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Endocrine Disruption and Human Prostate Cancer

Endocrine disrupting chemicals (EDCs) are present in our everyday life and many of them act to oppose androgens including the fungicide, Vinclozolin. Most of the studies on EDCs are conducted in rodents and have limited utility when extrapolating the findings to humans. In order to test the concept that Vinclozolin alters human prostate development and induces disease, we used our model system to study human prostate development and maturation over 8-12 weeks, comparable to the process that takes decades in men. Briefly we use rodent stroma to create a niche in which human stem cells are directed to become human prostatic epithelia. Task 1 sought to test if the rodent stromal niche (obtained after exposure to Vinclozolin) was altered so that differentiation and development to normal human prostate tissue was aberrant leading to a disease phenotype. Surprisingly, our results showed development of the prostatic phenotype was completely blocked and was not predicted. These novel findings are fundamental to understanding how normal prostatic differentiation proceeds and our publications show the essential role of stroma is perturbed by Vinclozolin, define the prostatic phenotype induced by Vinclozolin and demonstrate the involvement of NFkB signalling pathways.

Endocrine disrupting chemicals, prostate cancer, prostatitis
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INTRODUCTION

Endocrine disrupting chemicals (EDCs) are present in our everyday life and many of them act to oppose androgens including the fungicide, Vinclozolin. Most of the studies on EDCs are conducted in rodents and have limited utility when extrapolating the findings to humans. In order to test the concept that Vinclozolin alters human prostate development and induces disease, we used our model system to study human prostate development and maturation over 8-12 weeks, comparable to the process that takes decades in men. Briefly we use rodent stroma to create a niche in which human stem cells are directed to become human prostatic epithelia. Task 1 sought to test if the rodent stromal niche (obtained after exposure to Vinclozolin) was altered so that differentiation and development to normal human prostate tissue was aberrant leading to a disease phenotype. Surprisingly, our results showed development of the prostatic phenotype was completely blocked and was not predicted. These novel findings are fundamental to understanding how normal prostatic differentiation proceeds and our publications show the essential role of stroma is perturbed by Vinclozolin, define the prostatic phenotype induced by Vinclozolin and demonstrate the involvement of NFkB signalling pathways.

BODY

Research Outcomes associated with Tasks as outlined in the approved Statement of Work.

Aim:

The aim of the experiments described in Task 1, was to expose pregnant female rats to vinclozolin and test if the inductive and instructive properties of the prostate stroma is altered and disrupts differentiation of human prostate tissue derived from hESC.
Methods

Briefly, this involved the assignment of pregnant dams to one of four treatment groups: 100, 200 or 400 mg Vinclozolin/Kg/Day (in 2.5µl of corn oil/g body wt) or corn oil vehicle control. Dams transiently exposed by oral gavage from gestational days (GD) 14 to 19, and left to litter down. Vinclozolin exposure to male pups was confirmed by measuring anogenital distance (1).

Tissue recombinants generated from rodent derived prostate mesenchyme from male pups in the litters and combined with human embryonic stem cells. Tissues harvested for analysis after 12 weeks of growth and evidence of Vinclozolin treatment assessed using the following parameters:

Tissue size: Graft volume was determined by stereological method.

Histopathology: Tissue sections selected from the entire block stained for H&E and examined by microscopy. Evidence for specific lesions was examined to identify:

**PIN lesions:** Nucleolus size and nucleoli prominence determined as evidence of PIN lesions. Focal PIN lesions identified by up-regulation of AR, ERα and p63 and the down regulation of E-cadherin as previously described (2).

**Inflammation:** Areas of inflammation identified based on accumulation of neutrophils and lymphocytes and lymphoid aggregates in the stroma. (2).

**Epithelial hyperplasia:** Specific morphological criteria assessed (3) and accurately estimated using CAST software. Counting frames and systematic uniform random sampling methods were adapted from those used to estimate epithelial morphology in gut (4). Epithelial ‘branches’ were counted for a minimum of 6 sections taken through each graft. Counts were expressed per unit of section area, with the mean for each graft used for comparison.

**Altered secretory epithelial cell activity and reduced PSA:** Image analysis was performed to estimate conversion to PSA expressing glandular tissue as previously described (5). Entire grafts were sectioned and beginning from a randomly selected tissue section, a systematic
uniform sampling procedure applied to select 10% of the tissue throughout the explant. Using CAST software, area that was glandular/PSA positive was measured and expressed as a percentage of the total section area. The mean for each graft was derived from the mean values of at least 10 sections. The mean value of glandular tissue (PSA positive) for the graft type was obtained from not less than 4 grafts per group.

Results:

**Perturbations in normal human prostate differentiation in Vinclozolin + hESC recombinants**

Analysis of tissue recombinants at 12 weeks revealed the perturbation of normal human prostate tissue development and maturation using Vinclozolin exposed mesenchyme. The observed effect was not as predicted and showed a fundamental failure to allow normal prostate development and maturation (Figure 1). The absence of PSA expressing glandular tissue in Vinclozolin + hESC recombinants confirmed the absence of formation of human prostate tissue (Figure 1).

**Aberrant androgen receptor expression in Vinclozolin exposed mesenchyme**

The inductive and instructive potential of the mesenchyme and importance of mesenchymal androgen signaling in
normal prostate branching and development has been well established. Analysis of Vinclozolin exposed mesenchyme revealed a significant down regulation in androgen receptor (AR) expression (Figure 2).

Thus, the perturbation of normal prostatic differentiation in Vinclozolin + hESC recombinants is due to the reduced AR expression and consequential aberrant androgen signaling.

**Outcomes and conclusions:**
We predicted one of 2 outcomes. The predicted outcome was that Vinclozolin would perturb human prostate development or maturation and reduce graft size, reduce epithelial differentiation and secretory activity and may even have caused PIN lesions. This outcome was partly achieved, in that there was a failure of human prostatic graft development, attributal to the reduced AR expression. We know AR expression is crucial for prostate development and this would explain why the tissue failed to develop. However this outcome did not allow us to determine if there were any PIN lesions or evidence of inflammatory pathologies.

**Task 2:**

**Aims and approach**

The original aim was to determine if the effects of Vinclozolin were transgenerational and evident in the F2 generation, but the outcome of Task 1 required us to evaluate the nature of the failure of human prostate development from the tissue recombinants. Specifically this led us to determine the aberrant effects of *in utero* Vinclozolin exposure on the rodent prostate to identify the similarity to human prostatic inflammation or prostatitis.
Results.

Our data demonstrate rats transiently exposed to Vinclozolin develop prostatitis immediately post-puberty. At 2 months (8weeks) of age; the incidence of prostatitis in these relatively young animals was 100%. The early onset of prostatitis, is comparable to the onset of non-bacterial early-onset prostatitis in younger men, and implicates the EDC, Vinclozolin, as a causative factor in the etiology of prostatitis.

Specifically we report key observations:

*Post-pubertal prostatitis occurs in all of the Vinclozolin treated rats.*

The incidence of prostatic inflammation or prostatitis was 100% in post-pubertal rats treated in utero with Vinclozolin.

The prostates showed significant pathology compared to controls. Prominent, but focal, regions of inflammation were observed with an increase in the proportion of inflammatory cells, particularly leukocytes and including macrophages (ED1) surrounding the ducts and vasculature – see figure 3.

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence</th>
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<tbody>
<tr>
<td>Control</td>
<td>0/17</td>
</tr>
<tr>
<td>Vinclozolin</td>
<td>24/24</td>
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**Figure 3** The left hand panels show control prostate tissues with normal histology (H&E) and no macrophage infiltration as assessed by immunostaining for ED1. The top right hand panel is an H&E section of Vinclozolin treated prostate tissue showing evidence of inflammation. The bottom right...
hand panel shows brown staining representing localisation of a ED1 antibody and evidence of infiltration of macrophages.

**NFkB signalling pathway is implicated in the onset of prostatitis in post-pubertal rat tissue**

A number of genes activated and significantly up-regulated - Table 2- several of these genes are associated with NFkB signalling.

To determine if NFkB itself was upregulated, we examined if there was nuclear localisation of phosphorylated NFkB which only occurs upon activation.

<table>
<thead>
<tr>
<th>Fold-increase</th>
<th>Genes</th>
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<tbody>
<tr>
<td>&gt;5</td>
<td>Interleukin 1; Interleukin 6 receptor α; Interleukin 8 receptor α, macrophage migration inhibitory factor; CD40 ligand</td>
</tr>
<tr>
<td>&gt;10</td>
<td>Interleukin 6 signal transducer; Interleukin-17 precursor; Nitric oxide synthase 2; Toll-like receptors 1 and 4-6; Tumor necrosis factor (ligand) superfamily member 4</td>
</tr>
<tr>
<td>&gt;20</td>
<td>Transforming growth factor beta 1; Toll-like receptors 2, 3 and 9; Tumor necrosis factor superfamily member 2; Tumor necrosis factor receptor superfamily member 1a &amp; 1b</td>
</tr>
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</table>

**Figure 4:** left panel shows no evidence of phosphor-NFkB (Ser536) in control prostate tissue. In contrast, Figure 4: right panel shows evidence of nuclear NFkB (arrows) demonstrating activation of NFkB.

**Deregulation of epigenetic associated dimethyltransferase (DNMT) enzymes occurs with prostatitis in post-pubertal rat tissue**

**Figure 5** shows the relative levels of DNA methyltransferases in prostatic tissues from control (black
bars) and EDC treated (open bars) postpubertal rats. Significant increases (*p<0.05) observed in DNMT3a and 3b, indicative of epigenetic modification by DNA methylation due to EDC.

### KEY RESEARCH ACCOMPLISHMENTS

*Bulleted list of key research accomplishments emanating from this research.*

- Demonstration that Vinclozolin disrupts human prostate development in tissue recombinants composed of human ESCs and rodent stroma, implicating this EDC as a potential hazard to human prostate health.

- Demonstration that Vinclozolin causes prostatitis, implicating this EDC as a causative factor in prostatitis, a condition known to increase the risk of prostate cancer.

### REPORTABLE OUTCOMES

*Provide a list of reportable outcomes that have resulted from this research to include:*

**Manuscripts – Refer to Appendix 1 for full text**

Cowin PA, Foster P, Pedersen J, Hedwards S, McPherson S, Risbridger GP (2008) Early onset endocrine disruptor induced prostatitis in rodents (Accepted February 2008; Environmental Health Perspectives) Impact Factor: 5.86 *In Press*

**Abstracts of Presentations – Refer to Appendix 2 for full text**


**Degrees obtained that are supported by this award**

- To be included in doctoral thesis submitted by Ms Prue Cowin PhD (June 2008)

**Funding applied for based on work supported by this award**

Based on these data, application was made for Australian government funding from NH&MRC for a project grant.
CONCLUSION:
The role of endocrine disrupting chemicals (EDCs) in the early origins of adult prostate disease is of concern and controversy to the lay and scientific communities. These data are the first to unequivocally implicate the anti-androgenic activity of EDCs as causative factors in the aetiology of prostatitis in the rat, providing novel insight to the origins of this disease.

The majority (>90%) of prostatitis cases are ascribed to unknown (non-bacterial) origins and the symptoms, both acute and chronic, are common, bothersome and burdensome in terms of health-related quality-of-life (6, 7). The economic impact of prostatitis includes an estimated annual expenditure in the US of >$84 million for diagnosis and management, excluding subsequent pharmaceutical costs (8-10). As there are extensive gaps in our understanding of prostatitis aetiology, many of these current expenditures may be ineffective and a waste of resources. **These data are the first to unequivocally implicate EDCs as a causative factor and fill an important knowledge gap on the aetiology of prostatitis.**

Overall, the robust incidence of inflammation in 100% of young adult rats mimics more closely human non-bacterial prostatitis that occurs in young men. Ninety percent of prostatitis cases are of unknown cause and these data are the first to implicate anti-androgenic EDCs as a causative factor in the aetiology of this inflammatory disease of the prostate via activation of the classical NFκB inflammatory pathway. Whilst the level of Vinclozolin utilised in this study far exceeds that observed in the environment and projected human exposure, this study raises further concerns that *in utero* exposures to EDCs, with anti-androgenic activity have long range effects that include the development of prostatitis in early adult life, and provide further impetus to test the efficacy of treatments that block or abrogate NFκB signalling in the treatment of prostatitis.
REFERENCES

List all references pertinent to the report using a standard journal format (i.e. format used in *Science*, *Military Medicine*, etc.).


Appendix 1: Manuscript (in Press) in Environmental Health Perspectives

Early Onset Endocrine Disruptor Induced Prostatitis in the Rat

Prue A. Cowin\textsuperscript{1}, Paul Foster\textsuperscript{2}, John Pedersen\textsuperscript{3}, Shelley Hedwards\textsuperscript{1}, Stephen J. McPherson\textsuperscript{1}, and Gail P. Risbridger\textsuperscript{1}

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Running Title: Endocrine Disruptor Induced Prostatitis

Key Words: Anti-androgen, Endocrine Disruptors, Inflammation, Prostate, Prostatitis, Vinclozolin

Acknowledgements: We thank A. Mansell (Monash University, Australia) for insightful discussions and M. Richards (Monash University, Australia) for skilled technical assistance. The authors declare they have no competing financial interest.

Funding: This research was funded by the US Army Department of Defence, Prostate Cancer Research Program Exploration-Hypothesis Development Award (W81XWH-07-1-0126) (GPR) and supported [in part] by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences (PF).

Abbreviations:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AGD</td>
<td>Anogenital distance</td>
</tr>
<tr>
<td>AP</td>
<td>Anterior prostate</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign prostate hyperplasia</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>DP</td>
<td>Dorsal prostate</td>
</tr>
<tr>
<td>EDC</td>
<td>Endocrine disruptor</td>
</tr>
<tr>
<td>E</td>
<td>Estrogen</td>
</tr>
<tr>
<td>FgF10</td>
<td>Fibroblast Growth Factor 10</td>
</tr>
</tbody>
</table>
GD  Gestational day

LP   Lateral prostate

NFκB Nuclear factor kappa B

PCa  Prostate cancer

PIN  Prostatic intra-epithelial neoplasia

PIA  Proliferative inflammatory atrophy

PND  Post-natal day; prostate

SV   Seminal vesical

T    Testosterone

VP   Ventral
Outline of section headers:

Abstract

Introduction

Material and Methods

Animals

Treatment

Necropsy of male littermates

Hormone Analysis

Tissue Collection

Tissue Separation

Histology

mRNA Extraction

Oligo GEArray

Stereology

Wholemount Immunolabelling

Analysis of Branching Morphogenesis

Statistical Analysis
Results

Compound purification data

Post-natal day 0 litter data

Gross analysis of male off-spring following in utero treatment

Absence of perturbations in branching morphogenesis in neo-natal off-spring

Normal prostate development until puberty with early onset of prostate inflammation in post-pubertal off-spring

Epithelial aberrations in post-pubertal off-spring without evidence of pre-malignancy

Discussion and Conclusions

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Table 2: Effects of 6 day in utero administration of Vinclozolin on PND28 and PND56 reproductive organ weights

Table 3: Effects of 6 day in utero administration of Vinclozolin on key NFκB dependant inflammatory genes at post-natal day 56 compared to Control treatment

Figure Legends

Figures
Figure 1: Absence of perturbations in branching morphogenesis in neo-natal off-spring exposed in utero to Vinclozolin.

Figure 2: Serum Testosterone.

Figure 3: Early onset post-pubertal prostatitis in animals exposed in utero to Vinclozolin.

Figure 4: Epithelial attenuation in post-pubertal animals following in utero Vinclozolin treatment.
Abstract

**Background:** Androgens are critical for specifying prostate development with the fetal prostate sensitive to altered hormone levels and endocrine disrupting chemicals (EDCs) that exhibit estrogenic or anti-androgenic properties. Prostatic Inflammation (prostatitis) affects 9% of men of all ages and >90% of cases are of unknown aetiology. **Objectives:** This study aimed to evaluate effects of *in utero* exposure to the anti-androgenic EDC Vinclozolin during the period of male reproductive tract development, on neo-natal, pre- and post-pubertal prostate gland function of male off-spring. **Methods:** Fetal rats were exposed to Vinclozolin (100mg/kg/bw) or vehicle control (2.5ml/kg/bw) *in utero* from gestational day 14 to 19 via oral administration to pregnant dams. Male off-spring were aged to 0, 4 or 8 weeks before tissue analysis. **Results:** *In utero* exposure to Vinclozolin was insufficient to perturb prostatic development and branching, although androgen receptor and mesenchymal fibroblast growth factor 10 expressions were down-regulated. Prostate histology remained normal until puberty but 100% of animals displayed prostatitis post puberty (56 days). Prostatic inflammation was associated phosphorylation and nuclear translocation of NFκB and post-pubertal activation of pro-inflammatory NFκB dependant genes including the chemokine Interleukin-8 and cytokine Transforming Growth Factor β1. Significantly, inflammation arising from Vinclozolin exposure was not associated with the emergence of pre-malignant lesions, such as prostatic intra-epithelial neoplasia (PIN) or proliferative inflammatory atrophy (PIA) and hence mimics non-bacterial early-onset prostatitis that commonly occurs in young men. **Conclusions:** These data are the first to unequivocally implicate EDCs as a causative factor and fill an important knowledge gap on the aetiology of prostatitis.
Introduction

Environmental pollutants or industrial chemicals disrupt, and have the potential to alter, the action of gonadal steroid hormones by virtue of their anti-androgenic or estrogenic properties and in doing so, effect hormonal balance (11-13). Reproductive tract development during fetal and neonatal life is hormonally regulated and in an undifferentiated state, lacking compensatory homeostatic mechanisms to prevent adverse effects of endocrine disrupting chemicals (EDCs) (14, 15). Thus, the organisational effects of EDCs on the developing reproductive tract can be permanent and irreversible.

Dissimilar to prostate cancer (PCa) and benign prostate hyperplasia (BPH) that predominantly affect ageing men, prostate inflammation (prostatitis) affects 9% of men of all ages (10). The majority (>90%) of prostatitis cases are ascribed to unknown (non-bacterial) origins and the symptoms, both acute and chronic, are common, bothersome and burdensome in terms of health-related quality-of-life (6, 7). The economic impact of prostatitis includes an estimated annual expenditure in the US of >$84 million for diagnosis and management, excluding subsequent pharmaceutical costs (8-10). As there are extensive gaps in our understanding of prostatitis aetiology, many of these current expenditures may be ineffective and a waste of resources. Thus, it is imperative we better understand this disease, one that has received relatively little attention compared to BPH and PCa.

Whilst increased levels of developmental or environmental estrogens have been linked to the increased incidence of prostate disease (16, 17), chemicals with anti-androgenic activity are potentially of greater importance because androgens are critical to establishing the male phenotype. Vinclozolin (3-(3-5-dichlorophenyl)-5-methyl-oxazolidine-2,4-dione) is an anti-androgenic systemic dicarboximide fungicide used widely throughout Europe and the United States in the control of diseases caused by Botrytis cinerea, Sclerotinia sclerotiorum, and Moniliniam spp. Vinclozolin is degraded to several
metabolites: 2-[[3,5-dichlorophenyl]-carbamoyl]oxy]-2-methyl-3-butenolic acid (M1) and 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2), which are competitive antagonists of androgen receptor (AR)-ligand binding, rather than 5α-reductase enzyme inhibitors (18, 19). Vinclozolin has a half-life of 23 days when sprayed as Ronilan (a 50% mixture of Vinclozolin) on soil, with previous reports showing Vinclozolin exposure induces malformations such as cryptorchidism, hypospadias and Leydig cell hyperplasia, and permanent changes in sexually dimorphic structures, such as anogenital distance (AGD) and areola/nipple retention (20). These effects occur before hypothalamic-pituitary-gonadal axis formation and long after Vinclozolin has been cleared from the pup, thus are organisational effects rather than due to interruption of a feedback loop via the pituitary.

Recent interest in Vinclozolin arose from reports that transient embryonic exposure during embryonic gonadal sex determination (gestational days (GD) 8-14) appears to alter the male germ line epigenome and subsequently promotes transgenerational adult onset disease, including testis and immune abnormalities, prostate and kidney disease and tumour development (21). A preliminary report stated prostate disease, including inflammation and epithelial atrophy, occurs in aged rats (12-14 months), although the incidence of prostatic lesions across four generations of male rats was only 10% (22). These findings are interesting (although the low incidence of prostatic lesions is not compelling) and at the same time controversial because of the EDC purity and timing and route of its administration in utero.

Vinclozolin, when purchased commercially, requires purification and recrystallisation to obtain >99% purity and ensure any effects are not due to contaminants. Human exposure to Vinclozolin occurs by oral ingestion enabling metabolism to the more potent AR antagonists (M1 and M2). Direct
intraperitoneal administration runs the risk of producing effects not observed by the conventional oral route, such as uterine irritation and changes in uterine blood flow. The timing of Vinclozolin exposure also varies the effect on male reproductive tract development. A window of sensitivity for prostate development occurs when ARs are activated between GD 14-19, rather than during embryonic gonadal sex determination around GD8-14 (23). Commonly, the outcomes of any transient in utero treatments are examined in ageing animals. However, anti-androgen effects also manifest at other times including pre- and post-puberty, when hormone action is critical for normal prostate maturation and function. Altogether, these variations in treatment protocol may account for the low incidence of prostatic lesions reported by Anway et al who used intraperitoneally administered unpurified Vinclozolin, during GD8-14 and did not study outcomes until 12-14 months of age (21).

Therefore, the aim of this study was to evaluate effects of fetal exposure to purified Vinclozolin, administered orally to pregnant dams during the period of male reproductive tract development (GD 14-19), on pre- and post-pubertal prostate gland function in male off-spring.
Material and Methods

Animals

All animal procedures were conducted according to National Health and Medical Research Council (NHMRC) guidelines and animal experimentation ethics committee at Monash Medical Centre, Clayton, Australia (MMCA/2006/22). Animals were treated humanely and with regard for alleviation of suffering. Time mated female outbred Sprague-Dawley rats were obtained from Monash University Central Animal Services (Clayton, Australia) on gestational day (GD) 8 and housed at Monash Medical Centre Animal House (Clayton, Australia) under controlled 12 hour light-dark cycle and temperature conditions. Animals were fed *ad libitum*. GD 0 was the day plugs were observed in the vagina of mated females. Dams and offspring were housed together until weaning (post-natal day (PND) 21) when male litter mates were group-housed, ≤ 4 per cage. Dams and female offspring were euthanized humanely by CO₂ asphyxiation and not subjected to post mortem examination.

Treatment

The treatment regime was performed as previously described (23). On GD14 dams were weighed and animal allocation to treatment groups done by body weight (bw) randomization to ensure unbiased weight distribution among groups. Dams were assigned 1 of 2 treatment groups (0, 100mg.kg.bw Vinclozolin) (n=16 dams per group) and 1 of 3 time points of collection (PND 0, PND 28 or PND 56) (n=8 dams at PND 0; n=4 dams at PND 28 and PND 56 per treatment group). Dams were orally dosed using a micropipette daily at 1000hr from GD14 to 19 with 100mg/kg/bw body weight Vinclozolin (Research Triangle Park, NC, USA) or corn oil vehicle Control (Sigma; 2.5 ml/kg/bw) and examined for clinical signs of toxicity. The dose chosen corresponds to a level commonly used to investigate Vinclozolin effects on male reproductive tract formation, inducing an array of male reproductive tract
malformations at high incidence without maternal toxicity. As the pubertal period in the rodent is controversial, puberty has been defined in relation to functional puberty or the time at which sperm appear and serum Testosterone rises. This occurs around post-natal day 43 in rats (24, 25).

Necropsy of male littermates

Male offspring were collected at PND 0, 28 or 56, weighed, euthanized by decapitation (PND 0) or CO₂ asphyxiation (PND 28 and 56) and blood collected by cardiac puncture for hormonal analysis. External genitalia, including scrotum, prepuce and penis were visually examined and anogenital distance (AGD) measured with a calliper.

Hormone Analysis

Serum testosterone (T) levels were measured by ANZAC Research Institute (Sydney, Australia) as previously described (26).

Tissue Collection

Using a dissecting microscope (SZX12, Olympus Corporation, Tokyo, Japan) and dissecting tools ventral (VP), dorsal (DP), lateral (LP) and anterior (AP) prostate lobes, testes and seminal vesicles (SV) were dissected from PND 28 and 56 animals and wet weights recorded. For isolation of PND 0 prostates, urogenital tracts were removed and VPs micro-dissected in a modified watch glass (Maximov depression slide; San Francisco, CA), in the presence of dissecting media (basal medium of Dulbecco’s Modified Eagles Media (DMEM) and Hams F-12 (1:1 vol/vol) supplemented with
penicillin and streptomycin (5mls/ltr) and fungizome (20µg/ml) at pH 7.3). Pair matched organs were fixed in Bouin’s fixative or immediately frozen in liquid nitrogen and stored at -80°C.

**Tissue Separation**

PND 0 VP’s for epithelial and mesenchymal RNA analysis were digested in 1% trypsin (Difco, Detroit, MI) in Hank’s calcium and magnesium free Balanced Salt Solution (HBSS; Gibco, Invitrogen, Vic, Australia) for 60 minutes. Mesenchyme and epithelia were mechanically separated, immediately frozen in liquid nitrogen and stored at -80°C.

**Histology**

Fixed tissues were dehydrated, processed and embedded in paraffin. Serial 5µm sections were cut and mounted onto Superfrost Plus+ coated slides (Menzel-Glaser®, Germany). Tissue sections were stained with Harris’ haematoxylin and eosin (H&E) or used for immuno-histochemistry.

Immunohistochemistry was performed using the DAKO Autostainer Universal Staining System (DAKO A/S, Denmark) (27). Antibodies were purchased from Santa Cruz Biotechnology Inc.(CA, USA) to: AR (AR (N-20)), TGFβ1 (SC-146), TLR4 and Fgf10 (H-121:SC_7917); PCNA (clone PC10) DAKO Corporation, (Denmark); CD68 (ED1) Sapphire Bioscience Pty Ltd (NSW, Australia); phospho-NFκB p65 (Ser536) Cell Signaling Technology Inc. (MA, USA). Antibodies were used as previously described (28-30) or according to company specifications.
mRNA Extraction

Total RNA was extracted from prostate tissues using TRIzol Reagent (Invitrogen Life Technologies, Rockville, MD) according to manufacturer specifications and as previously described (27).

Oligo GEArray

Gene expression analysis was carried out using GEArray™ DNA Microarray (PND56: array no. ERN-011.2, PND0: EMM-014) (SuperArray Bioscience Corporation, MD, USA) according to manufacture’s directions. Analysis was conducted on a minimum of 4 samples per group in duplicate. Briefly, complementary deoxyribonucleic acid (cDNA) was synthesised from pure RNA using the TrueLabelling-AMP™ 2.0 kit (SuperArray Bioscience Corporation, MD, USA). cDNA was amplified followed by a 24 hour complimentary RNA (cRNA) synthesis reaction. cRNA concentration and purity was determined by UV Spectrophotometry. Following generation and purification of cRNA, array hybridisation was performed using the Oligo GEArray® HybPlate Basic Protocol (SuperArray Bioscience Corporation, MD, USA) according to manufactures directions. Briefly, arrays were subjected to pre-hybridisation before hybridization with the labelled target cRNA and incubated for 24 hours at 60ºC, then washed repeatedly in stringency washes. Detection was performed using the Chemiluminescent Detection Kit (SuperArray Bioscience Corporation, MD, USA). Briefly, arrays were incubated at room temperature in dilute AP-Streptavidin for 10 minutes, rinsed in buffer and incubated with a chemiluminescent detection chemical, CDP-Star®. Images were acquired immediately using X-ray exposure. X-ray images were captured using a scanner and saved as 16 bit TIFF images. Data analysis was completed using GEArray Expression Analysis Suite (SuperArray Bioscience Corporation, MD, USA), with expression normalised to a specific set of house-keeping genes.
Stereology

An unbiased estimate of the terminally differentiated secretory epithelial cell population and incidence of inflammation was obtained using stereological techniques, based on the Cavalieri principle (31) and as previously described in the testes and prostate (32-34). Stereological analysis was performed using a BX-51 microscope (Olympus Corp.) and a JVC TK-C1380 video camera (Victor Company of Japan Ltd, Japan) coupled to an IBM computer. Images were projected directly onto a video screen and utilising the CAST V1.10 software (Computer Assisted Stereological Toolbox) (Olympus Danmark A/S, Denmark.) tissue sections were mapped at x 40 magnification to define tissue boundaries. Beginning from a random point, sampling was conducted at predetermined intervals along x- and y-axes using a three-by-three point grid counting frame. A minimum of 10 sections per animal uniformly spaced throughout explants and 5 animals per group were utilised. To accurately differentiate terminally differentiated secretory epithelial cells, tissue sections stained for CKHMW were utilized, with positively and negatively stained CKHMW cells identified and percentages of positive and negative cells was determined. To determine the incidence of inflammatory lesions random fields were designated as positive or negative for inflammation. Abnormal inflammatory regions were classified as areas that displayed chronic inflammation as represented in Figure 3D.

Wholemount Immunolabelling

Branching morphogenesis analysis was conducted on PND 0 VPs. Individual VPs were placed in methanol and stored at -20°C. Immunolabelling was performed as previously described (35). Briefly, tissues were permeabilized in 0.2% (v/v) Triton X-100 (Sigma, St Louis, USA) and 5μg/ml Sodium Borohydride in PBS (pH 8.0) for 15 mins. Non-specific binding was blocked with ‘Superblock’ blocking buffer (Pierce, IL, USA) for 1 hour at room temperature, before overnight incubation at 4 °C
with a monoclonal mouse anti-human CKHMW (Dako Corporation, USA) (5 µg/ml immunoglobulin G (IgG). Tissues were incubated with 5 µg/ml goat anti-mouse IgG secondary antibody (F[ab]2 fragments) conjugated with fluorescein isothiocyanate (FITC) (Dako Corporation, USA) for 90 min at room temperature. VPs were mounted on slides using Vectorshield fluorescent mounting medium (Vector, CA, USA) with coverslips mounted on nail polish platforms, to maintain 3D patterns.

### Analysis of Branching Morphogenesis

Serial optical images of CKHMW stained wholmount tissues were generated at 2µm interframe steps using an Olympus confocal microscope, captured, and stored in 8 bit BMP format using Fluroview software (Olympus) as previously described (36). Confocal images were subsequently used to construct a 3D skeleton representing the original ductal pattern of the gland, using lines running through the centre of each ductal/epithelial branch. A full description of this process was previously reported for the study of branching morphogenesis in kidney (37, 38) and prostate (36). The resultant algorithm provides fully automated measurements of the branch length (in pixels and micrometres) and cumulative surface areas (in square pixels) of the individual ducts as they appear in all the frames. Total ductal length was calculated by adding together individual branch lengths from multiple ducts within a lobe. The value of the surface areas was multiplied by 1.5522 (pixel area), to convert it into micrometres, then by 2 (µm, interframe steps) to obtain total volume (in µm³) of individual ducts. Numbers of branch points, branches, and terminal tips were automatically generated by the software.
Statistical Analysis

All pup data was analysed individually and nested by dam to yield litter means. To test for significance of treatment effects litter was corrected for as a main effect variable using one-way analysis of covariance (ANCOVA) on SPSS (v.16 SPSS Inc., Chicago, USA) and data expressed as litter mean ± standard error of the mean (SEM). AGD and organ weights were analyzed with body weight as a covariate. Control and Vinclozolin were compared using an F test, with the significance threshold employed at a level of 5% (p<0.05). Analysis of Control and Vinclozolin stereological data was performed using a two-tailed paired t test, and using Prism 4.0 software (GraphPad Software, Inc., San Diego, CA).

Results

Compound purification data

Vinclozolin was obtained from BASF AG (Research Triangle Park, NC, USA) as Ronilan ® EG (a 50% Vinclozolin mixture) and purified and recrystallised. Catalogued as lot no. 357-141A, it was certified as being of >99% purity by ChemService Inc (West Chester, PA, USA).

Post-natal day 0 litter data

Gestating Sprague Dawley rats were transiently exposed to Vinclozolin (100 mg.kg.day) and compared to corn oil vehicle treated control (2.5ml.kg.day) from gestational day (GD) 14-19. Vinclozolin did not induce maternal toxicity or affect normal pregnancy as no dams presented dystocia or delivered late. Dam weight gain through the dosing period was not significantly different between groups (Table 1). Live litter size and sex ratio were not affected by Vinclozolin treatment, with sex confirmed at puberty (Table 1).
**Gross analysis of male off-spring following in utero treatment**

Male off-spring exposed *in utero* were analysed at post-natal day (PND) 0 (day of birth), PND 28 (pre-pubertal) or PND 56 (post-pubertal) for any weight differences or gross morphological abnormalities. Pup weights at PND 0 were significantly reduced between *in utero* Control and Vinclozolin treated groups (p<0.05) (Table 1), however no significant changes to body weight were demonstrated at PND 28 or 56 (p<0.05; Table 2). Anogenital distance (AGD) is a sensitive indicator of anti-androgenicity, and AGD was significantly reduced at all ages following *in utero* Vinclozolin exposure compared to Control (Table 1 and 2). Covariate analysis demonstrated the PND 0 AGD reductions were not due pup weight reductions. At PND 28, compared to Control, *in utero* Vinclozolin treatment did not significantly reduce testis, seminal vesicles (SV), ventral (VP), anterior (AP), dorsal (DP) and lateral (LP) prostate weights (p<0.05) (Table 2). Analysis of external genitalia revealed undescended testis in 35.93 ± 4.37% of pre-pubertal animals exposed *in utero* to Vinclozolin, compared with none in Control. At PND 56, *in utero* Vinclozolin treatment significantly reduced SV and VP weights and significantly increased AP weight (p<0.05) (Table 2). No significant differences in LP, DP and testis weights were observed (Table 2). Malformations of external genitalia included cleft prepuce, incomplete preputial separation, cleft phallus and hypospadias and observed in 47.02 ± 7.92% of post-pubertal animals exposed *in utero* to Vinclozolin compared to none in Control. Analysis of serum testosterone (T) levels revealed no significant differences between *in utero* treatment with Control and Vinclozolin at any post-natal age (Figure 1).

Whilst all prostate lobes were collected and analysed, only the ventral prostate (VP) is reported herein as in addition to being the most commonly reported lobe with respect to EDC exposures, it is the most
androgen sensitive lobe (39, 40) and it was predicted the actions of an anti-androgenic chemical would be more likely to induce effects in this lobe.

Absence of perturbations in branching morphogenesis in neo-natal off-spring

The inductive and instructive potential of the mesenchyme and importance of mesenchymal androgen signalling in normal prostate branching and development has been well established. To examine whether in utero Vinclozolin exposure perturbed normal mesenchymal signalling in offspring, gene array analysis of 113 common growth factors was performed on neonatal mesenchyme and revealed significant (>1.5 fold) down regulation of several mesenchymal genes including fibroblast growth factor (Fgf10) in prostates from animals exposed in utero to Vinclozolin compared to Control and confirmed by immuno-protein localisation (data not shown). Since estrogen exposure down regulates Fgf10 and perturbs normal prostate ductal branching (41), we investigated whether the anti androgen-induced reduction in mesenchymal Fgf10 was associated with developmental abnormalities. Using a computer-based method that allows temporal and spatial alterations in branching morphogenesis as a result of experimental manipulations to be examined branching morphogenesis in neonatal tissues was analysed. Despite mesenchymal Fgf10 reductions, prostate size was normal with no significant differences present in ductal number, length, volume or branch points in neonatal specimens between prostates from animals exposed in utero to Vinclozolin and Control (Figure 2).

Normal prostate development until puberty with early onset of prostate inflammation in post-pubertal off-spring

No gross morphological differences between in utero Vinclozolin and Control treatment were observed in PND28 (pre-pubertal) prostates (Figure 3A, B). However, analysis of prostate specimens at PND56 (post-pubertal) revealed the onset of prostate inflammation in in utero Vinclozolin exposed males
Prominent, but focal, regions of inflammation were observed in 100% of animals, with an increase in the proportion of inflammatory cells, particularly leukocytes and macrophages, surrounding the ducts and infiltrating into the vessels (Figure 3D). Increased macrophage infiltration was evident in prostates from animals exposed *in utero* to Vinclozolin, as demonstrated by immuno-localisation of ED1 (Figure 3E, F), although this was absent in control animals. *In utero* Vinclozolin treatment resulted in a significant increase (p<0.05) in the percentage of prostatic inflammatory lesions, identified by stereological analysis from 1.4 ± 0.80% to 16.8 ± 3.72% (Figure 3G).

Pro-inflammatory stimuli and immune responses are commonly controlled by the nuclear factor-kappa B (NFκB) family of transcription factors. In unstimulated cells, NFκB is sequestered in the cytoplasm of cells and activated when phosphorylated and translocated to the nucleus. Activation of NFκB in prostates exposed *in utero* to Vinclozolin was confirmed by nuclear immuno-protein localisation of phospho-NFκB p65 (Ser536) antibody which detects NFκB p65 only when phosphorylated at serine 536. In prostates exposed to vehicle control few immuno-positive cells were identified (Figure 3H, I).

Activation of NFκB subsequently induces transcription of many NFκB dependent genes, including those encoding inflammatory cytokines and chemokines. To determine the distinct pattern of gene expression following activation of NFκB signalling by Vinclozolin treatment, pathway specific gene array analysis of 113 key genes involved in the inflammatory response was performed. A significant (>1.5 fold) up- and down-regulation of 69 and 34 genes respectively was observed in prostate tissues of PND 56 animals exposed *in utero* to Vinclozolin compared with animals exposed *in utero* to the vehicle Control (Table 3). These data show increased activation of classic pro-inflammatory NFκB dependant genes including chemokines such as Interleukins (IL)-1α, IL-6 and IL-8) and cytokines such
as Transforming Growth Factor (TGF)-β and Tumour Necrosis Factor (TNF)-α, as well as other ligands and receptors including toll-like receptors (TLR) 1-6 and 9 and TNF receptors.

Several key NFκB dependant genes were selected to examine transcriptional activity by immuno-protein localisation, including TLR-4 and TGF-β1. Heightened expression of TLR-4, an important innate immune receptor, was confirmed by increased immuno-protein localisation, particularly in the stromal and peri-ductal compartments of tissues exposed in utero to Vinclozolin (Figure 3J, K). A significant up-regulation of TGF-β1 expression was observed in tissues exposed in utero to Vinclozolin (Figure 3L, M) correlating with a significant down regulation of the immuno-suppressive cytokine and TGF-β1 negative regulator IL-10 (Table 3).

**Epithelial aberrations in post-pubertal off-spring without evidence of pre-malignancy**

In addition to the 100% penetrance of prostatic inflammation observed in PND56 prostate specimens, in utero exposure to Vinclozolin also induced focal epithelial attenuation (reduction in epithelial cell height and thinning of ductal structure) in all animals. A reduction in epithelial AR was observed concurrently with a reduction in terminally differentiated secretory epithelia (Figure 4A, B). Loss of terminally differentiated secretory epithelia and epithelial attenuation was shown by immuno-localisation of the basal cell marker, CKHMW (Figure 4C, D), which showed a continuous layer of basal cells compared to a discontinuous layer observed in Control, and confirmed by stereological analysis (Figure 4E).
Prostatic inflammation associated with atrophy and proliferation has been reported as a pre-malignant lesion in men, known as proliferative inflammatory atrophy (PIA). Although PIA has not been confirmed in rodents, proliferative activity was examined by immuno-localisation of proliferating cell nuclear antigen (PCNA) (Figure 4F, G). In epithelial attenuated glands of tissues exposed \textit{in utero} to Vinclozolin there was an apparent loss of proliferative activity with a reduction in immuno-positive epithelial cells, demonstrating the absence of pathology comparable to PIA in these tissues. There was no evidence of other prostatic lesions, such as pre-malignant prostatic intraepithelial neoplastic (PIN) lesions.
Discussion and Conclusions

The role of endocrine disrupting chemicals (EDCs) in the early origins of adult prostate disease is of concern and controversy to the lay and scientific communities. These data are the first to unequivocally implicate the anti-androgenic activity of EDCs as causative factors in the aetiology of prostatitis in the rat, providing novel insight to the origins of this disease in which >90% of human cases are of unknown cause (10).

The longer term consequences of in utero Vinclozolin exposure include the development of gross malformations of the male reproductive tract such as the epididymis, vas deferens, seminal vesicles, prostate, external genitalia (hypospadias), cryptorchidism and testicular injury and permanent change in sexually dimorphic structures (23). A reduction in nuclear epithelial AR in prostate tissues from animals exposed in utero to Vinclozolin correlates with previous studies demonstrating rapid AR degradation following anti-androgen binding (19).

The immediate effects of reduced AR and mesenchymal Fgf10 expression in tissues exposed in utero to Vinclozolin do not result in any perturbations in prostate branching. This implies that other androgen-regulated paracrine factors produced by the mesenchyme were sufficient to compensate and induce normal differentiation and development. Furthermore, the absence of a branching effect following in utero treatment during primary gland genesis may indicate that Fgf10 may only act with concurrent chemical exposure in the neonate when secondary branching morphogenesis occurs. These findings contrast effects reported following neonatal estrogen exposure, in which reductions in mesenchymal Fgf10 inhibits branching morphogenesis (41).
Prostatic inflammation is a common feature of endocrine disruption by estrogentic and anti-androgenic chemicals (14, 42, 43). Our results demonstrate the absence of any morphological changes prior to puberty but an inflammatory response in all young post-pubertal (56 day old) prostates following \textit{in utero} anti-androgenic exposure. Activation of the NFκB inflammatory pathway was evident with a significant down regulation of AR expression. There is considerable evidence to show cross talk between AR and NFκB (44) thus it is reasonable to postulate that the persistent repression of AR signalling induced by Vinclozolin results in androgenic activity that is insufficient to suppress NFκB signalling pathways, resulting in inappropriate activation of NFκB and the emergence of prostatitis.

Whilst the exact mechanism by which estradiol exposure promotes an inflammatory response in the adult prostate has not yet been determined, the anti-androgen induced inflammation is associated with activation of the ‘canonical’ pro-inflammatory NFκB inflammatory signalling pathway and NFκB dependant genes.

There are further, important differences between EDCs that are antiandrogenic or estrogenic. In contrast to estrogen induced inflammation, we demonstrate the long-term effects of transient \textit{in utero} exposure to Vinclozolin failed to induce pre-malignancy. Inflammation and focal atrophy associated with increased proliferation has been described in human prostate specimens as proliferative inflammatory atrophy (PIA), and may be pre-malignant. The pathology arising from \textit{in utero} Vinclozolin treatment and described herein does show inflammation and focal epithelial attenuation but in the absence of increased proliferation. Therefore it was concluded that Vinclozolin did not induce the pre-malignant lesion PIA.
Overall, the robust incidence of inflammation in 100% of young adult rats mimics more closely human non-bacterial prostatitis that occurs in young men. Ninety percent of prostatitis cases are of unknown cause and these data are the first to implicate anti-androgenic EDCs as a causative factor in the aetiology of this inflammatory disease of the prostate via activation of the classical NFκB inflammatory pathway. Whilst the level of Vinclozolin utilised in this study far exceeds that observed in the environment and projected human exposure, this study raises further concerns that in utero exposures to EDCs, with anti-androgenic activity have long range effects that include the development of prostatitis in early adult life, and provide further impetus to test the efficacy of treatments that block or abrogate NFκB signalling in the treatment of prostatitis.
Table 1: Effects of 6 day *in utero* administration of Vinclozolin at PND 0

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Control</th>
<th>Vinclozolin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dams assigned</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Dams pregnant</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Dams delivered late</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dam weight gain through dosing period (g)</td>
<td>49.29 ± 10.38</td>
<td>53.33 ± 11.81</td>
</tr>
<tr>
<td>Live litter sizes</td>
<td>12.18 ± 2.26</td>
<td>12.83 ± 2.33</td>
</tr>
<tr>
<td>M/F ratio at birth</td>
<td>1.10 ± 0.67</td>
<td>1.61 ± 1.80</td>
</tr>
<tr>
<td>Pup wt at birth (g)</td>
<td>6.44 ± 0.07</td>
<td>6.16 ± 0.09*</td>
</tr>
<tr>
<td>AGD in male offspring at birth</td>
<td>4.36 ± 0.08</td>
<td>3.75 ± 0.10*</td>
</tr>
</tbody>
</table>

Note: Litter mean ± SEM. AGD, anogenital distance; wt, weight; g, grams; M/F, Male to Female.

*p<0.05
Table 2: Effects of 6 day *in utero* administration of Vinclozolin on PND28 and PND56 reproductive organ weights

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Control</th>
<th>Vinclozolin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PND 28</td>
<td>PND 56</td>
</tr>
<tr>
<td>Body (g)</td>
<td>70.97 ± 0.61</td>
<td>259.09 ± 7.21</td>
</tr>
<tr>
<td>AGD</td>
<td>25.55 ± 0.54</td>
<td>40.51 ± 0.91</td>
</tr>
<tr>
<td>VP (mg)</td>
<td>37.67 ± 1.45</td>
<td>223.74 ± 6.81</td>
</tr>
<tr>
<td>AP (mg)</td>
<td>4.56 ± 0.55</td>
<td>69.32 ± 3.67</td>
</tr>
<tr>
<td>LP (mg)</td>
<td>7.3 ± 0.49</td>
<td>45.68 ± 4.04</td>
</tr>
<tr>
<td>DP (mg)</td>
<td>9.1 ± 1.23</td>
<td>61.26 ± 4.42</td>
</tr>
<tr>
<td>SV (mg)</td>
<td>11.61 ± 0.96</td>
<td>507.94 ± 15.14</td>
</tr>
<tr>
<td>Testis (g)</td>
<td>0.525 ± 0.02</td>
<td>2.41 ± 0.06</td>
</tr>
</tbody>
</table>

*Note: Litter mean ± SEM. AGD, anogenital distance; VP, ventral prostate; AP, anterior prostate; LP, lateral prostate; DP, dorsal prostate; SV, seminal vesicle. *p<0.05
Table 3: Effects of 6 day *in utero* administration of Vinclozolin on key NFκB dependant inflammatory genes at post-natal day 56 compared to Control treatment

<table>
<thead>
<tr>
<th>UniGene</th>
<th>Symbol</th>
<th>Description</th>
<th>Fold Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rn.12300</td>
<td>II1a</td>
<td>Interleukin 1 alpha</td>
<td>5.05</td>
</tr>
<tr>
<td>Rn.9869</td>
<td>II1b</td>
<td>Interleukin 1 beta</td>
<td>2.02</td>
</tr>
<tr>
<td>Rn.1716</td>
<td>II6ra</td>
<td>Interleukin 6 receptor, alpha</td>
<td>5.16</td>
</tr>
<tr>
<td>Rn.12138</td>
<td>II6st</td>
<td>Interleukin 6 signal transducer</td>
<td>11.13</td>
</tr>
<tr>
<td>Rn.138115</td>
<td>II8ra</td>
<td>Interleukin 8 receptor, alpha</td>
<td>7.80</td>
</tr>
<tr>
<td>Rn.90347</td>
<td>II8rb</td>
<td>Interleukin 8 receptor, beta</td>
<td>4.29</td>
</tr>
<tr>
<td>Rn.92374</td>
<td>II9</td>
<td>Interleukin 9</td>
<td>3.52</td>
</tr>
<tr>
<td>Rn.10045</td>
<td>II9r</td>
<td>Interleukin 9 receptor</td>
<td>1.96</td>
</tr>
<tr>
<td>Rn.54465</td>
<td>Itgam</td>
<td>Integrin alpha M</td>
<td>3.34</td>
</tr>
<tr>
<td>N/A</td>
<td>LOC301289</td>
<td>Similar to Interleukin-17 precursor (IL-17) (Cytotoxic T lymphocyte-associated antigen 8) (CTLA-8)</td>
<td>13.43</td>
</tr>
<tr>
<td>Rn.2661</td>
<td>Mif</td>
<td>Macrophage migration inhibitory factor</td>
<td>9.19</td>
</tr>
<tr>
<td>Rn.10400</td>
<td>Nos2</td>
<td>Nitric oxide synthase 2, inducible</td>
<td>19.35</td>
</tr>
<tr>
<td>Rn.29157</td>
<td>Rac1</td>
<td>Ras-related C3 botulinum toxin substrate 1</td>
<td>9.89</td>
</tr>
<tr>
<td>Rn.40136</td>
<td>Tgfb1</td>
<td>Transforming growth factor, beta 1</td>
<td>27.30</td>
</tr>
<tr>
<td>Rn.107212</td>
<td>Tlr1</td>
<td>Similar to toll-like receptor 1 (LOC305354), mRNA</td>
<td>18.47</td>
</tr>
<tr>
<td>Rn.46387</td>
<td>Tlr2</td>
<td>Toll-like receptor 2</td>
<td>27.23</td>
</tr>
<tr>
<td>Rn.15273</td>
<td>Tlr3</td>
<td>Toll-like receptor 3</td>
<td>31.22</td>
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<tr>
<td>----------</td>
<td>------</td>
<td>---------------------</td>
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<tr>
<td>Rn.14534</td>
<td>Tlr4</td>
<td>Toll-like receptor 4</td>
<td>14.04</td>
</tr>
<tr>
<td>Rn.198962</td>
<td>Tlr5</td>
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<tr>
<td>Rn.163249</td>
<td>Tlr6</td>
<td>Toll-like receptor 6</td>
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<td>Rn.92495</td>
<td>Tlr9</td>
<td>Toll-like receptor 9</td>
<td>22.90</td>
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<tr>
<td>Rn.2275</td>
<td>Tnf</td>
<td>Tumor necrosis factor (TNF superfamily, member 2)</td>
<td>27.49</td>
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<tr>
<td>Rn.11119</td>
<td>Tnfrsf1a</td>
<td>Tumor necrosis factor receptor superfamily, member 1a</td>
<td>23.49</td>
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<tr>
<td>Rn.83633</td>
<td>Tnfrsf1b</td>
<td>Tumor necrosis factor receptor superfamily, member 1b</td>
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<tr>
<td>Rn.30043</td>
<td>Tnfsf4</td>
<td>Tumor necrosis factor (ligand) superfamily, member 4</td>
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<tr>
<td>Rn.44218</td>
<td>Cd40lg</td>
<td>CD40 ligand</td>
<td>9.51</td>
</tr>
<tr>
<td>Rn.9868</td>
<td>Il10</td>
<td>Interleukin 10</td>
<td>0.44</td>
</tr>
<tr>
<td>Rn.50003</td>
<td>Il17b</td>
<td>Interleukin 17B</td>
<td>0.79</td>
</tr>
<tr>
<td>Rn.11118</td>
<td>Il18</td>
<td>Interleukin 18</td>
<td>0.92</td>
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Figure 1: Absence of perturbations in branching morphogenesis in neo-natal off-spring exposed in utero to Vinclozolin. Analysis of (a) ductal number, length, volume or (b) branches, points or tips revealed no significant differences between Vinclozolin (open bar) and Control (solid bar) in utero exposed prostates. Mean ± SEM.

Figure 2: Serum Testosterone. No differences in serum testosterone between Vinclozolin (open bar) or Control (solid bar) in utero exposed at PND 0, 28 or 56. Mean ± SEM.

Figure 3: Early onset post-pubertal prostatitis in animals exposed in utero to Vinclozolin. Pre-pubertally no morphological differences between prostates of animals exposed in utero to Control (A) and Vinclozolin (B) were identified. Prostates from in utero exposed Control animals (C) showed extensive ductal branching and canalization with pseudo stratified columnar epithelial cells lining the ducts and continuous stromal sheaths surrounding the ducts. In in utero Vinclozolin exposed prostates (D) prominent, but focal, regions of inflammation are evident. Compared to Control in utero treatment (E) an increase in the proportion of macrophages surrounding the ducts and infiltrating into vessels is observed following in utero Vinclozolin treatment (F). Compared to control treatment (solid bar) a significant (p<0.05) increase in the percentage of prostatic inflammation is observed following in utero Vinclozolin exposure (open bar) (G). Compared to in utero Control exposed tissues (H) activation of the inflammatory NFκB pathway was evident in prostates following in utero Vinclozolin treatment with increased nuclear immuno-protein localisation of phospho-NFκB p65 (Ser536) (H and inset). Compared to in utero Control exposed prostates (I), in utero Vinclozolin exposed prostates showed NFκB dependant TLR-4 gene expression up-regulation (J) and an increase in TGFβ1 expression (E, F). Bar 50μm (A-D, G, H, K, L); 20 μm (E,F); 100 μm (I, J). Mean ± SEM.
Figure 4: Epithelial attenuation in post-pubertal animals following *in utero* Vinclozolin treatment. In tissues exposed *in utero* to Control (A) AR is localised predominately to epithelial cells. (B) A down regulation in epithelial AR expression is observed in *in utero* Vinclozolin exposed prostates. (C) A discontinuous layer of basal cells were identified in tissues Control exposed by immuno-localisation of CKHMW, compared with a continuous layer in prostates exposed *in utero* to Vinclozolin (D). A significant loss of terminally differentiated epithelial was confirmed by stereological analysis (E; Solid bar, Control exposed *in utero*; Open bar, Vinclozolin exposed *in utero*). Proliferative activity was examined by immuno-localisation of PCNA (F, G). In attenuated glands of prostate tissues from animals exposed *in utero* to Vinclozolin a reduction in immuno-positive epithelial cells was observed (G). Bar 100 μm (A,B); 50μm (C,D); 200μm (F,G); Mean ± SEM * p<0.05.
Appendix 2: Abstracts of Presentations

1. Southern Health Research Week, Clayton, Victoria Australia (Poster presentation)

Prostate disease begins in the womb: Early origins of prostatic inflammation caused by transient exposure to the endocrine disrupting chemical Vinclozolin.

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² National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA, 27709

The role of endocrine disrupting chemicals (EDCs) in the early origins of adult prostate disease is of concern and controversy to the lay and scientific communities. Environmental estrogens disrupt development and have been linked to the increased incidence of prostate disease in aging men. This study is the first to implicate the EDC Vinclozolin (an anti-androgen) as a causative factor. Early-onset prostate inflammation is evident in 100% of post pubertal (56 days) male off-spring exposed orally in utero during the sensitive period of male reproductive tract development (gestational days 14-19). Prostatic inflammation is due to activation of the NFκB inflammatory pathway with phosphorylation and activation of NFκB evident by increased nuclear translocation of phospho-NFκB p65 (Ser536). Pro-inflammatory NFκB dependant genes are also activated in post-pubertal animals following in utero Vinclozolin treatment including the chemokine Interleukin-8, which is associated with the increased
macrophage infiltration, and the cytokine Transforming Growth Factor (TGF)-β1, associated with the down regulation of the anti-inflammatory gene Interleukin-10. These are the first data to implicate EDC exposure as a causative factor of prostatic inflammation and, given the link between chronic inflammation and prostate cancer, warrant further regulatory investigation.
2. Lorne Cancer Conference. Lorne, Victoria Australia *(Poster presentation)*

**Prostate disease begins in the womb: Early origins of prostatic inflammation caused by transient exposure to the endocrine disrupting chemical (EDCs) – Vinclozolin.**

**Prue A. Cowin**¹, Paul Foster², Stephen McPherson¹, Gail P. Risbridger¹

¹ Centre for Urological Research, Monash Institute of Medical Research, Monash University, Clayton, Victoria, Australia, 3168

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EDC exposure as a causative factor of prostatic inflammation and, given the link between chronic inflammation and prostate cancer, warrant further regulatory investigation.
Fetal exposure to the anti-androgenic fungicide Vinclozolin has been shown to have adverse effects on male reproductive tract development. Recent studies have suggested the adult prostate may also be altered by exposure to Vinclozolin. However, it is not clear whether the reported prostatic pathology occurs earlier in development, nor is it clear whether growth regulatory pathways may be affected. Therefore, the aim of this study was to determine the effects of transient in utero exposure to Vinclozolin on the pre- and post-pubertal rodent prostate gland.

Fetal rats were exposed to Vinclozolin (100mg.kg bw) or corn oil vehicle control (2.5ml.kg bw) in utero for 6 days from gestational day 14 to 19 via oral administration to pregnant dams. This period corresponds to a critical window of male reproductive tract development, particularly the onset of appearance of androgen receptors in the urogenital sinus and the beginning of prostate budding. Male
pups were aged to 4 or 8 weeks (pre-pubertal; post-pubertal respectively) before tissue was collected for analysis. At 4 weeks of age *in utero* exposure to Vinclozolin resulted in no significant developmental or morphological abnormalities compared to control animals. Prostates of Vinclozolin-treated post-pubertal animals displayed apparent epithelial atrophy which was confirmed by subsequent stereological analysis and immunohistochemistry revealed a significant increase in the percentage of basal cells within epithelia of atrophic glands. Analysis of hormone receptor expression revealed reduced epithelial and increased stromal AR expression in Vinclozolin-treated tissues, although no differences in estrogen receptor alpha or beta expression were observed. An apparent increase in inflammatory cells was observed in Vinclozolin treated tissues and preliminary studies suggest a link with up regulation in the NFκB signaling pathway.

Overall, this study demonstrates that transient *in utero* exposure to an anti-androgenic chemical during a critical period of male reproductive tract development has the potential to induce prostate inflammatory atrophy by disrupting normal prostate development and inducing an inflammatory response which only becomes identifiable in post-pubertal animals implying aberrant androgenic response. This work is of particular significance as there is increasing literature suggesting a link between chronic inflammation and prostate cancer.
Prostate Inflammatory Atrophy induced following transient *in utero* exposure to the endocrine disruptor Vinclozolin

*Prue A. Cowin¹, Paul Foster², Stephen J. McPherson¹ and Gail P. Risbridger¹*

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Transient endocrine disruption induces prostate pathologies upon aging

**Risbridger GP\(^1\), Cowin PA\(^1\), McPherson SJ\(^1\), Foster P\(^2\)**

\(^1\)Centre for Urological Research, Monash Institute of Medical Research, Monash University, Melbourne, VIC, Australia

\(^2\)Research Triangle Park, National Institute of Environmental Health Sciences, North Carolina, United States

Normal development and differentiation of the prostate gland is regulated by androgenic and estrogenic hormones requiring a complex interplay between endocrine and cell-cell signaling. Although prostate disease occurs in later life, it is known that transient perturbations in hormone action or in the relative ratio of androgens to estrogens, result in pathologies in late life – long after the initial event. We and others have demonstrated the importance of androgens and estrogens in the maintenance of the epithelial stem cell niche and the pivotal role of the stroma in mediating these effects.

The fungicide Vinclozolin is an anti-androgen and an endocrine disrupting chemical (EDC), with adverse effects on male reproductive tract development. We show transient neonatal exposure to Vinclozolin results in the perturbation of hormone action and aberrant stromal-epithelial cell signaling that alters the prostatic stem cell niche. Epithelial cell pathologies occur at maturity; specifically prostatic inflammatory atrophy. Since chronic inflammation is linked to the onset of premalignant lesions, these results provide a mechanism for the long range effects of transient exposure to Vinclozolin on the prostate gland.
Appendix 3: Updated Biographical Sketch for Prof. Gail P. Risbridger

BIOGRAPHICAL SKETCH

NAME
Gail Risbridger

POSITION TITLE
Professor

EDUCATION/TRAINING

<table>
<thead>
<tr>
<th>INSTITUTION AND LOCATION</th>
<th>DEGREE (if applicable)</th>
<th>YEAR(s)</th>
<th>FIELD OF STUDY</th>
</tr>
</thead>
<tbody>
<tr>
<td>University of Sussex (UK)</td>
<td>B.Sc (Hons)</td>
<td>1971-1974</td>
<td>Biochemistry</td>
</tr>
<tr>
<td>Oxford University</td>
<td>Dip. Ed.</td>
<td>1974-1975</td>
<td>Education</td>
</tr>
<tr>
<td>University of Strathclyde (Glasgow)</td>
<td>M.Sc.</td>
<td>1976-1977</td>
<td>Reproductive Endocrinology</td>
</tr>
<tr>
<td>Monash University (Australia)</td>
<td>PhD</td>
<td>1977-1980</td>
<td>Reproductive Endocrinology</td>
</tr>
</tbody>
</table>

RESEARCH AND PROFESSIONAL EXPERIENCE:

Previous Employment:
1980-1981 Research Officer, NH&MRC (National Health and Medical Research Council), Royal Children's Hospital, Melbourne.
1981-1990 Senior Research Officer, Research Fellow (NH&MRC), Departments of Physiology & Anatomy, Monash University
1988-1994 Senior Lecturer, Dept Anatomy
1996 NH&MRC Principal Research Fellow, Institute of Reproduction & Development, Monash University
1998 -2001 Associate Professor, Faculty of Medicine, Monash University

Current Positions:
1996 NH&MRC Principal Research Fellow, Monash Institute of Medical Research, Monash University
1997 Associate Director Monash Institute of Medical Research
1996 Director for Centre for Urological Research, Monash Institute of Medical Research, Monash University
2000 Executive Committee Member, Australian Federal Government Centre for Excellence in Male Reproductive Health, Andrology Australia.
2001 Professor, Faculty of Medicine, Nursing and Health Sciences, Monash University
2006 Associate Dean, Research Centre & Institutes, Faculty of Medicine, Nursing and Health Sciences, Monash University
Other Experience and Professional Memberships

Federal Government public advisory committees.
1991-1997  NH&MRC, Regional Grants Interviewing Committee
1995-1997  NH&MRC Assigner’s Panel Member
1997 -  NH&MRC Program Grant Interviewing Committee
1998-2000  NH&MRC Chair of Discipline panel for Endocrinology & Reproduction,
2005-  NH&MRC Member of Grant Review Panels: Endocrinology, Cancer Biology
2006-  NH&MRC Member of Career Development Awards Panel
2001 - Andrology Australia, Member of Executive Committee of Management
2005 - Prostate Cancer Foundation of Australia, Member of Executive Scientific Advisory Group
2005 -  Australian Prostate Cancer Consortium - APCC, Executive Committee of Management
2006-  Medical & Scientific Committee of the Cancer Council Victoria, Member

Editorial Boards
1992-1995  Member of Editorial Board, Molecular & Cellular Endocrinology
2001-6  Member of Editorial Board, Endocrinology
2001-  Section Editor, Reproductive Biology; Cell and Tissue Research
2003-  Section Editor, Journal of Molecular Endocrinology

International & National Endocrine Society Positions
1994 -1998  Endocrine Society of Australia, Council Member and Secretary
2005 - Women in Endocrinology Board member and Awards Committee member
2006-8  Member of Steering Committee for US Endocrine Society Meetings

University Positions
1993 -  Member of Directors Management Group, Monash Institute of Medical Research
1994-1996  Member of Bachelor of Medical Science Committee, Faculty of Medicine, Monash University.
1995 -1997  Member of Senior Women’s Advancement Scheme, Vice Chancellors/Equal Opportunity office
1996 -1998  Member Strategic Planning Committee, Monash Institute of Medical Research
1996 -1998  Chairman, OHS Committee Zone 11, Monash University
1996 -1999  Member, Faculty Medicine Affirmative Action Committee
1993 - 2000  Member of Standing Committee on Animal Services, Monash University
1997 -  Member of Advisory Board Subcommittee for Commercialization
1998-2000  Member, Faculty of Medicine Research Committee
2000-  Chair of the MIRD Postgraduate Student Committee
2000-2003  Co-ordinator BSc Honours Program MMC4000
2001-2003  Member Faculty Research Degrees Committee
2004 -  Member Faculty of Medicine Research Management Committee
2006-  Deputy Chair, Faculty Research Committee
2006-8  Associate Dean, Research Centres and Institutes

Honours & Awards
2006   Asia-Oceana Medal - British Endocrine Society (for Contribution to Endocrinology)
2006  Postgraduate Supervision Award, Monash University, Special Commendation,
2005   Industry Engagement Award, Monash University
2003   Fulbright Senior Scholar Award - Department of Urology, Columbia University, New York
2001   Monash - Kings College Fellowship - London
2000   Silver Jubilee Prize – Monash University, Faculty of Medicine
1997   Academy of Science - Royal Society Exchange Program Award.
1991   British Council: Academic Links and Interchange Scheme Award
1991   Finnish Academy Science Award Visiting Scientist
1977-1979  Monash University Graduate Scholarship
1975-1977  MRC (UK) Postgraduate Scholarship

Successful Grants

Major National Grants
2006-   NH&MRC Fellowship Grant & SEO, ID 384104 - Risbridger
Role: PI   Funding: $130,000 p.a.
2005 – 2007  NH&MRC Program Grant Control of Reproductive Processes ID 334011 - De Kretser, Risbridger, Hedger, Hearn, Jenkin, Loveland, O’Bryan, Wallace
Role: Co-PI   Funding: $1,545,075 p.a.
2005 – 2009  NH&MRC Enabling grant Australian Prostate Cancer Collaboration (APCC) Bio-Resource (currently known as the Commonwealth Bank Australian Prostate Cancer BioResource in partnership with Andrology Australia) ID 290542 - Clements, Tilley, Sutherland, Risbridger
Role: CI   Funding: $2,100,000

Major International Grants
2008-2010  Risbridger, Taylor
USA Army Medical Research & Material Command (DOD) Grant
Idea Development Award #PC073444 Using Human Stem Cells to Study the Role of the Stroma in the Initiation of Prostate Cancer
Role: PI   Funding: US$154,882.37/year
2008 – 2010  Stuart Ellem, Risbridger
USA Army Medical Research & Material Command (DOD) Grant
Prostate Cancer Training Award #PC073307 Linking Estrogen, Prostatitis and Prostate Cancer
Role: Mentor   Funding: Total US$144,480
2007  Risbridger
USA Army Medical Research & Material Command (DOD) Grant Concept Award #PC060377 Endocrine Disruption and Human Prostate Cancer
Reports on a novel, reliable, and reproducible model system that can be used to study human prostate development and maturation and that can be used to test the potential effects of endocrine disruption chemicals (EDCs) on human prostate tissues.
Funding: Total $US96,644
2006- present  Risbridger, de Kretser, Simpson & McLachlan, O’Bryan
Schering AG
Melbourne Male Network is a joint venture between MIRD Centre for Urological Research and Prince Henry’s Institute of Medical Research & Division of Andrology &
Gynecology, Schering AG. It was funded following a bid for funds from the International Pool of Funds for Corporate Research at Schering AG.

Role: Lead PI  Funding: AUD$1,061,855

2007-2008  
Balanathan & Risbridger  
**USA Army Medical Research & Material Command (DOD) Grant**  
ID New Investigator Award #PC060112  
New Action of Inhibin Alpha Subunit in Advanced Prostate Cancer.  
To investigate a potentially new role for inhibin alpha subunit (INHA) in promoting prostate cancer growth and metastasis.  
Funding: Total $US113,614

2006-2008  
McPherson & Risbridger  
**USA Army Medical Research & Material Command (DOD) Grant**  
ID New Investigator Award #PC050653  
Is hormonal induction of prostate carcinogenesis due to declining androgens in late life and/or increased estrogen in early life?  
Role: CoI  
Total Funding: $US219,334

**Other Grants:**

2008 - 2010  
Prostate Cancer Foundation Australia (PCFA) & Cancer Australia (Co-Funded)  
NHMRC/PCFA Priority Call Grant “MicroRNAs in prostate cancer; Novel biomarkers and potential therapeutic targets”  
Role: CI B  
Funding: $648,000

2008 - 2011  
Prostate Cancer Foundation Australia (PCFA)  
**Young Investigator Grant.** Project ID: PCFAY01 “Molecular profiling and plasticity of prostate cancer stem cells with disease progression”  
Role: Mentor  
Funding: $300,000

2008 – 2009  
GlaxoSmithKline Australia – GSKA Post Graduate Support Grant. New action of inhibin alpha subunit in advanced prostate cancer  
Role: Mentor  
Funding: $25,000

2008  
Faculty of Medicine, Nursing & Health Sciences, Monash University – Monash Strategic Grants. Project ID: ECD040 “Biological Mechanisms underlining the tumour suppressive and pro-metastatic role of inhibin-α subunit in the changing tumour microenvironment”  
Role: Mentor  
Funding: A$35,000

2007  
Australian Research Council (ARC) - Linkage Infrastructure, Equipment and Facilities Scheme. Project ID: LE0883078 “Liquid Chromatography Tandem Mass Spectrometry Steroid Analysis Facility.”  
Role: CI  
Funding: $356,000

2007  
ANZ Trustees – Medical Research & Technology in Victoria – The William Buckland Foundation. Dual – or multi-functionality of inhibun-α subunit in prostate cancer progression  
Role: Mentor  
Funding: $15,000

2007  
The Cancer Council Victoria – Cancer Research Vacation Studentship. Endocrine Disrupting chemicals and early origins of prostate cancer  
Role: Mentor  
Funding: $1,500

2007  
Monash University – 2007 Near Miss Grant for NHMRC Projects – Deputy Vice Chancellor (Research)  
Role: PI  
Funding: $10,000
2007  
Monash University – 2007 Near Miss Grant for NHMRC Projects – Faculty of Medicine, Nursing & Health Sciences  
Role: PI  Funding: $10,000

2007 – 2010  
The Coulson Group Pty Ltd – “PhD Scholarship”. Risbridger, G  
Role: PI  Funding: $81,000

2007 –  
Harold and Core Brennen Benevolent Trust – The Role of Cancer Associated Fibroblasts (CAFs) in Prostate Carcinogenesis  
Role: CI  Funding: $30,000

2007 –  
ANZ Trustees – James & Vera Lawson Trust – The role of cancer associated fibroblasts (CAFS) in prostate carcinogenesis  
Role: PI  Funding: $10,000

2006-2008  
Cancer Council Victoria Project Grant: Early origins of prostate cancer. Risbridger G, McPherson S  
Role: PI  Funding: $203,000

2006  
Dorothy Hill Memorial Trust “Prof. G Cunha International Visiting Fellowship”  
Role: PI  Funding: $12,800

2006 –  
Fuji-Xerox Community Services Award – Equipment Grant : Double Header Microscope Attachment  
Role: PI  Funding: $5,000

2006 -  
The Eirene Lucas Foundation - Equipment Grant : Double Header Microscope Attachment  
Role: PI  Funding: $9,000

2006 -  
H&L Hecht Trust – Role of Stroma in Carginogenesis  
Role: PI  Funding: $20,000

2006  
Thomas & Rosalinda Ditchfield Medical Research Trust: “Is activin C a marker of early stage liver disease?” Risbridger G, Gold E.  
Role: PI  Funding: $12,800

2006  
J & R McGauran Trust.”Is activin C a marker of early stage liver disease?” Risbridger G, Gold E.  
Role: PI  Funding: $14,500

2006  
Oliver-Affleck Fund “Is activin C a marker of early stage liver disease?” Risbridger G, Gold E.  
Role: PI  Funding: $17,200

2005 – 2006  
Helen McPherson Smith Trust – Investigation of the Biological effects of increased activin C. Risbridger, G and Gold, E.  
Role: PI  Funding: $35,000

Publications

From a total of >130 publications, the following lists those since 2000


17. **Risbridger, GP.** and Butler, C, 2006 Activins and Leydig cell development, differentiation and disease (Chapter 22 ) from Contemporary Endorinology. In The Leydig Cell in Health and Disease, p323-331, Humana Press.


23. Butler CM, Gold EJ, **Risbridger GP**. (2005) Should activin bC be more than a fading snapshot in the activin/TGFb family album? Cytokine & Growth Factor Reviews 16 (4-5): 377-85 (IF 11.5)


32. Ball EMA, **Risbridger, GP** 2003 Epigenetic regulation of Inhibin alpha subunit gene in prostate cancer cell lines. Journal of Molecular Endocrinology. 32 (1): 55-67(IF 4.3)Cited 3


36. Ilic D, **Risbridger G**, Green S. 2004 Searching the internet for information on prostate cancer screening: an assessment of quality. Urology (64) 1, p112-116 (IF 2.7)


39. Ball EMA, **Risbridger GP**, 2003 New perspectives on Growth Factor-sex steroid interaction in the prostate Cytokine & Growth Factor Reviews, Vol 14/1 p 5-16 (IF 11.5)Cited 1

40. Mellor SL, Ball EMA, O’Connor AE, Ethier J-F, Cranfield M, Schmitt JF, Phillips DJ, Groome NP, **Risbridger GP.** 2003 Activin β, subunit heterodimers provide a new mechanism for regulating activin levels in the prostate. Endocrinology, 144 (10) 4410-4419 (IF 5.2)Cited 3


