prostate could be in part the result of improper development or differentiation of the epithelial cells during maturation of the prostatic ducts. Although early in development (i.e., P15) the prostate morphologically appeared normal, prostate epithelial cell atrophy was seen as early as P30 in a small percentage of the F2 generation animals examined. Inhibition of proper epithelial cell development and differentiation has been associated with the initiation of adult onset prostate disease [30,31]. The abnormal epithelial cell function could not be attributed to altered androgen levels since no affects on serum testosterone levels were observed. The inconsistent affects on body and prostate weights suggests the affects observed are not physiologically relevant, but likely due in part to animal variations. The affects on the epithelial cells observed are postulated to be due to abnormal programming of cellular differentiation [30,31].

Endocrine disrupting chemicals administered to neonatal pups have been shown to influence prostate development [12,15,17,32]. Prostate development requires androgens for proper differentiation. Anti-androgenic compounds such as vinclozolin and flutamide have been shown to inhibit the development of the prostate [15,17]. Furthermore, compounds influencing estrogen receptor activity that have been shown to alter the development of the prostate include; estrogen, BPA [12,33], and methoxychlor [32]. These estrogenic compounds may act indirectly since neonatal exposure to estrogenic compounds down regulates the androgen receptor in the prostate [34]. Interestingly, a recent study with BPA [12] demonstrated abnormal prostate development may be induced in part through an epigenetic mechanism. These environmental compounds have been shown to influence prostate disease in the males exposed to the compounds. However, the results shown here demonstrate increased abnormalities (i.e., atrophy, hyperplasia) in animals not directly exposed.

Previously, no study using embryonic or postnatal exposure has shown a transgenerational phenotype in the developing prostate. In the current study, the embryonic exposure during gonadal sex determination, E8–E14 in rat, to the antiandrogenic compound vinclozolin induced a transgenerational prostate phenotype through four generations. Although four generations with the same disease frequency clearly establishes a transgenerational phenomenon [22], a fifth generation was considered, but the experimental protocol avoided

any sibling breedings to avoid any inbreeding artifacts and the colony population was insufficient to generate a fifth generation. Prostate abnormalities were observed in the F3 generation VOC male offspring, but not in the RVOC males. This suggests that the transmission of the transgenerational phenotype was transmitted through epigenetic alterations of the male germ cell, similar to the previous reports on a testis transgenerational phenotype [1,6].

This transgenerational phenotype does not involve normal Mendalian characteristics and appears to in part involve an epigenetic reprogramming of the male germ-line [1,5]. The altered epigenome provides a potential mechanism for the transgenerational changes in the transcriptome observed in the current study. Prostate disease has been associated with changes in the epigenome [35,36] and increases the prostate susceptibility to prostate carcinogenesis [12]. Alteration in the epigenetic programming of the male germ-line and induction of new imprinted-like genes is hypothesized to be involved in the transgenerational phenotype [1,5,7]. The proposed mechanism is that the endocrine disruptor vinclozolin exposure during embryonic gonadal sex determination alters the epigenetic (i.e., DNA methylation) programming of the male germ-line to induce altered DNA methylation causing changes in the epigenome that allows the phenotype to become transgenerational [1,7,22]. The altered epigenome could regulate the transcriptome in a transgenerational manner, as shown in the current study for the prostate transcriptome. Although these previous studies and the non-Mendelian nature of the transgenerational prostate disease observed support this epigenetic mechanism, the specific epigenetic alterations that occur during prostate development remain to be elucidated.

Prostate disease is associated with several types of abnormalities. Common types of prostate disease are benign hyperplasia and prostate cancer [37]. A much less and unappreciated disease state in the prostate is atrophy of the prostate ducts [38]. Atrophy of the prostate ducts is commonly observed in aging men [39] and is associated with acute and chronic inflammation [38]. Inflammation induced atrophy of the prostatic ducts has been associated with the development of cancer and benign prostate hyperplasia (BPH) [38,40]. The prostate disease phenotype observed in the current study involves prostate epithelium atrophy, inflammation and prostatitis, and hyperplasia. Therefore, critical parameters of prostate disease were observed in the transgenerational adult onset prostate disease observed.

Several genes that had differential regulation between the control and vinclozolin F3 generation males have been shown to be associated with BPH and cancer. *NK3* is a transcription factor whose expression has been shown to decrease during prostate cancer [41,42]. Additionally, mice lacking a functional *NK3* develop prostate dysplasia and intra-epithelial neoplasia [42]. It has been discussed that the loss of *NK3* expression is due to chronic inflammation [38]. However, the regulation and function of *NK3* in prostate cancer progression remains unknown. Msp is a small protein secreted by the prostate epithelial cells and has been targeted as a marker for prostate cancer [43,44]. It is unknown what role *Msp* plays in prostate cancer development and progression. Tumor necrosis factor receptor superfamily 6 (FADD) is a cytoplasmic protein involved in regulating the apoptosis pathway. A loss of FADD protein activity has been associated with tumor progression [45]. The observation that a number of the genes altered in the transgenerational prostate

transcriptome are associated with prostate cancer helps confirm the relevance of the transgenerational induced prostate disease observed.

The microarray analysis from whole ventral prostate tissue and ventral prostate epithelial cells revealed a number of related gene categories being altered. Interestingly, the category of immune response genes had 49 of the 51 candidates up-regulated in the vinclozolin F3 generation ventral prostate. It has previously been proposed that an increase in immune response can lead to prostate hyperplasia and cancer formation [46,47]. Some of the aged animals examined had increased prostatitis. Therefore, the transgenerational effects on the immune related genes may correlate with the prostate disease observed. Prostate duct atrophy has also been proposed to be a causal factor in the development of age related prostate disease [38,48]. Several of the functional gene categories and altered signaling pathways (e.g., apoptosis, calcium, and WNT) associated with the altered F3 transgenerational transcriptome could be involved in the epithelial cell atrophy observed. Although the changes in the F3 generation whole ventral prostate transcriptome could in part be due to alterations in cell populations (i.e., loss of epithelial cells), the changes observed in the F3 generation ventral prostate epithelial cells confirms a transgenerational reprogramming of the transcriptome. This transgenerational model will be valuable in elucidating the molecular mechanisms potentially involved in adult onset prostate disease. The correlation of the altered prostate transgenerationl transcriptome to individual animals and specific disease phenotype was not performed in the current study. Such analysis of individuals will be useful in the identification of early stage diagnostic markers for prostate disease and provide potentially new therapeutic strategies.

The current study demonstrates that embryonic exposure to vinclozolin during gonadal sex determination promotes a defect in development that leads to adult onset disease in ventral prostate. This phenotype was heritable through four generations and is transgenerationally transmitted through the male germ-line. The atrophy of the prostate ducts is associated with increased prostatitis and hyperplasia in aged rats of the vinclozolin generation males. Microarray analyses of isolated F3 generation ventral prostate epithelial cells and whole ventral prostate tissue identified a transgenerational alteration in the transcriptome that was promoted by an embryonic exposure of an F0 mother to vinclozolin. These transgenerational changes in gene expression now provide candidate genes and cellular pathways for the future study of atrophic prostate ducts leading to adult onset prostate disease. Combined observations suggest the ability of an environmental compound (i.e., vinclozolin) to promote an epigenetic transgenerational disease phenotype and reprogramming of the transcriptome may be an important factor to consider in prostate disease etiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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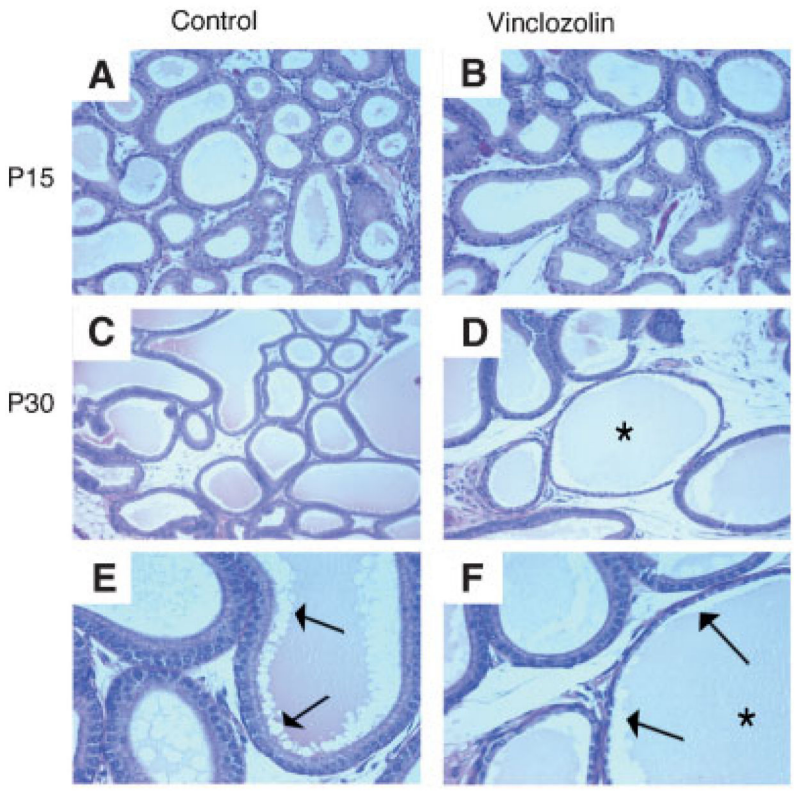

Fig. 1.

Ventral prostate morphology from P15 control (**A**) and vinclozolin (**B**) and P30 control (**C**,**E**) and vinclozolin (**D**,**F**) F2 generation rats. (A–D) Magnification 200× and (E,F) magnification 400×. Arrows indicate ductal epithelium. Asterisks (*) indicate prostate ducts with atrophic/degenerated epithelial cells. Micrographs are representative of 4 controls and 8 vinclozolin P15 samples and 12 control and 14 vinclozolin P30 samples.

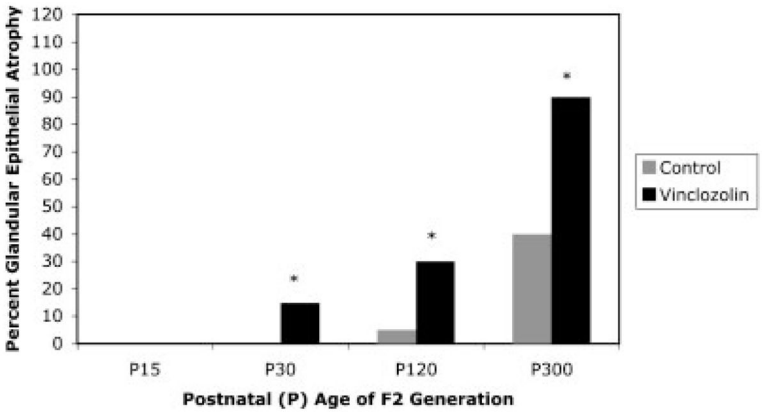

Fig. 2.

The percent of animals affected containing atrophic ducts in the ventral prostate sections from F2 generation control (gray bars) and vinclozolin (black bars) from postnatal day P15 (control n =4 and vinclozolin n =8), P30 (control n =12 and vinclozolin n =14), P120(control n =10 and vinclozolin n =10), and P300 (control n =21 and vinclozolin n =23) rats. Asterisks (*) indicate statistically significant differences from control by Fisher's Exact test.

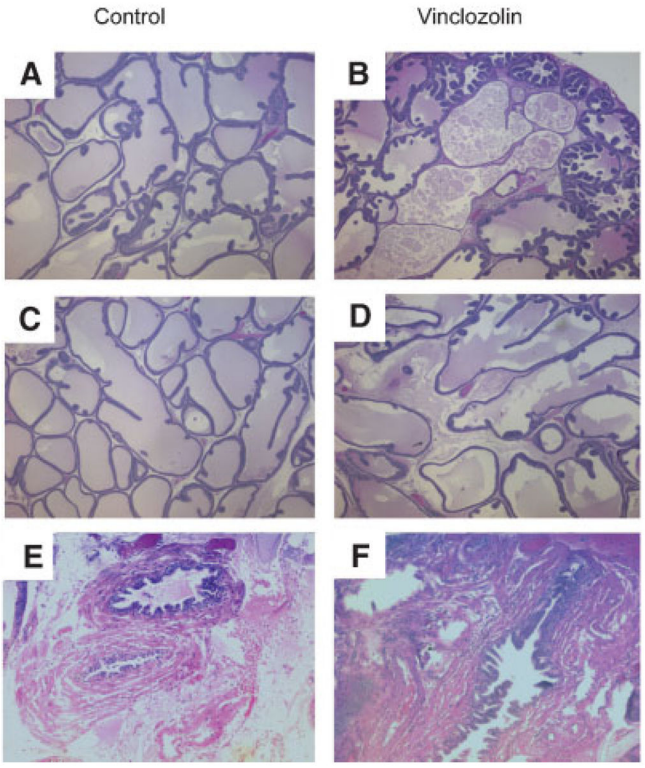

Fig. 3.

Ventral prostate morphology from P120 control (**A**,**C**,**E**) and vinclozolin (**B**,**D**,**F**) F2 generation rats. Ventral prostate cross-sections of distal (A,B), intermediate (C,D), and proximal (E,F) regions. Asterisks (*) indicate atrophic ducts. Magnification 100× (A–D)and200× (E,F). Micrographs are representative of 10 controls and 10 vinclozolin samples.

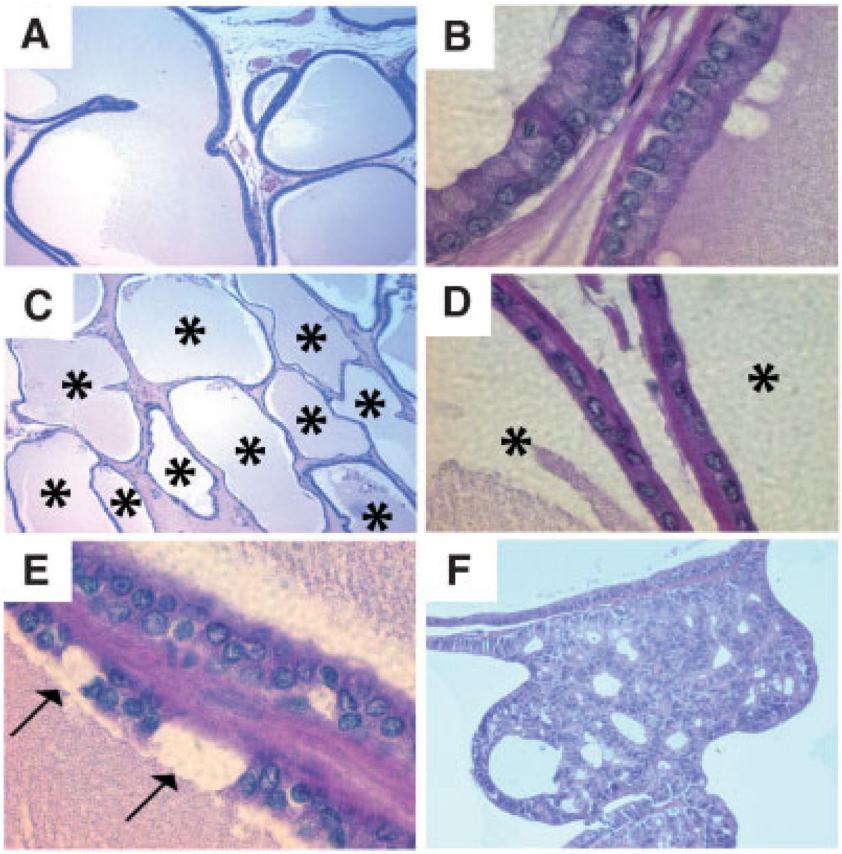

Fig. 4.

Ventral prostate morphology from control (**A**,**B**) and vinclozolin (**C**–**F**) P300 F3 generation rats. F: Regions of epithelial cell hyperplasia. Asterisks (*) indicate atrophic ducts. Arrows denotes region of vacuoles in the basal epithelium (E). Magnification 100× (A,C,F) and 1000× (B,D,E). Micrographs are representative of 5 controls and 12 vinclozolin samples; except (F), which is 1 control and 4 vinclozolin samples.

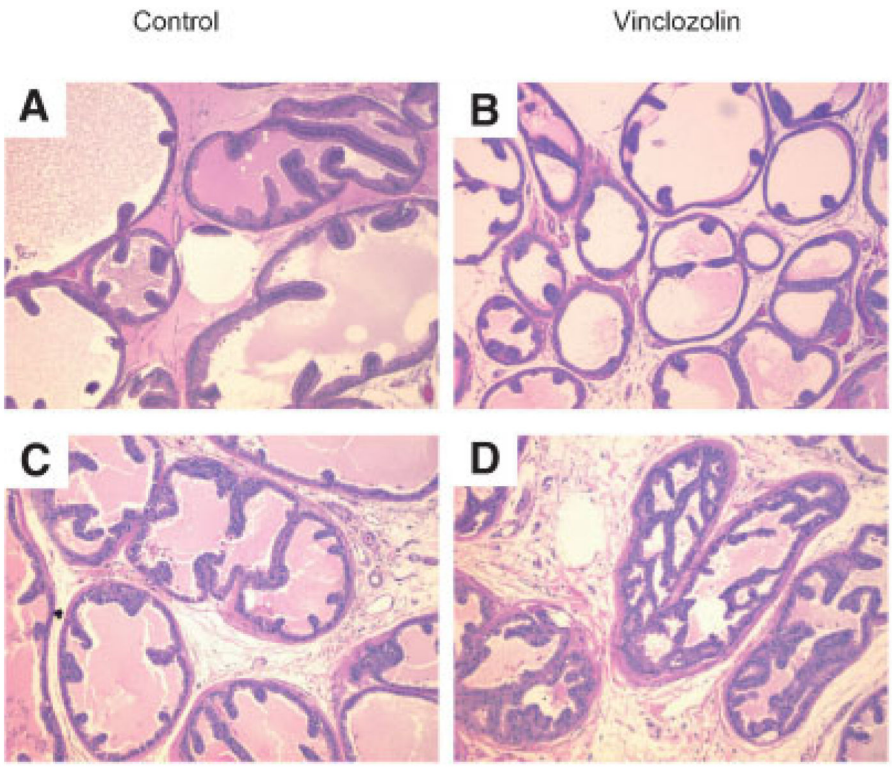

Fig. 5.

Lateral (**A**,**B**) and dorsal (**C**,**D**) prostate morphology from control (A,C) and vinclozolin (B,D) P300 F3 generation rats. Magnification 200×. Micrographs are representative of 10 controls and 12 vinclozolin samples.

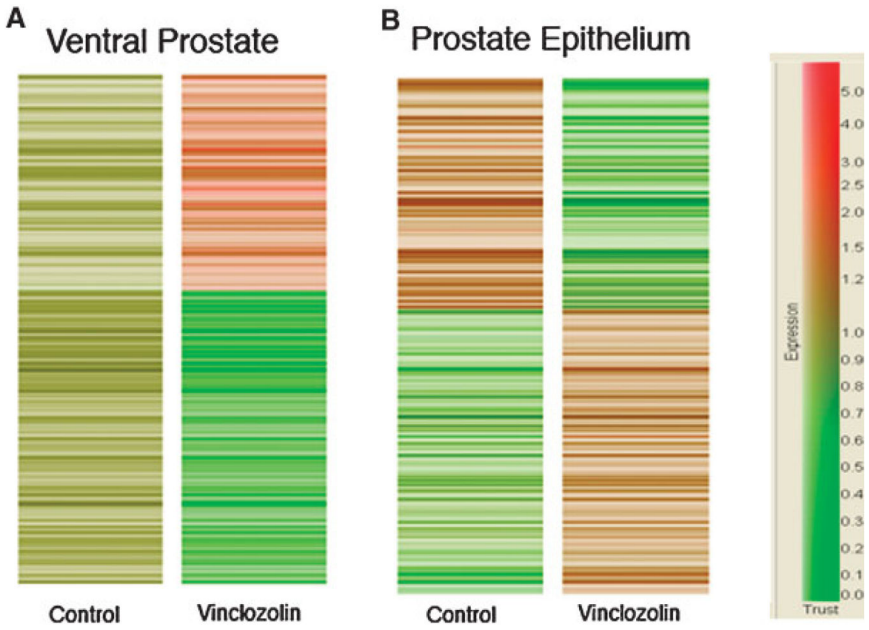

Fig. 6.

Dendrogram analyses of the microarray regulated gene data from whole ventral prostate tissue(**A**)and isolated epithelial cells(**B**)from F3 generation rats. Dendrogram were produced in Gene spring using an unsupervise cluster analysis. Dendrogram comprised from 954 regulated gene list from ventral prostate tissue (A) and 259 regulated gene list from isolated epithelial cells (B). Green color indicates a decrease in expression and red color indicates an increase in expression, scale provided.

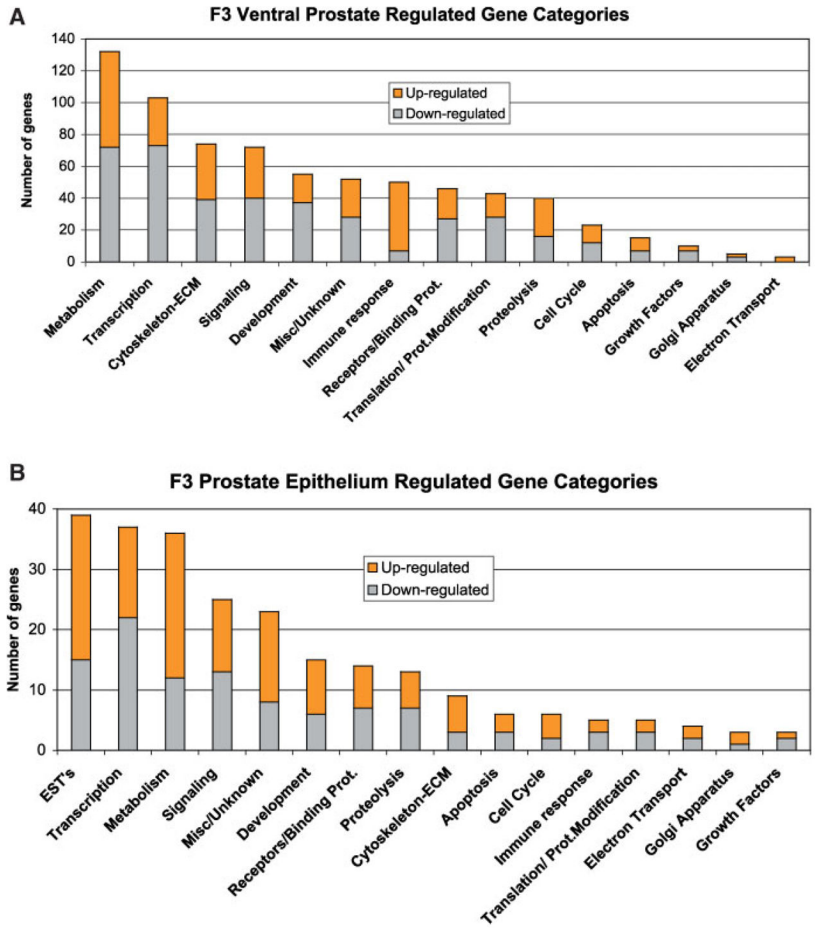

Fig. 7.

Functional categorization of the regulated gene lists. Each gene was separated into a functional family and graphed to indicate numbers of genes up- and down-regulated.(**A**) 954 gene list from ventral prostate tissue and (**B**) 259 epithelial list.

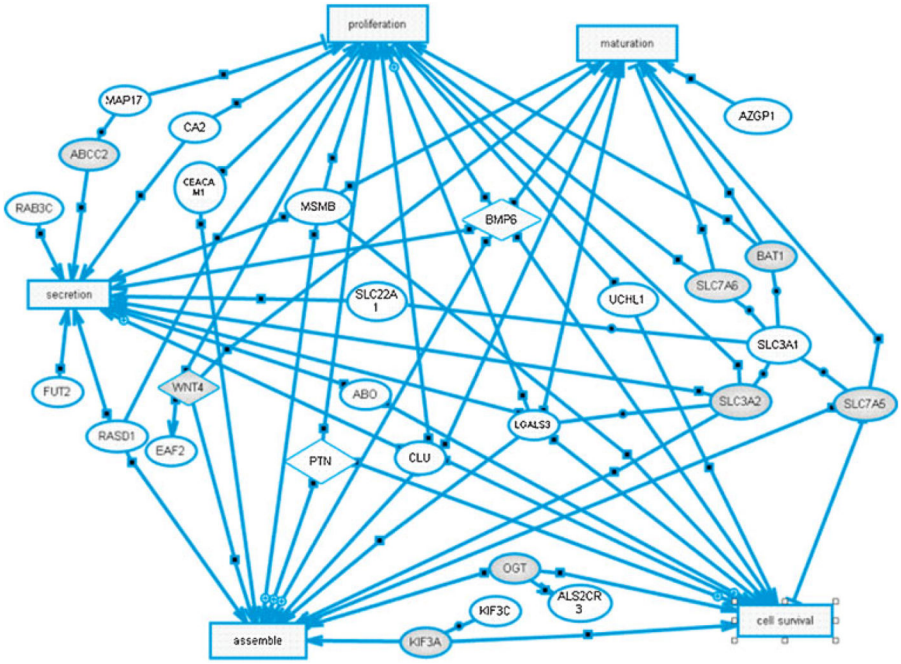

Fig. 8.

Functional connectivity analyses of the common 55 regulated gene lists between total prostate and epithelial cells. Cellular processes associated with each gene in the 55 list were determined based on the number of arrows connected to each box (connectivity). Shown are only the boxes that had more then 5 connections with genes from the 55 common regulated gene list. The shaded genes listed are not in the 55 gene list but provide bridging connections, while the white genes listed are in the 55 gene list.

TABLE I

Body, Ventral Prostate Weights, and Serum Test osterone of Young Adult Rats (P70–P120) and Aged Adult Rats (P180–P420)

Generations	n	Body (g)	Ventral prostate (g)	Ventral prostate/body ratio	Testosterone (ng/mL)
A: Young adult rats (P70–P120)					
F1 Control	9	513 ± 12.6	0.97 ± 0.07	1.70 ± 0.11	3.93 ± 0.39
F1 Vinclozolin	9	517 ± 12.0	0.81 ± 0.09	1.57 ± 0.17	4.57 ± 0.40
F2 Control	10	415 ± 25.1	0.59 ± 0.06	1.41 ±.10	5.35 ± 1.14
F2 Vinclozolin	10	414 ± 27.4	0.71 ± 0.08	1.67 ± 0.11	4.78 ± 1.14
F3 Control	8	551 ± 14.9	1.08 ± 0.08	1.95 ± 0.11	3.65 ± 0.42
F3 Vinclozolin	14	537 ± 15.8	1.21 ± 0.07	2.56 ± 0.11	3.62 ± 0.30
B: Aged adult rats (P180–P420)					
F1 Control	6	676 ± 26.0	1.19 ± 0.05	1.77 ± 0.05	1.86 ± 0.45
F1 Vinclozolin	8	635 ± 17.3	1.39 ± 0.09	2.20 ± 0.14	1.76 ± 0.24
F2 Control	21	592 ± 18.2	1.08 ± 0.05	1.86 ± 0.09	1.02 ± 0.07
F2 Vinclozolin	23	711 ± 21.1 *	1.43 ± 0.08 *	2.03 ± 0.11	1.15 ± 0.12
F3 Control	5	518 ± 21.1	1.11 ± 0.08	2.15 ± 0.17	1.21 ± 0.20
F3 Vinclozolin	12	613 ± 28.0 *	0.89 ± 0.06 *	1.47 ± 0.09 *	0.88 ± 0.12
F4 Control	13	607 ± 27.2	1.58 ± 0.08	2.60 ± 0.16	1.77 ± 0.30
F4 Vinclozolin	8	622 ± 24.6	1.75 ± 0.10	2.84 ± 0.18	1.64 ± 0.20
VOC	19	629 ± 25.7	1.32 ± 0.10	2.07 ± 0.04	0.88 ± 0.09 **
RVOC	5	699 ± 6.96	1.55 ± 0.02	2.22 ± 0.04	1.77 ± 0.26

* P >0.05 from corresponding control value.

** P >0.05 from corresponding RVOC value.

TABLE II

Prostate Disease Associated Genes

Gene name	From control fold change	Refs.
Ventral prostate list		
Cyclin D1	1.95	49
Early growth response 1	0.3	50
FtsJ homolog 2(predicted)(MAD1L1)	1.5	51
Met proto-oncogene	0.42	52
Beta microseminoprotein	0.39	43
NK-3 transcription factor (predicted)	0.29	41
Tumor necrosis factor receptor superfamily 6	0.61	45
Epithelial cells list		
Chemokine receptor (LCR1, Cxcr4)	0.63	53
Glutathione-*S*-transferase pi1	1.63	54
Beta microseminoprotein	1.63	43
NK-3 transcription factor (predicted)	1.55	41
Tumor necrosis factor receptor superfamily 6	0.63	45