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Distinct transcriptional profiles of the female, male and finasteride-induced feminized male anogenital region in rat fetuses

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ABSTRACT

A short anogenital distance (AGD) in males is a marker for incomplete masculinization and a predictor of adverse effects on male reproductive health. For this reason, AGD is used to assess the endocrine disrupting potential of chemicals for risk assessment purposes. The molecular mechanisms underpinning this chemically induced shortening of the AGD, however, remains unclear. Although it is clear that AR-mediated signaling is essential, evidence also suggest the involvement of other signaling pathways. This study presents the first global transcriptional profile of the anogenital tissue in male rat fetuses with chemically induced short AGD, also including comparison to normal male and female control animals. The anti-androgenic drug finasteride (10mg/kg bw/day) was used to induce short AGD by exposing time-mated Sprague Dawley rats at gestation days (GD) 7-21. The AGD was 37% shorter in exposed male fetuses compared to control males at GD21. Transcriptomics analysis on anogenital tissues revealed a sexually dimorphic transcriptional profile. More than 350 genes were found to be differentially expressed between the three groups. The expression pattern of four genes of particular interest (*Esr1, Padi2, Wnt2 and Sfrp4*) was also tested by RT-qPCR analyses, indicating that estrogen and Wnt2 signaling play a role in the sexually dimorphic development of the anogenital region. Our transcriptomics profiles provide a stepping-stone for future studies aimed at characterizing the molecular events governing development of the anogenital tissues, as well as describing the detailed Adverse Outcome Pathways for short AGD; an accepted biomarker of endocrine effects for chemical risk assessment.

Keywords: Anogenital distance, transcriptome, gene array, endocrine disruptors, risk assessment, reproduction

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INTRODUCTION

In mammals, the two sexes are morphologically indistinguishable until the bipotential gonads differentiate into either testes or ovaries in response to genetic cues during early fetal organogenesis (Svingen & Koopman, 2013). Formation of testes lead to the production of androgens that, to a large degree, drive development of the male accessory sex organs as well as general masculinization of the fetus (Jost, 1954). In the absence of high androgen levels, as in females, feminization of the fetus takes place. Thus, the balance between masculinization and feminization of the fetus is largely controlled by androgen signaling.

During development the perineum and surrounding tissues are responsive to androgen signalling. The anlage for the perineal muscles (levator ani/bulbocavernosus (LABC) muscle complex) are present in both sexes, but androgen action prompts the growth and differentiation of the muscles only in the male fetus (Cihak et al, 1970; Ipulan et al, 2014; MacLean et al, 2008). Under normal conditions, the distance between the anus and the genitals, the anogenital distance (AGD), becomes twice as long in males as in females (Hotchkiss & Vandenbergh, 2005; Salazar-Martinez et al, 2004). The programming of AGD occurs during a short timespan during fetal development (embryonic day 15.5-18.5 in rats and gestation week 8-14 in humans) and is often referred to as the masculinization programming window (MPW) (van den Driesche et al, 2017; Van den Driesche et al, 2012; Welsh et al, 2008). If androgen signaling is disrupted during the MPW, it can have severe consequences for androgen-dependent tissues and organs, including the AGD; a morphometric change regarded as a feminization effect (Schwartz et al, 2018).

Since the AGD is so clearly different between the two sexes, and because it is directly dependent upon fetal androgen action, it has emerged as a promising biomarker of incomplete masculinization.

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Although a short AGD is not necessarily harmful in itself, it is associated with male reproductive disorders in both rodents and humans. This group of disorders is often described as part of the testicular dysgenesis syndrome (TDS) hypothesis and range from genital malformations at birth (hypospadias and cryptorchidism) to low serum testosterone, low sperm quality and infertility in adulthood (Skakkebaek et al, 2001). For this reason, measurement of male AGD at birth can be used as a marker to retrospectively evaluate endocrine disruption during fetal life and hence predict future reproductive health outcomes (Schwartz et al, 2018).

The measurement of the AGD is currently incorporated into several OECD test guidelines for reproductive toxicity to test for endocrine disrupting potential of existing and emerging chemicals (OECD, 2012; OECD, 2016a; OECD, 2016b; OECD, 2018). It is unclear, however, how various chemicals affect the AGD at the molecular level. Such knowledge could aid in the development for predictive, non-animal test methods for future risk assessment of chemicals. To start characterizing these mechanisms, we profiled the transcriptome of the anogenital tissues in both male and female rat fetuses, as well as in male fetuses with a short AGD induced by *in utero* exposure to the anti-androgenic prostate cancer drug finasteride, a type II 5α-reductase inhibitor that blocks the conversion of testosterone to dihydrotestosterone (DHT) (Clark et al, 1990; Imperato-McGinley et al, 1992).

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METHODS

Test compound

The test compound used in this study was finasteride (purity reported by the manufacturer > 98%, CAS No.: 98319-26-7). Corn oil was used as control compound and vehicle (product number: C8267-2.5L). Both compounds were purchased from Sigma-Aldrich (Copenhagen, Denmark).

Animal study

Twelve time-mated nulliparous, young adult Sprague Dawley rats with a body weight of approximately 240±30 g were supplied at GD3 (NTac:SD, SPF, Taconic Europe, Ejby, Denmark). The day of vaginal plug detection was designated GD1. On GD4, dams were distributed into 2 groups of 6 animals with similar body weight distributions. Animals were housed in pairs until GD17 and thereafter individually. The animals were housed under standard conditions in semi-transparent polysulfone (PSU) type III cages (PSU 80-1291HOOSU Type III, Tecniplast, Buguggiate, Italy) (15x27x43 cm) with Aspen wood chip bedding (Tapvei, Gentofte, Denmark), Enviro Dri nesting material (Brogaarden, Lynge, Denmark) and Tapvei Arcade 17 (Aspen wood) shelters (Brogaarden). They were placed in an animal room with controlled environmental conditions: 12h light-dark cycles with light starting at 9 p.m., temperature 22 ± 1°C, humidity 55 ± 5%, 10 air changes per hour.

All animals were fed a standard diet with Altromin 1314 (soy- and alfalfa-free, Altromin GmbH, Lage, Germany). Acidified tap water (to prevent microbial growth) in PSU bottles (84-ACBTO702SU Tecniplast) were provided ad libitum. The PSU bottles and cages as well as the aspenwood shelters (instead of plastic) were used to reduce the risk of migration of bisphenol A that

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potentially could confound the study results. From GD7-21, dams were weighed daily and dosed by oral gavage by qualified animal technicians with a stainless steel probe 1.2x80 mm (Scanbur, Karlslunde, Denmark) with either vehicle control (corn oil) or finasteride (10 mg/kg bw/day) at a constant volume of 2 ml/kg bw per day.

Cesarean sections GD 21

Dams were decapitated under CO_2/O_2-anesthesia at GD21 and fetuses collected by caesarean section. Uteri were taken out and weighed, and the number of live fetuses, resorptions and implantations were registered. Prior to decapitation of the fetuses, their body weights were recorded. AGD was measured as the distance between the genital papilla and the anus, and measured in all fetuses by the same, blinded technician using a stereomicroscope with a micrometer eyepiece. The AGD index (AGDi) was calculated by dividing AGD by the cube root of the body weight. The anogenital tissue was isolated from the fetuses by dissection under a stereomicroscope. The tissue was trimmed to include only the genital tubercle, the perineal tissue and the very base of the tail (Figure S1). The tissue was placed in RNAlater (Qiagen, Hilden, Germany) and stored at -80˚C until further analysis. The animal experiments were carried out at DTU Food (Mørkhøj, Denmark) with ethical approval from the Danish Animal Experiments Inspectorate (license number 2015-15-0201-00553) and by the in-house Animal Welfare committee.

RNA extraction and gene array analysis

For the finasteride-exposed males, we selected fetuses with an AGD markedly shorter than control males for RNA analysis. Total RNA was isolated from the perineal tissue (n=4-6 pups/group) using RNeasy mini kits, including on-column DNaseI treatment, according to manufacturer's instructions (Qiagen, Hilden, Germany). The RNA purity and quantity was analyzed using the Agilent 2100

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Bioanalyzer system and Eukaryote Total RNA Nano assay (Agilent Technologies, Santa Clara, USA) according to manufacturer's instructions, and only samples with a RIN-score >7 were included for further analyses except for one sample that fell just short of 7. Sample RIN-scores are reported in the sample overview in Table S2. The Affymetrix Rat Gene 2.0 ST Array was performed by the Center for Genomic Medicine (Copenhagen University Hospital, Denmark). The gene array data were normalized with the Robust Multi-Array Average (RMA) method (Irizarry et al, 2003) implemented in the statistical software R (version 3.4.2) using the Brainarray custom Chip Description Files (CDF) so that intensity values are not summarized for each probe set but directly for each Entrez Gene ID (Dai et al, 2005).

Data and software availability

Raw data and transcriptomic signatures were uploaded to the NCBI Gene Expression Omnibus (GEO) (Barrett et al, 2013), to the TOXsIgN (Darde et al, 2018) and to Mendeley-Data repositories under the accession numbers GSE122494, TSP765 and doi:10.17632/bbtmz7wp4v.1, respectively.

Gene filtration and clustering

Data analysis was performed using the AMEN suite of tools (Chalmel & Primig, 2008). Briefly, genes showing a signal higher than a given background cutoff (median of the normalized data set cutoff 6.5483) and at least a 1.5 fold change in at least one pairwise comparison were selected as described in Figure S2. Gene array quality control assessment data can be found in Figure S3. To define a set of 364 genes displaying significant statistical changes across comparisons, the empirical Bayes moderated t-statistics was performed using the limma package (F-value adjusted using the Benjamini & Hochberg (BH) False Discovery Rate approach, $p \leq 0.05$ (Smyth, 2004). The resulting

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genes were then partitioned into two broad expression clusters (termed Cluster 1 and Cluster 2) using the k-means algorithm.

Functional analysis

The enrichment analysis module implemented in AMEN was used to identify gene ontology terms (biological processes, molecular functions, and subcellular components) and KEGG pathways significantly associated with each expression cluster by calculating Fisher's exact probability using the Gaussian hypergeometric function (FDR-adjusted p-value ≤ 0.01 number of probes in a given group associated with a given annotation term ≥ 5).

Synthesis of cDNA and RT-qPCR analysis

cDNA was synthesized from 500 ng total RNA using a random primer mix (New England Biolabs, Ipswich, MA, USA) and the Omniscript kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Quantitative RT-PCR (RT-qPCR) reactions were run in technical duplicates on an QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Thermo Fischer Scientific) in 11µl reactions containing: 3µl diluted (1:20) cDNA, TaqMan Fast Universal Mastermix (2X) (Life Technologies, Carlsbad, CA, USA) and TaqMan Gene Expressions Assays (Life Technologies). TaqMan assays were: *Wnt2* (Wingless-type MMTV integration site family member 2, Rn01500736_m1), *Sfrp4* (Secreted frizzled-related protein 4, Rn00585549_m1), *Esr1* (Estrogen receptor 1, Rn01640372_m1), *Padi2* (Protein-arginine deiminase type-2, Rn00568155_m1), *Srd5a2* (5α-reductase, Rn00575595_m1*), Ddx3* (DEAD-box polypeptide 3, Rn04224517_u1) and *Trdn* (Triadin, Rn00572292_m1). Cycling conditions were as follows: an initial step of 95°C for 20 sec followed by 45 two-step thermal cycles of 95°C for 1 sec and 60°C for 20 sec. The relative transcript abundance was calculated using the $2^{-\Delta CT}$ method using *ActB* (β-Actin, Rn00667869_m1)

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and *Hprt1* (Hypoxanthine guanine phosphoribosyl transferase, Rn01527840) as normalizing genes. Selection of normalizing genes were based on a RT^2 Profiler PCR Arrays (Qiagen, Hilden, Germany) run on the same tissue (unpublished data), where *ActB* and *Hrpt1* showed stable expression.

Statistics

AGD was analyzed using fetal weight as a covariate and fetal body weights were analyzed using the number of offspring per litter as covariate. For all analyses, the litter was the statistical unit. Statistical analyses were adjusted using litter as an independent, random and nested factor.

Data on weight, dams, AGD and AGDi, were analyzed using one-way ANOVA followed by Dunnett's post hoc test, using the statistical software SAS® (SAS Enterprise Guide 6.1, SAS Institute, Inc., Cary, NC, USA). For data presentation, group mean ± SEM was calculated from 6 litters/group based on litter means. Statistical analysis of the effect of finasteride on cryptorchidism was done using Fisher's Exact Test.

Analysis of RT-qPCR data was done by one-way ANOVA with Dunnet's post-hoc test using the statistical software GraphPad Prism 5 (GraphPad Software, San Diego California, USA). In cases of non-equal variance between groups, data were log-transformed prior to one-way ANOVA analysis, while the graphs still represent the untransformed data. For data presentation, mean ± SEM was calculated from 4-6 pups/group.

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RESULTS

Finasteride-induced short AGD in male offspring

Pregnant Sprague Dawley rats were exposed to 10 mg/kg bw/day finasteride from GD7 to GD21. We assessed maternal body weights, weight gain, and uterine weights of exposed dams for potential maternal toxicity and observed no significant treatment-related differences, nor any signs of maternal toxicity (Table S1). At GD21, the body weight of exposed males was lower ($p<0.05$) than that of the control males, while the exposed females only tended ($p=0.055$) to have a lower body weight than the control females (Table 1). AGD was on average 37% shorter ($p<0.01$) in exposed males when compared to control males (Figure 1). To confirm that this was not a function of lower body weight of the exposed males, AGDi was calculated and was 35% lower ($p<0.05$) in exposed males compared to controls. Fetal exposure to finasteride did not affect female AGD or AGDi (Table 1). In addition, one litter had three cryptorchid male fetuses, while two litters had one cryptorchid male fetus; in total 5 fetuses ($p=0.062$).

Distinct transcriptome profiles in control male, control females and finasteride-exposed males

We next performed gene array analysis to investigate the transcriptional profile of the three groups. For the group comprising finasteride-exposed males, we selected those with a markedly shorter AGD. A total of 364 differentially expressed genes (DEGs) were identified (Table S2) and subsequently partitioned into two broad expression clusters (Figure 2). One cluster (Cluster 1) comprised 319 genes that were highly expressed in the control females compared to the control males and the second (Cluster 2) comprised 45 genes with reversed expression pattern compared to Cluster 1. In both clusters, the gene expression profile of the exposed males was intermediary to the male and female control groups. Venn diagrams were made to visualize the up- and downregulated

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genes between control females or exposed males and control males (Figure 3A), as well as between control males or exposed males and control females (Figure 3B). The exposed males and control females shared ~63% of the DEGs (Figure 3A), while the overlap in DEGs was only ~2% between the exposed males and control males (Figure 3B). Despite exposed males being intermediary to the control males and control females, the exposed males are more closely related to the control females than the control males at the transcriptional level. The lists of genes from the resulting comparisons are available in Table S3.

Functional analysis showed no enriched terms for Cluster 2. In Cluster 1, there were 173 enriched terms (Table S4) of which the most relevant biological processes and pathways were related to development, muscle development, metabolism and calcium signaling (Figure 2). These included genes linked to cell development (57 were associated, 26 were expected by chance; 57/26, p=2.64e-07), cell differentiation (89/54, p=1.24e-05), skeletal muscle tissue development (35/2 p=3.4e-28), neuromuscular junction development (6/1, p=0.0001), ATP metabolic process (11/3, p=0.002), regulation of fatty acid oxidation (5/1, p=0.002), the glycolysis/glyconeogenesis pathways (6/1, p=0.009), and the calcium signaling pathway (11/3, p=0.001).

Distinct expression profiles validated by RT-qPCR

Among the differentially expressed genes, we initially selected three genes for further validation by RT-qPCR. These were the Y-chromosome linked DEAD-box helicase 3 (*Ddx3*), 5α-reductase (*Srd5a2*) and Triadin (*Trdn*). The three genes were chosen because they were the ones showing the biggest difference in expression levels between the three groups (control male, control female and exposed males). The differences should therefore be detectable in the less sensitive RT-qPCR analysis. *Ddx3* was only detected in males, with expression lower ($p<0.05$) in exposed versus

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control males (Figure 4). *Srd5a2* expression was lower ($p<0.01$) in control females compared to control males, while *Trdn* expression was higher ($p<0.05$) in control females compared to control males (Figure 4). These data corroborated the expression pattern observed in the global gene array.

Sexually dimorphic expression of Wnt2, Sfrp4, Esr1 and Padi2

We selected another four DEGs we believe could be possible key factors in the differential development of the anogenital region based on known functions. In accordance with the gene array data, expression of *Wingless-type MMTV integration site family member 2* (*Wnt2*) (Figure 5), a factor of the Wnt signaling pathway, was significantly lower ($p<0.01$) in control females compared to control males. In addition, an inhibitor of Wnt signaling, *Secreted frizzled protein 4* (*Sfrp4*) was higher ($p<0.01$) in control females compared to control males.

Expression of *Estrogen receptor 1* (*Esr1*) was higher ($p<0.05$) in exposed males compared to control males (Figure 5). This finding was corroborated by the higher expression ($p<0.001$) of the *Esr1* regulator *Padi2* in exposed males compared to control males (Figure 5). Expression of *Padi2* was also higher ($p<0.01$) in control females compared to control males.

DISCUSSION

In males, a short AGD is an indication of incomplete fetal masculinization. It is associated with adverse male reproductive health effects. Consequently, AGD in fetuses or newborn rodents is used as a biomarker in biological and toxicological research, as well as in regulatory toxicity testing (OECD, 2012; OECD, 2016a; OECD, 2016b; OECD, 2018). Despite its utility in determining anti-androgenicity, we still lack molecular insight into the mechanisms controlling anogenital

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development and how it relates to the AGD. This hampers the development of potential new, non-animal test methods for future risk assessment of chemicals.

In this study, we aimed to investigate the transcriptome of the anogenital region in males with short AGD and compare it to that of both control males and control females. We selected a model compound, finasteride, to induce short AGD in male offspring. Finasteride is a synthetic anti-androgen used in humans to treat benign prostatic hyperplasia or prostate cancer and acts by inhibiting type II 5α-reductase, thereby blocking the conversion of testosterone to DHT (Clark et al, 1990; Imperato-McGinley et al, 1992).

We observed a significantly shorter AGD of 37%, as well as a few male fetuses with cryptorchidism in the exposed group at GD21. These results are consistent with previous *in utero* studies showing several adverse effects on male reproduction after fetal exposure to finasteride (Bowman et al, 2003; Christiansen et al, 2009; Clark et al, 1993; Clark et al, 1990; Hib & Ponzio, 1995; Imperato-McGinley et al, 1992).

With our transcriptomics analysis on tissue from the anogenital region, we found that the control males and control females had distinct transcriptional profiles, whereas the transcriptome of the exposed males with a short AGD were intermediary to that of the two control groups. Importantly, comparison of the DEGs showed that the exposed males were more closely related to the control females. The main biological processes affected were related to cell development and differentiation, muscle development, metabolism and calcium signaling. This corroborates the general view that the development of the anogenital region, and in particular the muscle cells, is sensitive to the masculinizing effects of androgens. It further suggests a general feminization of the

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finasteride-exposed males, and shows for the first time that short male AGD is associated with marked transcriptional changes in cells of the anogenital region. In addition, RT-qPCR showed that the genes *Ddx3, Srd5a2, Trdn, Padi2, Wnt2* and *Sfrp4* were differentially expressed between control males and control females.

Esr1 expression was higher in finasteride-exposed males compared to control males, but similar to control females. In addition, expression of the *Esr1* co-factor *Padi2* (Zhang et al, 2012) was higher in both exposed males and control females when compared to control males. This suggests that estrogen signaling may play a role in feminization of the anogenital region. In support of this, a polymorphism in the coding region of ESR1 is associated with short AGD in humans (Sathyanarayana et al, 2012). Polymorphisms in ESR1 in humans are associated with hypospadias, cryptorchidism and reduced fertility (Ban et al, 2008; Safarinejad et al, 2010; Watanabe et al, 2007), disorders that are all thought to be associated with short AGD (Skakkebaek et al, 2001). Furthermore, chemicals with an assumed estrogenic mode of action, such as bisphenol A (Kim et al, 2001; Matthews et al, 2001) and butyl paraben (Byford et al, 2002; Routledge et al, 1998) can induce short AGD in male offspring (Boberg et al, 2016; Christiansen et al, 2014; Zhang et al, 2014), as can treatment with diethylstilbestrol in mouse fetuses (Stewart et al, 2018). Other studies, however, failed to find an effect on AGD following exposure to these two chemicals (Boberg et al, 2008; Ferguson et al, 2011; Howdeshell et al, 2008; Tinwell et al, 2002), a discrepancy that could be due to differences in study design or statistical power. Alternatively, as both chemicals also display anti-androgenic properties *in vitro* (Chen et al, 2007; Rosenmai et al, 2014), the effect on AGD could instead be anti-androgenic, or disruption to the androgen-estrogen balance, or by other mechanisms still to be determined.

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Our gene array revealed that members of the Wnt-signaling pathway are expressed in a sexually dimorphic manner in the rat anogenital region at GD21. Expression of *Wnt2* was higher in control males compared to control females, while expression *Sfrp4*, an inhibitor of the Wnt signaling pathway, showed the opposite pattern. To our knowledge, this is the first evidence linking Wnt signaling to anogenital development, albeit expression of *Wnt4* and the Wnt signaling receptor *Fzd4* has been shown to be affected by global knock-out of *Ar* in male gastrocnemius muscle (MacLean et al, 2008). Since the perineal LABC muscle complex is highly androgen-responsive (Rand & Breedlove, 1992), it supports the view that finasteride-induced disruption of androgen signaling might also affect Wnt signaling in this muscle, but with Wnt being downstream of AR signaling.

A drawback to our study is that the analysis was performed on GD21 offspring. It is likely that many of the molecular events leading to a sexually dimorphic AGD takes place at an earlier stage and would not be captured in our analysis. As measurements of AGD at early stages such as GD17-19 are impractical, GD21 was chosen to make sure that we had induced short male AGD, as well as allowing for the selection of individual fetuses that had most pronounced shorter AGD relative to controls. This latter point is important in that effects on AGD typically are seen at the population level, but with large individual variation in both exposed and control animals. We therefore selected those GD21 fetuses that presented with obviously shorter AGD and matched them to control fetuses presenting with AGD close to the mean value. We allowed this bias to accommodate our objective of detecting transcripts affected in male fetuses displaying feminized AGD and to circumvent potential issues with larger individual variations at the molecular level were exposed males without a markedly shorter AGD to be included. This inclusion would be necessary if the focus was specifically at elucidating the mechanisms of action of finasteride, whereas this study has a specific

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focus of detecting differentially regulated transcripts in feminized male AGD more generally. Nevertheless, it is possible to detect sexual differences at the molecular level already at embryonic day 15.5 in mice, corresponding to approximately GD17-18 in rats (Ipulan et al, 2014). Thus, future analyses of the male and female anogenital region at different time points during development will elucidate exactly at which stage during fetal life Wnt and estrogen signaling becomes sexually dimorphic. In addition, future studies on feminized males following chemical exposure should include earlier time points to investigate the early anogenital development, which would require a high number of litters per dose group to account for the variation in AGD. Such analyses may provide further clues as to the role of these pathways in controlling the sex specific development of the AGD.

In this study, we used the 5α-reductase inhibitor finasteride to induce short AGD in male offspring. This was done specifically to investigate the changes in the anogenital region in response to an androgen signaling inhibitor with a known mechanism of action. As there is evidence to suggest that other chemicals may affect AGD by other mechanisms (Schwartz et al, 2018) it will be relevant to investigate whether exposure to such chemicals result in similar transcriptional changes in the anogenital region as those we observed in this study, or if the profiles will prove to be different.

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Declaration of Interests

The authors declare that they have no conflict of interest concerning this work.

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Table 1: Litter data. Data represent group means, based on litter means ± SD after exposure GD7-21. Male AGD (mm) was analysed with fetal weight as covariate Exposed: Finasteride, 10 mg/kg bw/day. AGD index = AGD divided by cube root of body weight. Bold values are statistically different from Control. *: Significantly different from Control, $p < 0.05$. **: Significantly different from Control, $p < 0.01$. ¤: Tends to be different from control, $p = 0.055$. #: One litter had three cryptorchid males, while two litters had one each, $p=0.062$ (Fisher's Exact test).

GD21 Caesarean section	**Control**	**Exposed**
No. of litters	N=6	N=6
No. of fetuses/litter	14.0 ± 2.4	12.5 ± 2.4
Body weight male (g)	4.3 ± 0.4	**3.9 ± 0.3***
Body weight female (g)	4.2 ± 0.3	3.6 ± 0.5$^{¤}$
Male AGD (mm)	3.64 ± 0.2	**2.28 ± 0.1****
Male AGD index	2.24 ± 0.03	**1.45 ± 0.08***
Cryptorchid male fetuses	0	5$^{\#}$
Female AGD (mm)	1.81 ± 0.09	1.70 ± 0.11
Female AGD index	1.13 ± 0.04	1.11 ± 0.04

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FIGURE LEGENDS

Figure 1: Finasteride-induced short anogenital distance. Anogenital distance (mm) of control males (Control), finasteride exposed males (finasteride, 10mg/kg bw/day GD7-GD21). Results are shown as Mean ± SEM, n=6 litters/group, with each dot representing one fetus. **: Significantly different from control, $p < 0.01$(with bw as covariate).

Figure 2: Distinct transcriptome profiles. Heatmap representation of gene expression in the perineal tissues from control males, finasteride-exposed males (finasteride, 10mg/kg bw/day GD7-GD21) and control females. Genes (rows) were partitioned according to their expression profiles in the distinct samples (columns) into two broad expression cluster containing 319 (Cluster 1) and 45 (Cluster 2) genes, respectively. The standardized log2-transformed intensity signals are represented in the scale bar. n=4-6 litters/group. The most relevant enriched GO and KEGG terms are indicated at the right side of the heatmap. For each term, the p-value is indicated as well as the number of genes associated with each term and compared to the number of genes that would be expected to be found by chance. See also Table S2 and Table S4.

Figure 3: Venn diagrams of differentially expressed genes (DEGs). A: Venn diagrams based on the list of upregulated and downregulated genes when control females and exposed males (finasteride, 10mg/kg bw/day GD7-GD21) were compared to control males. **B:** Venn diagrams based on the list of upregulated and downregulated genes when control males and exposed males (finasteride, 10mg/kg bw/day GD7-GD21) were compared to control females. See also Table S3.

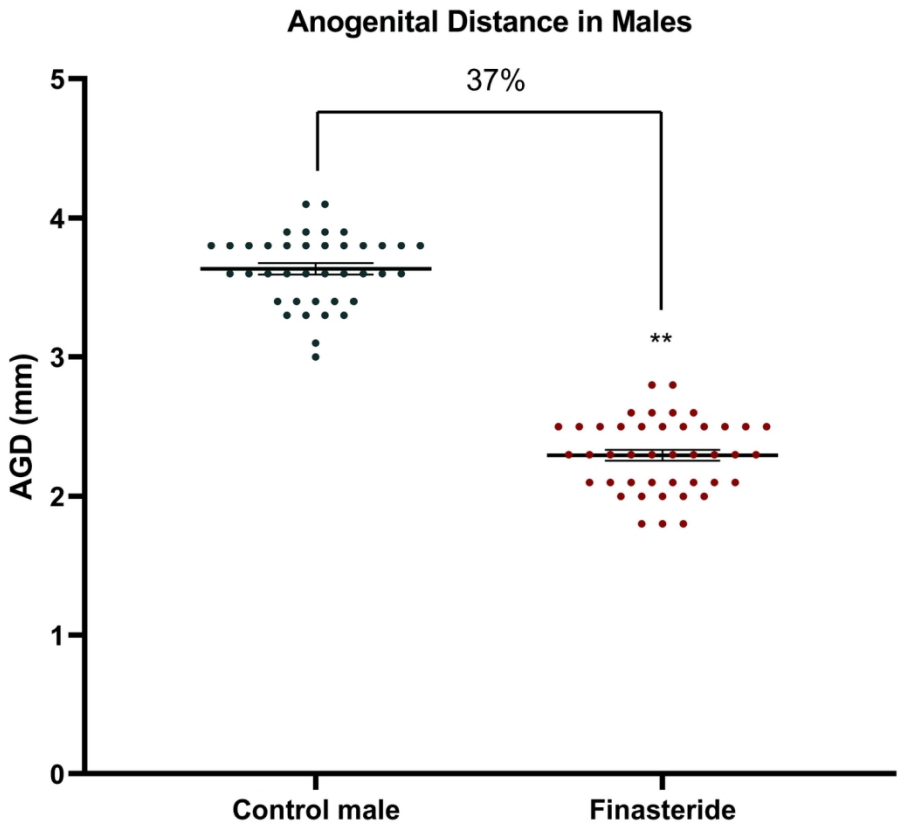
Figure 4: RT-qPCR validation of gene array. RT-qPCR validation of the three chosen genes. n=4-6. Results are $2^{-\Delta CT}$ values shown as mean±SEM. Finasteride: Exposed to finasteride, 10mg/kg

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bw/day GD7-GD21 *: Significantly different from control males, $p < 0.05$. **: Significantly different from control males, $p < 0.01$.

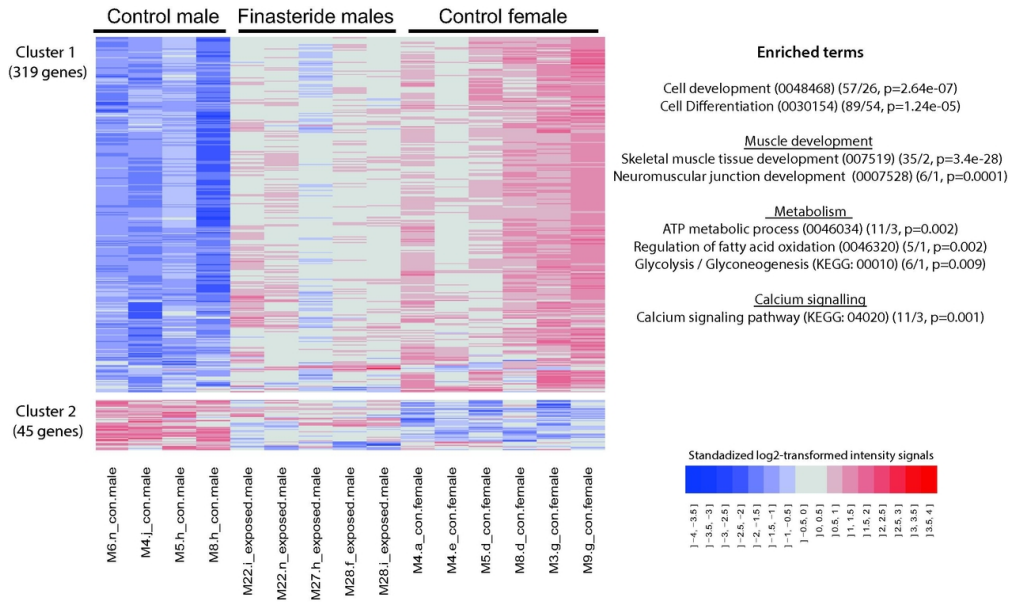
Figure 5: Expression of Wnt2, Sfrp4, Esr1, and Padi2. RT-qPCR validation of *Wnt2*, *Sfrp4*, *Esr1*, and *Padi2*. n=4-6. Results are $2^{-\Delta CT}$ values shown as mean±SEM. Finasteride: Exposed to finasteride, 10mg/kg bw/day GD7-GD21 *: Significantly different from control, $p < 0.05$. **: Significantly different from control males, $p < 0.01$. ***: Significantly different from control males, $p < 0.001$.

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Finasteride-induced short anogenital distance. Anogenital distance (mm) of control males (Control), finasteride exposed males (finasteride, 10mg/kg bw/day GD7-GD21). Results are shown as Mean ± SEM, n=6 litters/group, with each dot representing one fetus. **: Significantly different from control, $p < 0.01$(with bw as covariate).

140x123mm (300 x 300 DPI)



Heatmap representation of gene expression in the perineal tissues from control males, finasteride-exposed males (finasteride, 10mg/kg bw/day GD7-GD21) and control females. Genes (rows) were partitioned according to their expression profiles in the distinct samples (columns) into two broad expression cluster containing 319 (Cluster 1) and 45 (Cluster 2) genes, respectively. The standardized log2-transformed intensity signals are represented in the scale bar. n=4-6 litters/group. The most relevant enriched GO and KEGG terms are indicated at the right side of the heatmap. For each term, the p-value is indicated as well as the number of genes associated with each term and compared to the number of genes that would be expected to be found by chance. See also Table S2 and Table S4.

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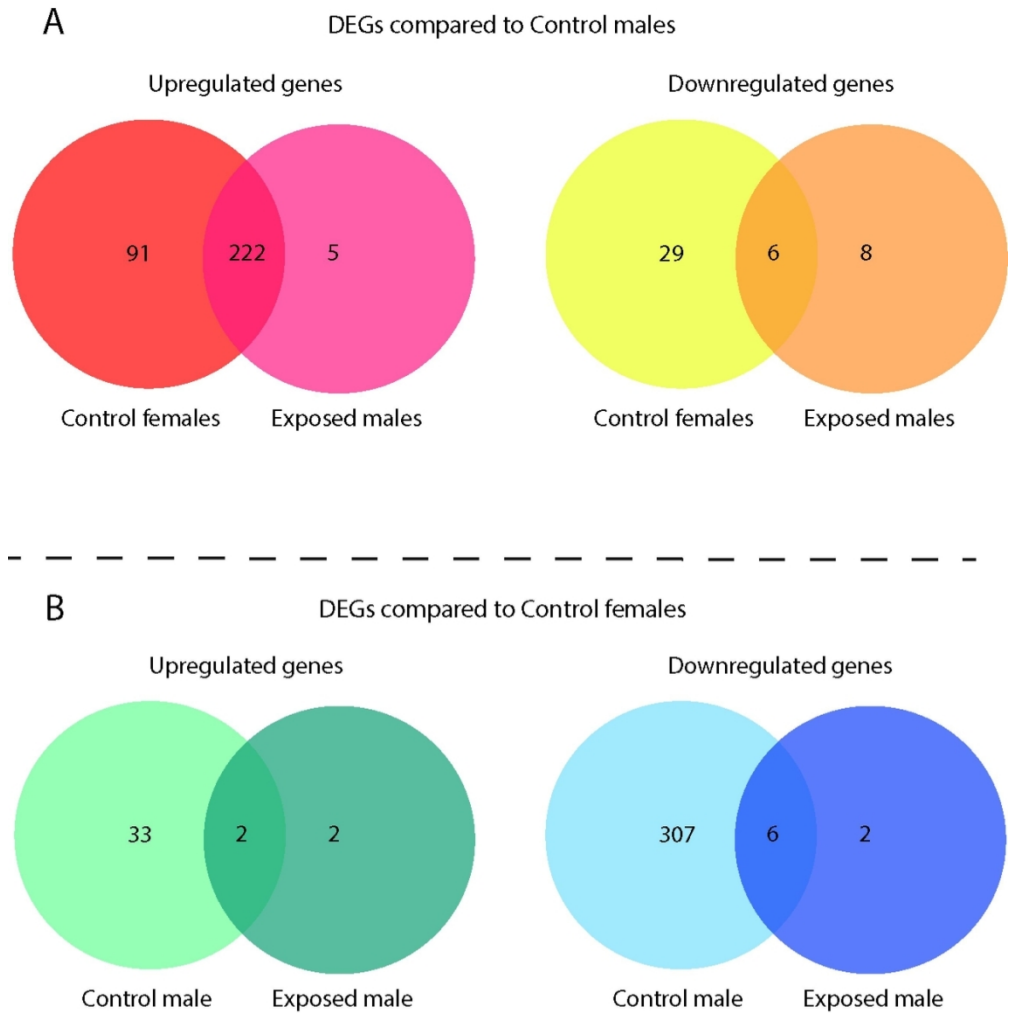
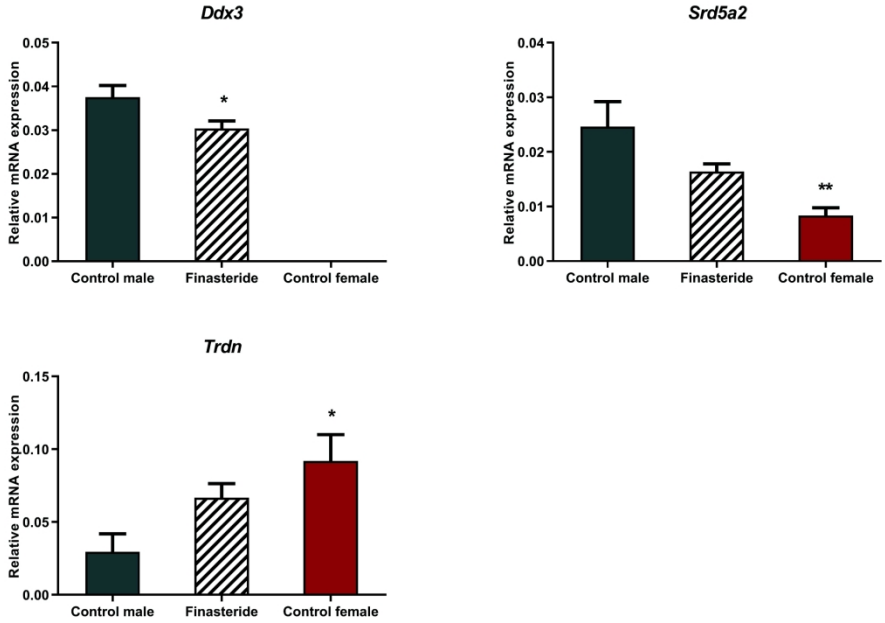


Figure 3: Venn diagrams of differentially expressed genes (DEGs). A: Venn diagrams based on the list of upregulated and downregulated genes when control females and exposed males were compared to control males. B: Venn diagrams based on the list of upregulated and downregulated genes when control males and exposed males were compared to control females. See also Table S3.

111x112mm (300 x 300 DPI)



RT-qPCR validation of the three chosen genes. n=4-6. Results are 2-ΔCT values shown as mean±SEM. Finasteride: Exposed to finasteride, 10mg/kg bw/day GD7-GD21 *: Significantly different from control males, $p < 0.05$. **: Significantly different from control males, $p < 0.01$.

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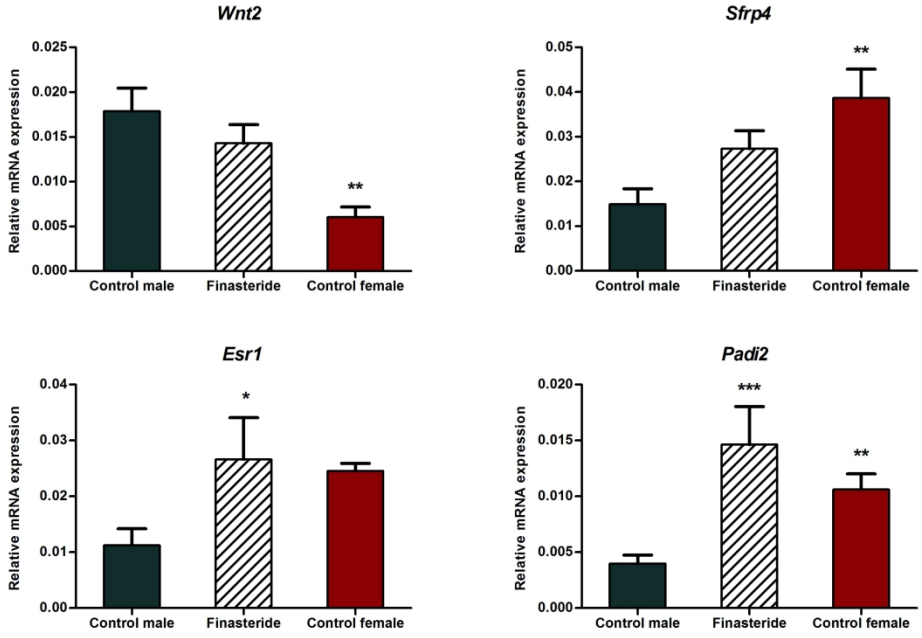


Figure 5: Expression of Wnt2, Sfrp4, Esr1, and Padi2. RT-qPCR validation of Wnt2, Sfrp4, Esr1, and Padi2. n=4-6. Results are 2-ΔCT values shown as mean±SEM. *: Significantly different from control, $p < 0.05$. **: Significantly different from control, $p < 0.01$. ***: Significantly different from control, $p < 0.001$.

262x180mm (300 x 300 DPI)